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ACID–BASE REGULATION IN TADPOLES OF *RANA CATESBEIANA* EXPOSED TO ENVIRONMENTAL HYPERCAPNIA

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Summary

Tadpoles of *Rana catesbeiana* were exposed to different levels of environmental hypercapnia. The acid–base regulatory response differed from that in adult amphibians in showing a high degree of pH compensation in the extracellular fluid (65–85 %) and complete compensation in the intracellular fluid (tail muscle and liver) within 24 h. Hypercapnia induced a massive transfer of HCO_3^- equivalents and Ca^{2+} from the tadpoles to the environment, which lasted some 4–6 h. Bicarbonate accumulated in the body fluids came mainly from internal buffer sources (probably $CaCO_3$ in lime sacs and/or skin deposits). It is suggested that the large bicarbonate efflux from the animal is a consequence of the dissolution of $CaCO_3$ stores and the delayed adjustment of bicarbonate-retaining mechanisms. Re-exposure of tadpoles to hypercapnia after 1–3 weeks of normocapnic recovery only affected transepithelial fluxes of acid–base equivalents marginally, suggesting that mobilisable $CaCO_3$ stores were depleted during the first exposure to hypercapnia and that they were not refilled. The $CaCO_3$ stores may normally be mobilised during the slowly developing internal hypercapnia that occurs during metamorphosis.

Key words: acid–base regulation, hypercapnia, pH compensation, fixed buffer resources, transfer of acid–base equivalents, *Rana catesbeiana*.

Introduction

In adult amphibians, exposure to environmental hypercapnia generally leads only to a partial (0–30 %) extracellular pH compensation (Boutilier *et al.* 1992). Aquatic species such as *Xenopus laevis* (Toews and Boutilier, 1986), *Siren lacertina*, *Amphiuma means* (Heisler *et al.* 1982) and *Necturus maculosus* (Stiffler *et al.* 1983) do not elevate plasma [HCO_3^-] during hypercapnia. *Siren lacertina* and *Amphiuma means* do, however, compensate the hypercapnic acidosis in the intracellular compartments to a high degree (Heisler *et al.* 1982). The priority for intracellular pH regulation makes sense since it is the intracellular space that contains pH-sensitive enzymes.

Tadpoles experience a slowly developing internal hypercapnia during their metamorphosis when a transition from water- to air-breathing occurs. The potential respiratory acidosis associated with the rise in P_{CO_2} is almost completely compensated in the extracellular fluid (Just *et al.* 1973). Intra- and extracellular acid–base regulