A PROTEIN TITRATION HYPOTHESIS FOR THE TEMPERATURE-DEPENDENCE OF TISSUE CO_2 CONTENT IN REPTILES AND AMPHIBIANS

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Summary

Whole-body CO_2 stores are known to increase with cooling in reptiles and amphibians ($-\Delta[CO_2]/\Delta T$). The aim of this study was to determine the mechanism(s) producing this inverse relationship. The $\Delta[CO_2]/\Delta T$ coefficients were determined for eight reptilian and one amphibian species and were found to differ by a factor of approximately 10, from -0.21 mmol kg^{-1} °C^{-1} in the Mediterranean spur-thighed tortoise *Testudo graeca* to -0.02 mmol kg^{-1} °C^{-1} in the bullfrog *Rana catesbeiana*. The $\Delta[CO_2]/\Delta T$ coefficients were correlated with values in the literature for *in vivo* plasma $\Delta pH/\Delta T$ coefficients ($\Delta[CO_2]/\Delta T = -0.18 - 8.24 \Delta pH/\Delta T$; $r^2 = 0.87$). Plasma electrolyte concentrations (Na$^+$, K$^+$, Ca^{2+}, Mg^{2+}, Cl$^-$, inorganic phosphate, SO$_4^{2-}$ and lactate), [protein], [CO_2], P_{CO_2} and pH were measured in chronically cannulated resting black racer snakes *Coluber constrictor*. When the temperature was reduced from 30 to 10 °C, pH increased slightly (by -0.0028 pH units °C^{-1}), P_{CO_2} decreased by 7 mmHg, [CO_2] increased by 3.2 mmol l^{-1} and [HPO$_4^{2-}$+H$_2$PO$_4^-$] increased by 0.7 mmol l^{-1}. Concentrations of protein and of the remaining electrolytes were not significantly different ($P>0.05$) at 30 and 10 °C. Net plasma protein charge, calculated from the principle of electroneutrality (the sum of the cations in mequiv = the sum of anions in mequiv), was -0.48 mequiv g^{-1} protein at 30 °C and -0.38 mequiv g^{-1} protein at 10 °C. This 21 % decrease was attributed to the increases in [CO_2] (i.e. carbonic acid) and inorganic phosphate concentration. Between 30 and 10 °C, skeletal muscle pH and [CO_2] in *C. constrictor* increased (by -0.009 units °C^{-1} and -0.125 mmol kg^{-1} °C^{-1}, respectively), [Na$^+$] and [Cl$^-$] each decreased by approximately 12 mequiv l^{-1}, and [K$^+$] and the percentage of water did not change significantly. It is concluded that the increase in whole-body CO_2 stores with cooling in reptiles and amphibians results from the passive effects of temperature changes upon the ionization constants of proteins and the active adjustment of P_{CO_2} (ventilatory regulation), so that $-\Delta pK$ is greater than $-\Delta pH$. Active transmembrane ion-exchange processes do not appear to be involved.

Key words: *Coluber constrictor*, reptiles, amphibians, temperature, respiratory exchange ratio, oxygen consumption, carbon dioxide production, acid–base balance, pH, protein charge, tissue carbon dioxide content, electrolytes, skeletal muscle.

Introduction

Previously we reported changes in whole-body CO_2 stores, estimated from transients in CO_2 elimination, with temperature changes ($\Delta CO_{2WB}/\Delta T$) in three species of reptiles and one amphibian species (Stinner and Wardle, 1988; Stinner *et al.* 1994*a*; Stinner, 1982). The $\Delta CO_{2WB}/\Delta T$ coefficient varied from -0.18 mmol kg^{-1} °C^{-1} in the black racer snake *Coluber constrictor* to -0.05 mmol kg^{-1} °C^{-1} in the cane toad *Bufo marinus*. The absolute value of the plasma $\Delta pH/\Delta T$ coefficient also differs among these species and is low in *C. constrictor* compared with *B. marinus* (-0.003 units °C^{-1} *versus* -0.015 units °C^{-1} between 30 and 10 °C, respectively; Stinner *et al.* 1996; Boutilier *et al.* 1987). This inverse relationship between the $\Delta CO_{2WB}/\Delta T$ and $\Delta pHpl/\Delta T$ coefficients suggests an explanation for the thermal dependence of CO_2 stores. When temperature is changed, the change in [CO_2] is related to the nonbicarbonate buffer value (β_{NB}), the change in the ionization constant of the nonbicarbonate buffers (ΔpK) and ΔpH by the expression $\Delta[HCO_3^-] = \beta_{NB}(\Delta pK - \Delta pH)$ (Heisler and Neuman, 1980; Heisler, 1986*a*). Thus, if β_{NB} and $\Delta pK/\Delta T$ are similar among species, $\Delta[HCO_3^-]$ (and hence the change in total [CO_2]) will be greatest in species exhibiting the smallest change in pH. For example, the $\Delta pK/\Delta T$ coefficient for 4-methyl-imidazole is -0.023 units °C^{-1} (Figge *et al.* 1991*a*) and so, when body temperature changes, the absolute value of $\Delta pKim-\Delta pH$ is much greater in *C. constrictor* than in *B. marinus* (0.019 *versus* 0.007 units °C^{-1}). Consequently, there would be considerably more nonbicarbonate buffering of CO_2, with concomitant changes in net imidazole charge, in *C. constrictor* than in *B. marinus*.

This study tests the hypothesis that the increase in whole-body CO_2 stores with cooling results from titration of proteins by carbonic acid. We estimated $\Delta[CO_2]_{WB}/\Delta T$

coefficients in eight additional species of reptiles and one additional amphibian species from measurements of the respiratory exchange ratio (rate of CO_2 elimination/rate of O_2 uptake), and compared $\Delta[CO_2]_{WB}/\Delta T$ coefficients with values from the literature for $\Delta pHpl/\Delta T$ coefficients. In addition, we estimated net plasma protein charge in *C. constrictor* at 30 and 10 °C from the principle of electroneutrality. Strong ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, lactate), inorganic phosphate, protein concentration, total $[CO_2]$, P_{CO_2} and pH were measured in plasma at the two temperatures in resting chronically cannulated black racer snakes under steady-state conditions. The interspecific $\Delta CO_{2WB}/\Delta T$ coefficients were found to be strongly correlated with the interspecific $\Delta pHpl/\Delta T$ coefficients, and net plasma protein charge in *C. constrictor* fell by 21 %. Furthermore, between 30 and 10 °C, $[CO_2]$ of skeletal muscle in *C. constrictor* increased, without corresponding changes in intracellular $[Na^+]$, $[K^+]$ or $[Cl^-]$. The findings of this study support the hypothesis that the thermal dependence of whole-body CO_2 stores in reptiles and amphibians results from the titration of protein by CO_2.

Materials and methods

Animals

Animals were purchased from commercial suppliers, except for painted turtles (*Chrysemys picta*) and snapping turtles (*Chelydra serpentina*) which were collected locally. They were housed in the animal facility at The University of Akron, where room temperature was maintained at 25–30 °C. The cages contained light bulbs set to a 12 h:12 h L:D cycle that provided basking sites at 35–40 °C. Approximately once weekly, the snakes, American alligators (*Alligator mississippiensis*), savannah monitors (*Varanus exanthematicus*), snapping turtles and bullfrogs (*Rana catesbeiana*) were fed on mice and rats. The desert iguanas (*Dipsosaurus dorsalis*), painted turtles and Mediterranean spur-thighed tortoises (*Testudo graeca*) were fed on canned dog food, fruit and vegetables several times each week. All animals used in this study appeared to be in excellent health. The animals were starved for 1 week prior to experimentation in order to avoid metabolic, acid–base and electrolyte changes associated with the digestion and absorption of food.

Metabolism studies

Net changes in whole-body CO_2 stores caused by changes in body temperature were assessed indirectly from measurements of the respiratory exchange ratio (RE). Rates of O_2 consumption ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$) in undisturbed animals were determined in a sealed recirculating apparatus housed inside a darkened temperature-controlled cabinet (Precision Scientific, model 815, ±1 °C). The details of this apparatus and the procedure followed have been described previously (Stinner *et al.* 1994*a*). Briefly, animals were allowed to adjust to the experimental apparatus for 24 h. The chamber was then sealed, and air was recirculated at

2.1–2.5 l min^{-1}. A portion of the recirculated air was continuously passed through a Drierite column and O_2 and CO_2 analyzers (Ametek S-3A and CD-3A; Ametek, Pittsburgh, PA, USA). The analyzers were calibrated with dry, CO_2-free room air and a 5 % CO_2 (balance N_2) certified gas mixture. The metabolism chamber was flushed with room air when the O_2 levels had fallen to no lower than 19 % and/or the CO_2 levels had risen to a maximum of 1.0 %, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were calculated using the equations for closed-system respirometry given by Stinner *et al.* (1994*a*). Net changes in whole-body CO_2 stores caused by changing the temperature were estimated from the differences between nonsteady-state and steady-state (control) RE values (see Stinner, 1982, for details of the integration procedure). The animals remained inside the metabolism apparatus for up to 3 weeks. They appeared to be in excellent condition at the end of these studies. The temperatures used and masses of each species are listed in Table 1.

Surgery and blood analyses

The dorsal aorta of 49 *C. constrictor* (mean mass 217±87 g) was occlusively cannulated (PE 50 or PE 10) as described by Stinner *et al.* (1996). After a recovery period of at least 72 h, the snakes were placed inside individual chambers within a darkened Precision temperature-controlled cabinet. The trailing ends of the cannulas extended outside the cabinets so that blood could be sampled without disturbing the snakes. Additional details of this apparatus and the blood sampling procedure (see below) are given in Stinner *et al.* (1996). The snakes were left undisturbed for 24 h at 30 °C and 20 °C and for 48 h at 10 °C before blood sampling began to allow them to achieve steady state with respect to whole-body CO_2 stores.

For P_{CO_2}, pH and $[CO_2]$ measurements, a train of four heparinized microhematocrit tubes was connected to a cannula and filled with a total volume of approximately 300 μl of blood. The capillary tube train was then immediately sealed and immersed in iced water. All blood sample analyses of P_{CO_2}, pH and $[CO_2]$ were completed within 1 h of collection. P_{CO_2} and pH were measured on blood from two and one of the microcapillary tubes, respectively, using Radiometer BMS-3 electrodes thermostatted to the snake's body temperature and PHM 72 acid–base analyzers (Radiometer, Copenhagen, Denmark). The P_{CO_2} electrode was calibrated using a 1.39 % CO_2 mixture supplied by mass-flow controllers (Tylan Corp., Torrance, CA, USA). The pH electrode was calibrated using Radiometer precision buffers. Plasma $[CO_2]$ was determined in duplicate on the fourth microcapillary tube using a Capni-con 3a CO_2 analyzer (Cameron Instrument Co., Port Aransas, TX, USA). pK_1' was calculated for each blood sample using the solubility coefficients given by Severinghaus (1965) (see Table 3). Snakes at 10 °C were sampled once each day for four consecutive days and snakes at 30 °C were sampled once each day for 2 days. Replicate values for each snake were then averaged before calculating group means ± S.D.

For analyses of ion concentrations, approximately 450 μl of

Table 1. *Oxygen consumption rates of animals kept at constant temperature for at least 24 h (temperature ≥30 °C) or for at least 48 h (temperature ≤15 °C)*

Species	Body mass (g)	Rate of oxygen consumption ($ml\,kg^{-1}\,h^{-1}$)					
		40 °C	35 °C	30 °C	15 °C	10 °C	5 °C
Savannah monitor (4) *Varanus exanthematicus*	654±100		75.9±3.7		8.4±2.0		
Mediterranean spur-thighed tortoise (5) *Testudo graeca*	642±138			32.1±1.7		4.0±2.6	
Coachwhip snake (4) *Masticophis flagellum*	488±86			54.1±11.3		5.2±0.9	
American alligator (4) *Alligator mississippiensis*	180±55			65.5±2.1		7.7±2.1	
Painted turtle (4) *Chrysemys picta*	509±88			66.9±6.4		5.2±1.5	
Snapping turtle (7) *Chelydra serpentina*	2,460±1,682			38.7±9.5		3.6±0.8	
Western diamondback rattlesnake (5) *Crotalus atrox*	1,256±1,103			48.1±18.2		6.0±2.7	
Desert iguana (5) *Dipsosaurus dorsalis*	47.2±11.8	142.5±18.2			8.6±1.6		
Bullfrog (6) *Rana catesbeiana*	608±65			72.9±6.4			3.7±0.8

Values in parentheses are the number of animals sampled.
Values for body mass and $\dot{V}_{O_2}$ are means ± S.D.

whole blood was collected directly into heparinized microcapillary tubes and immediately centrifuged at 2000 *g* for 5 min in an Eppendorf microcentrifuge. For lactate analysis, 100 μl of the plasma was immediately deproteinized by adding 200 μl of a cold 8 % perchloric acid solution. The mixture was centrifuged at 15 000 *g* for 10 min, and the supernatant was then frozen for no more than 1 week before plasma [lactate] analysis using an enzymatic test kit (Sigma no. 726-uv/826-uv) and Coleman Jr II model 6/20 spectrophotometer. The remaining plasma was frozen for future determination of ion concentrations.

Plasma [Na⁺] and [K⁺] were analyzed in duplicate using flame photometry (Coleman model 51; Bacharach, Inc., Pittsburgh, PA, USA). Plasma [Cl⁻] was measured (six replicates) using a Digital chloridometer (Buchler Instr., Lenexa, KS, USA). Total plasma osmolality was determined in duplicate using a model 5500 vapor pressure osmometer (Wescor, Inc, Logan, UT, USA). Means of the replicates were used to calculate group means ± S.D. for tabulation. Total plasma Ca²⁺, Mg²⁺, inorganic phosphate and protein concentrations were determined using Sigma diagnostic test kits (procedure nos 587, 595, 360-uv and 690, respectively; Sigma Diagnostics, St Louis, MO, USA). Calibration curves were constructed for [protein] from Sigma protein standards provided with the diagnostic kit, for [Ca²⁺] and [phosphate] from Sigma standards no. 360-11, and for [Mg²⁺] from Sigma standards no. 595-11. [SO₄²⁻] was determined using high-pressure liquid chromatography (Dionex HPLC 2010i; Dionex Corp., Sunnyvale, CA, USA).

Skeletal muscle

Skeletal muscle [CO₂] was measured using the technique described by Stinner *et al.* (1994*a*). Twelve black racer snakes (mean mass 158±93 g) and 42 bullfrogs (mean mass 353±84 g) were individually housed inside Precision temperature cabinets at 30 or 10 °C, as described above. The bullfrogs had access to water (10–15 cm deep) as well as dry areas. After at least 48 h at 30 °C and 96 h at 10 °C, animals were gently taken by hand from the temperature cabinets and decapitated. The spinal cord of the bullfrogs was immediately pithed, and their deep body temperature was taken using a Schultheis quick-reading cloacal thermometer. Duplicate pieces of skeletal muscle weighing 0.2–0.3 g were swiftly harvested and added to 0.7 ml of 0.1 mol l⁻¹ NaOH for soaking overnight in sealed bottles. [CO₂] of the solution was measured for each bottle in duplicate using a Capni-con 3a CO₂ analyzer. The four [CO₂] measurements for each muscle were then averaged before calculation of group means ± S.D.

Analysis of intracellular pH (pHi) in 19 black racer snakes (mean mass 170±88 g) was performed on muscle homogenates after metabolic inhibition according to the method of Pörtner *et al.* (1990). The snakes were housed inside Precision temperature cabinets for at least 48 h at 30 °C and 96 h at 10 °C. The cabinets were then briefly opened, and gauze soaked in isoflurane was dropped into the chambers. The chambers were then sealed for 15–20 min. The anesthetized snakes were removed and immediately decapitated. Four muscle samples (a combination of the transversospinalis, longissimus dorsi and iliolumbaris) were quickly taken from the mid-region of each

snake, freeze-clamped and stored in liquid N_2 for up to 2 h. Additional muscle samples were taken for electrolyte and water content determinations (see below). Frozen muscle samples were rapidly transferred to a porcelain mortar half-filled with liquid N_2 inside an ice chest and ground to a fine powder using a pre-cooled pestle. Grinding typically took 1 min. Approximately 150 mg of the powder was rapidly strained under the liquid N_2 atmosphere of the mortar and transferred to a 500 μl Eppendorf microcentrifuge tube containing 200 μl of ice-cold inhibitor solution (150 mmol l^{-1} potassium fluoride, 5 mmol l^{-1} nitrilotriacetic acid; pH approximately 7.00). The transferred powder was quickly covered with the inhibitor solution and mixed with a needle in order to release the N_2 bubbles from the thawed tissue powder. The microcentrifuge tube was then filled with the inhibitor solution and capped. The mixture was agitated for 3 s using a vortex mixer, centrifuged for 20 s at maximal speed in an Eppendorf centrifuge, and stored in ice for no more than 1 h. The supernatant pH was repeatedly measured using a Radiometer BMS-3 pH electrode thermostatted to the snake's body temperature and a PHM 72 acid–base analyzer. At least three pH measurements from each centrifuge tube were averaged, and the pH of the four muscle samples from each snake was averaged before calculation of group means ± S.D. In two snakes held at 10 °C and three snakes held at 30 °C, additional samples of the muscle powder were prepared and analyzed as described above, except that pH was measured at 30 °C and at 10 °C, respectively. The difference in supernatant pH between the 10 and 30 °C samples taken from the same ground muscle powder was used to determine the passive component of the temperature-dependent adjustment of pHi.

Muscle samples weighing approximately 0.25 g were analyzed for [Na$^+$], [K$^+$] and [Cl$^-$] according to the method of Boutilier *et al.* (1986). Four samples from each of 25 *C. constrictor* (mean mass 167±80 g) were placed in tared vials containing 4 ml of 0.1 mol l^{-1} HNO$_3$, weighed, and gently shaken for 24 h at room temperature. Dissolved [Na$^+$], [K$^+$] and [Cl$^-$] were determined as described above for plasma. Four additional muscle samples from 21 of the snakes were weighed and dried overnight to a constant mass at 60 °C.

Statistics

Comparisons between values were performed using paired *t*-tests, Student's *t*-tests and least-squares regression. Significance was assumed when $P<0.05$. Means ± S.D. are reported for group values.

Results

Metabolism and whole-body CO_2 stores

Metabolic rates ($\dot{V}_{O_2}$) for the nine species studied are listed in Table 1. These rates are for animals kept for at least 24 h at the specified body temperatures. They do not necessarily represent minimal resting values because the animals were occasionally active inside the metabolic chambers, especially at 30–40 °C. This probably accounts for the high thermal quotients ($Q_{10}>3$) evident in Table 1 (e.g. Q_{10} for *C. picta* of 3.6).

Cooling and heating the animals produced transient decreases and increases, respectively, in the respiratory exchange ratio. In eight of the nine species studied, steady-state RE values were not significantly affected by temperature
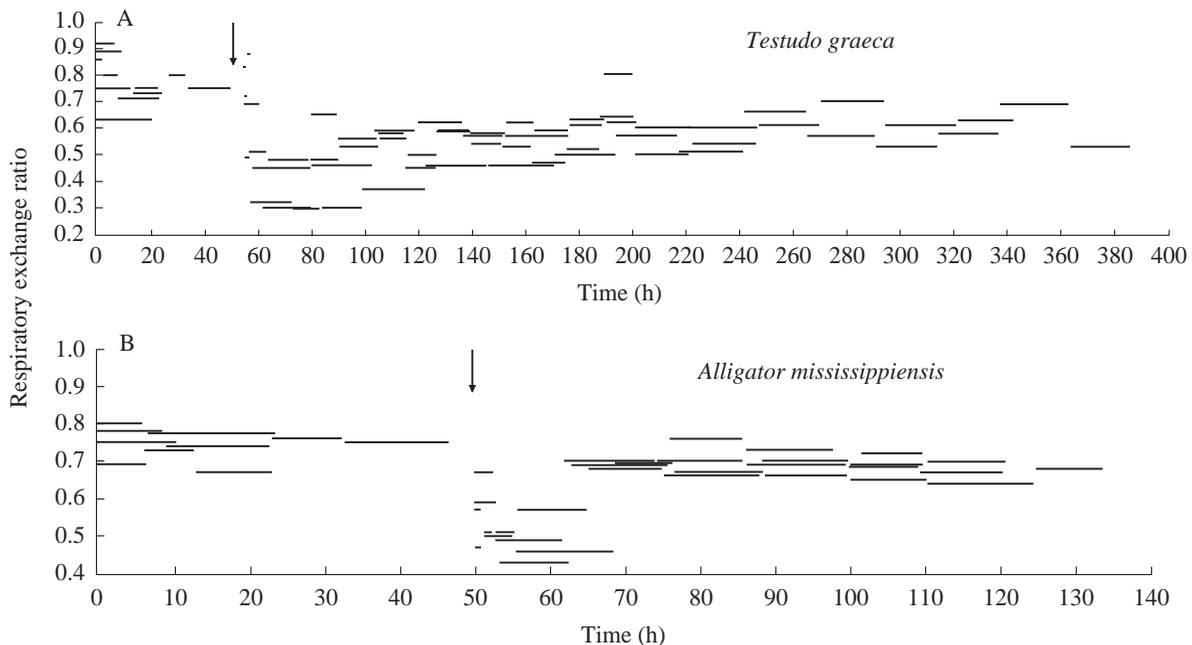

Fig. 1. Effect of lowering the temperature from 30 to 10 °C upon the respiratory exchange ratio in *Testudo graeca* (*N*=5) and *Alligator mississippiensis* (*N*=4). The length of the horizontal bars corresponds to the duration of individual measurement periods. The arrow at 50 h marks the time at which ambient temperature was lowered from 30 to 10 °C. The change in air temperature took 1.5–2 h to complete.

(P>0.05). However, in *T. graeca*, mean steady-state RE at 30 °C (0.78±0.08) was significantly higher than mean RE measured after 150 h at 10 °C (0.61±0.06), suggesting that the animals still had not returned to steady state. Estimates of changes in whole-body CO_2 stores in *T. graeca* at 10 °C were made using the 30 °C steady-state RE values. As expected, there were large differences among species, as illustrated by comparing *T. graeca* with *A. mississippiensis* (Figs 1, 2). Cooling *T. graeca* from 30 to 10 °C reduced the RE to approximately 0.3–0.5, which was then followed by a gradual increase in RE (Fig. 1A). Heating the tortoises back to 30 °C resulted in a sharp increase in RE to well above 1.0, and even above 2.0 in one tortoise, and then a return to steady state by approximately 12 h (Fig. 2A). The effect of temperature changes upon the RE was smaller in *A. mississippiensis*. Lowering body temperature from 30 to 10 °C lowered the RE

to approximately 0.6–0.7, and steady state returned within approximately 20 h (Fig. 1B). Raising the temperature back to 30 °C increased the mean RE to a little above 1.0, and RE then returned to steady state within 5 h (Fig. 2B).

Calculated changes in whole-body CO_2 stores are listed in Table 2, and range from -0.21 ± 0.10 mmol kg^{-1} °C^{-1} in *T. graeca* to -0.02 ± 0.02 mmol kg^{-1} °C^{-1} in *R. catesbeiana*. In addition to the nine species measured in this study, Table 2 contains $\Delta CO_2/\Delta T$ coefficients for four species taken from earlier reports and $\Delta pHpl/\Delta T$ coefficients available for 10 of the species. There is a significant correlation (r^2=0.87, P<0.01) between $\Delta CO_{2WB}/\Delta T$ and $\Delta pHpl/\Delta T$ (Fig. 3). Least-squares linear regression yields $\Delta CO_2/\Delta T=-0.18-8.24\Delta pHpl/\Delta T$, where $\Delta CO_2/\Delta T$ is in mmol kg^{-1} °C^{-1} and $\Delta pH/\Delta T$ is in pH units °C^{-1}. The equation predicts that a $\Delta pHpl/\Delta T=-0.022$ units °C^{-1} will result in no changes in whole-body CO_2 stores with changes in temperature (*x*-intercept of the line) and that a $\Delta pHpl/\Delta T=0.0$ units °C^{-1} will result in a whole-body change in CO_2 stores of -0.18 mmol kg^{-1} °C^{-1} (*y*-intercept of the line).
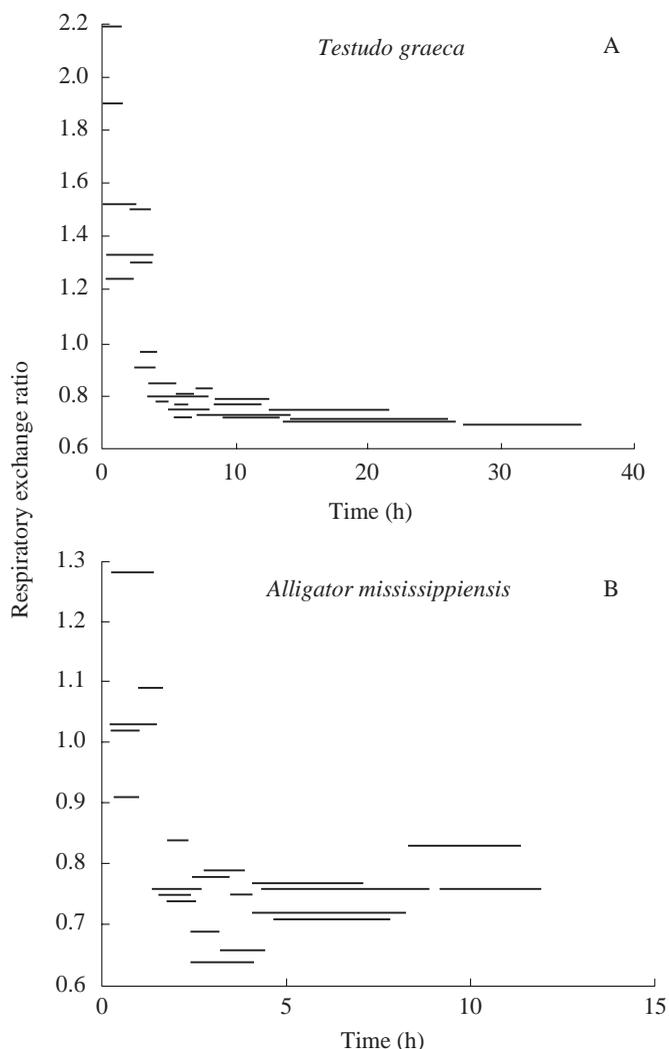

Fig. 2. Effect of raising the temperature from 10 back to 30 °C upon the respiratory exchange ratio in five *Testudo graeca* and four *Alligator mississippiensis*. Symbols as in Fig. 1. Air temperature was raised at the conclusion of the measurements at 10 °C (see Fig. 1) and took 1.5–2 h to reach 30 °C.

Table 2. *Changes in whole-body CO2 stores and plasma pH with changes in body temperature*

Species	$\Delta[CO_2]/\Delta T$ (mmol kg^{-1} °C^{-1})	$\Delta pH/\Delta T$ (units °C^{-1})
Mediterranean spur-thighed tortoise *Testudo graeca*	-0.21 ± 0.10 (5)	
Black racer snake *Coluber constrictor*	-0.18 ± 0.07 (4)	-0.0028
Savannah monitor *Varanus exanthematicus*	-0.12 ± 0.03 (4)	-0.005
Coachwhip snake *Masticophis flagellum*	-0.11 ± 0.07 (4)	
Slider turtle *Trachemys scripta*	-0.11 ± 0.04 (2)	-0.010
American alligator *Alligator mississippiensis*	-0.07 ± 0.04 (4)	-0.0125
Painted turtle *Chrysemys picta*	-0.07 ± 0.05 (4)	-0.011
Bullsnake *Pituophis catenifer*	-0.05 ± 0.01 (4)	-0.0157
Cane toad *Bufo marinus*	-0.05 ± 0.04 (6)	-0.015
Snapping turtle *Chelydra serpentina*	-0.05 ± 0.04 (7)	-0.018
Desert iguana *Dipsosaurus dorsalis*	-0.04 ± 0.04 (5)	-0.0145
Western diamondback rattlesnake *Crotalus atrox*	-0.04 ± 0.06 (5)	
Bullfrog *Rana catesbeiana*	-0.02 ± 0.02 (6)	-0.0206

$\Delta[CO_2]/\Delta T$ values are reported as means ± S.D.
Numbers in parentheses are the number of animals sampled.
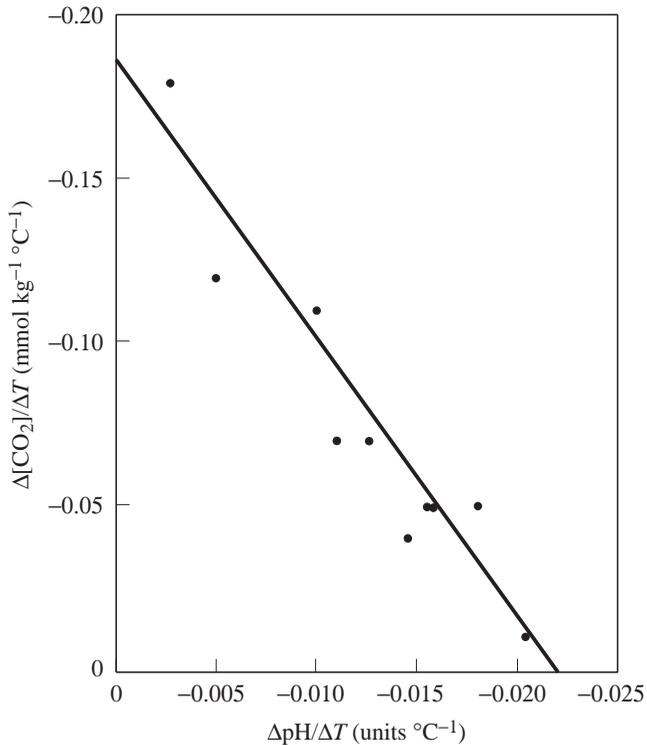$\Delta pH/\Delta T$ values were taken from Jackson (1986) and Table 3 for *Coluber constrictor*.

Fig. 3. Thermal dependence of whole-body CO_2 stores *versus* thermal dependence of plasma pH in 10 species of reptiles and amphibians. Values are taken from Table 2. The line represents the least-squares regression ($y=-0.18-8.24x$, $r^2=0.87$, $P<0.01$).

Plasma CO_2, pH and electrolytes

Although mean plasma pH in *C. constrictor* was higher by 0.056 units at 10 *versus* 30 °C (Table 3), the difference was not significant ($P<0.1$). P_{CO_2} was 7.1 mmHg lower, and total plasma [CO_2] was 3.2 mmol l^{-1} higher, at 10 *versus* 30 °C. The values for P_{CO_2} and pH at 30 °C listed in Table 3 also include measurements for five snakes from previous work in this laboratory (Stinner and Wardle, 1988). Plasma [HCO_3^-] and dissolved [CO_2] were estimated from mean total [CO_2] and mean P_{CO_2} using the solubility coefficients listed in Table 3. Between 30 and 10 °C, [HCO_3^-] increased by 3.1 mmol l^{-1} and dissolved [CO_2] remained virtually unchanged. pK_1' was slightly but significantly higher (0.111 units) at 10 °C.

Inspection of Table 4 reveals that plasma electrolyte concentrations were largely unaffected by cooling the snakes from 30 to 10 °C. Only [phosphate] changed significantly, increasing by 0.7 mmol l^{-1}. However, at 20 °C, the concentrations of several electrolytes (Na^+, K^+, Mg^{2+} and phosphate) were significantly different from those at 10 and/or 30 °C. The higher [Na^+] at 20 °C appeared to have been accompanied by a higher [Cl^-]. There was no effect of temperature upon [protein]. Protein charge (in mequiv l^{-1}) was calculated as (the sum of the concentrations of Na^+, K^+, $2\times Ca^{2+}$ and $2\times Mg^{2+}$) minus (the sum of the concentrations of Cl^-, lactate, $2\times SO_4^{2-}$, $2\times HPO_4^{2-}$ and $H_2PO_4^-$). The concentrations of HPO_4^{2-} and $H_2PO_4^-$ were estimated using the Henderson–Hasselbalch equation, mean pH values from

Table 3. *Steady-state values for systemic arterial plasma pH, carbon dioxide and oxygen in the snake* Coluber constrictor *at 30 and 10 °C*

Variable	30 °C	10 °C
pH	7.531±0.042 (10)	7.587±0.079 (8)
P_{CO_2} (mmHg)	21.5±2.5* (10)	14.4±2.1 (8)
Total [CO_2] (mmol l^{-1})	22.1±2.8* (26)	25.3±2.0 (17)
α_{CO_2} (mmol l^{-1} mmHg^{-1})	0.0356	0.0629
pK_1'	6.069±0.016* (5)	6.180±0.023 (6)
[HCO_3^-] (mmol l^{-1})	21.3	24.4
Dissolved [CO_2] (mmol l^{-1})	0.8	0.9
P_{O_2} (mmHg)	75	17
α_{O_2} (mmol l^{-1} mmHg^{-1})	1.4×10^{-3}	1.99×10^{-3}
Dissolved [O_2] (mmol l^{-1})	0.10	0.03

pH, P_{CO_2}, total [CO_2] and pK_1', are reported as means ± S.D.; numbers in parentheses are the number of animals used; *indicates values significantly different ($P<0.05$) from those at 10 °C.

Dissolved [CO_2] and [HCO_3^-] were calculated from the mean values of total [CO_2] and P_{CO_2}.

The solubility coefficients (α) for CO_2 and O_2 were taken from Severinghaus (1965) and Christoforides *et al.* (1969), respectively.

P_{O_2} values were predicted from an equation in Stinner and Wardle (1988).

1 mmHg=0.1333 kPa.

Table 3 and a pK of 6.791 at 37 °C (Siggaard-Anderson, 1974). The van't Hoff equation [$pK=(\Delta H°/4.576)(1/T_2-1/T_1)$] was used to correct pK to 30 and 10 °C, where $\Delta H°=982$ cal mol^{-1} (Edsall and Wyman, 1958). Protein charge decreased from −0.48 mequiv g^{-1} protein at 30 °C to −0.38 mequiv g^{-1} protein at 10 °C (Fig. 4), largely because of the increases in total [CO_2] (carbonic acid) and inorganic [phosphate].

Skeletal muscle

Between 30 and 10 °C, [CO_2] increased significantly by approximately 2.5 mmol kg^{-1} in the three muscles studied in *C. constrictor* (Table 5). This contrasts sharply with the pattern found in *R. catesbeiana* (Table 6): in 14 skeletal muscles, only one exhibited a significant increase in [CO_2] with cooling. Intracellular muscle pH in *C. constrictor* increased significantly with cooling ($\Delta pHi/\Delta T=-0.009$ units °C^{-1}; Table 7). The passive component was $\Delta pHi/\Delta T=-0.013\pm0.001$ units °C^{-1}. Muscle [Na^+] and [Cl^-] each decreased significantly by approximately 12 mequiv l^{-1}, but there were no significant differences in [K^+] or in percentage water content in snakes at 30 and 10 °C (Table 7).

Discussion

The temperature-dependence of whole-body CO_2 stores is

Table 4. *Steady-state levels of systemic arterial plasma electrolytes in the snake* Coluber constrictor

Variable	30 °C	20 °C	10 °C
[Na⁺] (mmol l⁻¹)	154.5±3.5† (18)	158.2±5.4* (13)	153.9±5.9 (20)
[K⁺] (mmol l⁻¹)	3.7±0.6† (13)	3.1±0.3* (13)	3.9±0.5 (13)
[Ca²⁺] (mmol l⁻¹)	2.9±0.4 (26)	2.7±0.3 (13)	2.8±0.3 (13)
[Mg²⁺] (mmol l⁻¹)	1.2±0.2† (11)	1.5±0.3* (13)	1.2±0.35 (13)
[Cl⁻] (mmol l⁻¹)	114.3±4.0 (9)	118.3±4.7 (9)	114.7±4.5 (9)
[Phosphate] (mmol l⁻¹)	1.2±0.3*·† (13)	1.6±0.4* (13)	1.9±0.4 (13)
[Protein] (g l⁻¹)	47.7±10.9 (29)	45.5±11.1 (13)	44.8±9.0 (17)
[SO₄²⁻] (mmol l⁻¹)	2.3±0.6 (6)	2.8±1.2 (8)	2.6±0.6 (7)
[Lactate] (mmol l⁻¹)	1.0±0.5 (13)		0.8±0.2 (6)
Osmolality (mmol kg⁻¹)	307±22 (14)		

Values are means ± S.D., numbers in parentheses are the number of animals sampled.

Comparisons of values were performed using paired *t*-tests or *t*-tests for two means, and significance was assumed when *P*<0.05; * indicates values that are significantly different from those at 10 °C; † indicates values that are significantly different from those at 20 °C.
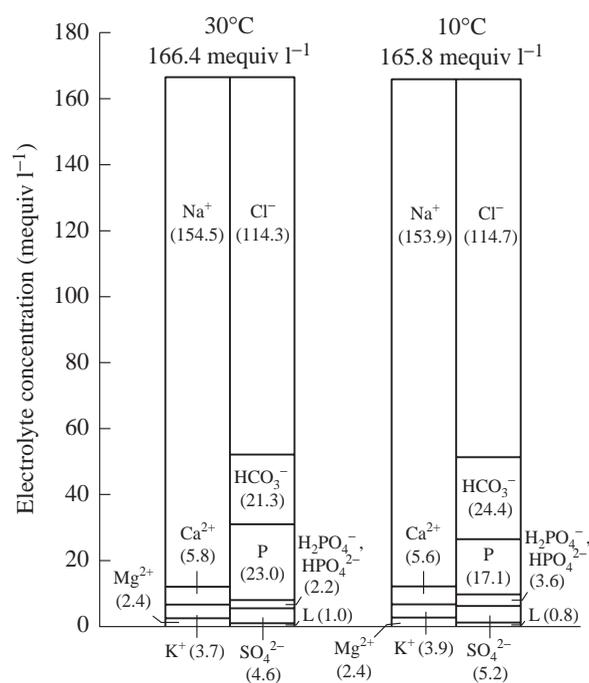

Fig. 4. Plasma electrolyte concentrations (in mequiv l⁻¹) in the snake *Coluber constrictor* kept at 30 °C for 24 h or more and at 10 °C for 48 h or more. Values were calculated from the concentrations (in mmol l⁻¹) listed in Tables 3 and 4. Note that the increase in [HCO₃⁻] between 30 and 10 °C was accompanied by a reduction in net negative protein charge, indicating titration of plasma proteins by carbonic acid. See text for additional explanation. L, lactate; P, protein.

Table 5. *Steady-state skeletal muscle CO₂ levels in black racer snakes* Coluber constrictor

	[CO₂] (mmol kg⁻¹)	
Muscle	30 °C	10 °C
Transversospinalis	11.2±1.5	13.9±1.4*
Longissimus dorsi	10.5±1.3	13.7±1.9*
Iliolumbaris	10.3±2.1	11.9±0.9*

Values are reported as means ± S.D.
Six snakes were sampled at each temperature.
*Indicates a value significantly different from that at 30 °C.

Table 6. *Steady-state skeletal muscle CO₂ level as a function of body temperature in bullfrogs* Rana catesbeiana

	[CO₂] (mmol l⁻¹)	
Muscle	28.1±0.6 °C	9.5±1.5 °C
Submaxillary	15.4±1.2	16.3±1.5
Rectus abdominis	12.8±1.2*	14.9±1.5
Oblique (external plus internal)	13.2±2.0	14.3±1.1
Coccygeo-iliacus	10.1±1.0	10.2±1.3
Longissimus dorsi	11.1±1.0	10.7±1.2
Iliolumbaris	12.8±1.6	12.7±1.7
Anconeus	12.5±1.4	13.0±1.2
Extensor carpi ulnaris	13.1±1.9	13.7±1.5
Pectoralis	12.1±1.8	11.9±1.3
Triceps femoris	13.8±3.7	13.0±2.6
Semimembranosus	13.1±3.5	13.2±2.6
Gracilis major	13.2±3.7	13.3±2.6
Sartorius	13.5±3.9	14.6±2.9
Gastrocnemius	13.3±3.6	13.1±2.9

Values are reported as means ± S.D.; each muscle was sampled from seven animals at each temperature and no more than five muscles were sampled from each animal.

*Indicates values that are significantly different (*P*<0.05) from those at 9.5 °C.

strongly correlated with changes in plasma pH per unit change in temperature in reptiles and amphibians (Table 2; Fig. 3): species exhibiting relatively small absolute ΔpH/Δ*T* coefficients experience greater changes in whole-body CO₂ stores. An explanation for this relationship is that, with differences between Δp*K* and ΔpH, there is titration of nonbicarbonate buffers by CO₂ (see Introduction). Assuming that the ΔpHpl/Δ*T* coefficients correspond to whole-body ΔpH/Δ*T* coefficients, as reported by Bickler (1982) and by Cameron and Kormanik (1982), the absolute value of the slope in Fig. 3 (8.24 mmol kg⁻¹ pH unit⁻¹) then represents a whole-body nonbicarbonate buffer value and

Table 7. *Steady-state values for skeletal muscle pH, electrolyte levels and water content in the black racer snake* Coluber constrictor

Variable	30 °C	10 °C
pH		7.189±0.049* (10)
[Na$^+$] (mmol kg^{-1})	50.2±8.2 (13)	37.8±6.4* (12)
[K$^+$] (mmol kg^{-1})	77.4±8.1 (13)	80.0±7.1 (12)
[Cl$^-$] (mmol kg^{-1})	36.8±7.6 (13)	25.1±4.6* (12)
Water (%)	78.7±1.7 (9)	78.2±2.0 (12)

Values are means ± S.D.; numbers in parentheses are the number of animals sampled.

Each muscle sample was a combination of the transversospinalis, longissimus dorsi and iliolumbaris.

*Indicates values that are significantly different (*P*<0.05) from those at 30 °C.

the *x*-intercept (−0.022 units °C^{-1}) represents the change in the mean whole-body ionization constant. Hence, $\Delta CO_{2WB}/\Delta T=8.24(-0.022-\Delta Hpl/\Delta T)$, which fits the general expression $\Delta[HCO_3^-]=\beta_{NB}(\Delta pK-\Delta pH)$, but with the addition of dissolved CO_2 (the effect of which is minor; see Table 3). While this explanation is speculative, it is interesting to note that the mean temperature coefficient for biological imidazole-like buffers is $\Delta pK/\Delta T\approx-0.021$ units °C^{-1} (Edsall and Wyman, 1958), which is very close to the −0.022 units °C^{-1} obtained in the present study. This implies that the postulated nonbicarbonate buffering is dominated by proteins (as opposed to phosphates, where $\Delta pK/\Delta T=-0.0025$ units °C^{-1}; Edsall and Wyman, 1958).

Increases in whole-body CO_2 stores with cooling in reptiles and amphibians obviously represents CO_2 (and hence carbonic acid) retention. However, within a given tissue compartment (e.g. extracellular fluid), a rise in [HCO$_3^-$] may not be accompanied by increases in [H$^+$] because the protons can be removed by ion-exchange mechanisms (e.g. by the kidney, stomach and skeletal muscle cells). If this were to occur, then there must be adjustments in the levels of other ions in order to preserve electroneutrality with the rise in [HCO$_3^-$]. The data contained in Tables 3 and 4 and Fig. 4 for plasma in *C. constrictor* clearly show that ion-exchange mechanisms were not involved. There were no changes in strong ion levels (Na$^+$, K$^+$, Mg^{2+}, Cl$^-$, Ca^{2+} SO$_4^{2-}$ and lactate) or protein concentration, and phosphate concentration (a weak acid) actually increased with cooling. Thus, the 3 mmol l^{-1} increase in [HCO$_3^-$] was accompanied by an equivalent increase in [H$^+$]pl that was buffered by the plasma proteins. The rise in phosphate concentration also titrated the plasma proteins. Consequently, net protein charge fell by 21 %, from −0.48 mequiv g^{-1} protein at 30 °C to −0.38 mequiv g^{-1} protein at 10 °C. Plasma protein charge at 37 °C and pH 7.4 in humans is −0.6 mequiv g^{-1} protein (Figge *et al.* 1991*b*). Plasma strong ion levels have been shown to be independent of temperature in other air-breathing ectotherm species (Bickler, 1984; Herbert and Jackson, 1985; Douse and Mitchell, 1991). The data in Table 3 also show that the change in [CO$_2$]pl

cannot be explained by simple increases in the solubility coefficients (α_{O_2}, α_{CO_2}) with cooling. Depending upon the *in vivo* steady-state P_{O_2} and P_{CO_2}, the increase in α_{O_2} and α_{CO_2} could potentially result in an accumulation of dissolved O_2 and dissolved CO_2. This would have the effect of adding to the pulmonary O_2 uptake and reducing the pulmonary CO_2 elimination with cooling. Hence, the depression in RE and elevation in CO_2 stores could be interpreted on the basis of the solubilities of the respiratory gases. However, inspection of Table 3 reveals that between 30 and 10 °C there was no change in dissolved [CO$_2$]pl and dissolved [O$_2$]pl decreased, because of the reductions in P_{O_2} and P_{CO_2}.

In previous reports, increases in extracellular CO_2 stores at lower temperatures were attributed to ion-exchange mechanisms generating HCO$_3^-$ (which is equivalent to removal of H$^+$) (Stinner and Wardle, 1988; Stinner *et al.* 1994*a*, 1996). This view now appears to be incorrect. The suggestion of ion exchange was based upon the lengthy time courses for accumulation of CO_2 in *C. constrictor* (35 h to reach steady state when cooled from 30 to 10 °C, and 60 h to reach steady state when cooled from 30 to 5 °C) and the relatively large changes in [CO$_2$]pl which could not be accounted for by estimated extracellular fluid nonbicarbonate buffering (Stinner *et al.* 1996). However, the estimates of [HCO$_3^-$] generated from extracellular fluid nonbicarbonate buffers did not include $\Delta pK/\Delta T$, and it was assumed that interstitial fluid is free of proteins. This assumption is not consistent with the high protein concentrations reported for interstitial fluid and lymph in ectotherms (30–100 % of plasma protein concentration; Hargens *et al.* 1974; Hillman *et al.* 1987). In mammals, the interstitial colloid osmotic pressure in loose connective tissue is approximately 50 % of the plasma colloid osmotic pressure (Aukland and Reed, 1993). Thus, the estimated extracellular fluid β_{NB} was too low. Extracellular β_{NB} for *C. constrictor* can be obtained from the [HCO$_3^-$]pl and pHpl values listed in Table 3 and an assumed $\Delta pK/\Delta T$ of −0.022 units °C^{-1}, where $\beta_{NB}=\Delta[HCO_3^-]/(\Delta pK-\Delta pH)$. The resulting calculation yields an extracellular fluid β_{NB} of 8.1 mmol l^{-1} pH unit^{-1}, which is close to the value of 6.2 mmol l^{-1} pH unit^{-1} measured in the toad *B. marinus* (Boutilier *et al.* 1979). Hence, we conclude that nonbicarbonate buffering and $\Delta pKpr/\Delta T$ account for the rise in [HCO$_3^-$] with cooling in *C. constrictor*.

A similar pattern for CO_2 was found in skeletal muscle tissue of *C. constrictor* cooled from 30 to 10 °C. [CO$_2$] increased by approximately 2.5 mmol kg^{-1}, which is close to that measured in plasma. Skeletal muscle [Na$^+$] and [Cl$^-$] each decreased significantly by approximately 12 mequiv l^{-1}, but the small increase in [K$^+$] was not significant (*P*>0.4; Table 7). These changes in whole-muscle electrolyte levels suggest that there was a decrease in the fraction of extracellular fluid volume relative to intracellular fluid volume with cooling, as reported for *B. marinus* (Stinner *et al.* 1994*a*). Extracellular fluid, relative to intracellular fluid, is high in Na$^+$ and Cl$^-$ but low in K$^+$. Hence, a decrease in the fraction of extracellular fluid will produce a decrease in whole-muscle [Na$^+$] and [Cl$^-$], and an

increase in whole-muscle [K$^+$]. We conclude that the increase in [CO$_2$] within the muscle resulted from an increase in [H$_2$CO$_3$] rather than from active ion exchange across the sarcolemma. The absolute value of the skeletal muscle ΔpHi/ΔT coefficient was greater than the ΔpHpl/ΔT coefficient (0.009 *versus* 0.003 units °C^{-1}, respectively). In terms of our protein titration hypothesis, the higher absolute ΔpH/ΔT coefficient in skeletal muscle resulted from its higher β_{NB} (e.g. 19.8 mmol kg^{-1} fresh mass pH unit^{-1} in *B. marinus* skeletal muscle; Pörtner, 1990) compared with β_{NB} for extracellular fluid (approximately 8 mmol kg^{-1} pH unit^{-1} in *C. constrictor*). This higher buffering capacity is, however, partly offset by a lower absolute ΔpK/ΔT coefficient. In *C. constrictor*, the passive ΔpH/ΔT coefficient (and hence ΔpK/ΔT) is -0.013 pH units °C^{-1} compared with a plasma passive ΔpH/ΔT coefficient of approximately -0.019 pH units °C^{-1} (Reeves, 1976). The result in skeletal muscle, relative to extracellular fluid, is that when cooled the passive component elevates pHi less but then the superimposed CO$_2$ retention lowers pHi very little so that the net ΔpHi/ΔT coefficient is greater than the ΔpHpl/ΔT coefficient.

In the discussion above, Fig. 3 was viewed as a whole-body nonbicarbonate buffer curve. Support for this conclusion in *C. constrictor* comes from the nearly identical value for the change in plasma [CO$_2$] per unit change in plasma pH (-8.3 mmol l^{-1} pH^{-1}; Table 3) and the Δ[CO$_2$]$_{WB}$/ΔpHpl coefficient in Fig. 3 (-8.24 mmol l^{-1} pH unit^{-1}). The Δ[CO$_2$]$_{pl}$/ΔT in *C. constrictor* was not associated with ion exchange, but was instead apparently the result of the change in pK of the protein buffers (physicochemical buffering) and the change in P_{CO_2} (ventilatory regulation). The same was also apparently true for the intracellular fluid of skeletal muscle. When cooled, reptiles and amphibians increase their air convection requirement and thus lower blood P_{CO_2} (Ultsch and Jackson, 1996). CO$_2$ is lipid-soluble, it readily diffuses across cell membranes, and intracellular fluid P_{CO_2} decreases in a parallel fashion. The Δ[CO$_2$]/ΔT coefficients of skeletal muscle in *C. constrictor* and in *R. catesbeiana* parallel the Δ[CO$_2$]/ΔT coefficients of plasma ([CO$_2$]$_{pl}$ is independent of temperature in *R. catesbeiana*; Reeves, 1972). Also, changes in extracellular pH appear to correspond to changes in whole-body pH (Bickler, 1982; Cameron and Kormanik, 1982). Consequently, it is reasonable that the Δ[CO]$_{2WB}$/ΔpHpl coefficient of Fig. 3 is similar in magnitude to the Δ[CO$_2$]pl/ΔpHpl coefficient measured in *C. constrictor* (Table 3). Alternatively, the close agreement between the slope of Fig. 3 and β_{NB} estimated from Table 3 may simply be coincidental.

The absence of changes in strong ion levels refers to steady-state conditions, i.e. after the change in whole-body CO$_2$ stores has been completed. Presumably, the ΔpK occurs virtually instantaneously with the ΔT so that, during the nonsteady-state period, there are transient adjustments in plasma and intracellular fluid strong ion levels, such as the rise in plasma lactate concentration observed in *C. constrictor* during cooling (Stinner *et al.* 1996). However, when comparing steady-state

conditions, the ΔpK/ΔT accounts for CO$_2$ retention in plasma and cells, despite hyperventilation and a lowering of P_{CO_2} with cooling. According to this hypothesis, individual differences in the Δ[CO$_2$]/ΔT coefficients among tissues, as found in *B. marinus* (Stinner *et al.* 1994*a*), result from differences in β_{NB} and/or differences in (ΔpK$-\Delta$pH).

In summary, on the basis of the findings of this study, titration of proteins with cooling occurs because the rising air convection requirement does not lower P_{CO_2} sufficiently to maintain a constant (ΔpK$-\Delta$pH)/ΔT, i.e $-\Delta$pK is greater than $-\Delta$pH. *C. constrictor* at 10 °C compared with 30 °C maintains a lower P_{CO_2} by hyperventilating, but [CO$_2$] is higher (Tables 3, 5) because of the thermally induced upward shift in pK. This protein titration hypothesis does not support general theories of acid–base regulation in ectotherms that are based upon preserving the same net protein charge irrespective of body temperature (i.e. constant relative alkalinity, alphastat and *z*-stat; Austin *et al.* 1927; Howell *et al.* 1970; Reeves, 1972; Cameron, 1989). In the present study, only bullfrogs appear to conform closely to the alphastat model, which predicts constant tissue CO$_2$ content. What effect, if any, CO$_2$ retention and changes in protein charge have upon enzyme activities and the physiology of these animals is unknown (Heisler, 1986*b*). There is a growing body of evidence that CO$_2$ retention (acidification) and low P_{O_2} (see Table 3) are potent metabolic inhibitors in ectotherms (Boutilier *et al.* 1997; Guppy *et al.* 1994). Furthermore, mammalian hibernators exhibit a transient reduction in RE when entering torpor and a transient elevation in RE during arousal (Nestler, 1990). *C. constrictor* is native to all 48 contiguous North American states and extends into southern Canada and northern Mexico. Black racer snakes have a wide temperature range and are even somewhat active in hibernacula below 5 °C (Stinner, 1987; Sexton and Hunt, 1980). By contrast, bullfrogs (*R. catesbeiana*) show minimal changes in whole-body CO$_2$ stores, but they also occupy a wide range in North America and are active at near-freezing temperatures in ice-covered ponds during winter (Stinner *et al.* 1994*b*). The possible relationship between CO$_2$ retention, protein charge and behavior is an important topic that deserves careful study in the future.

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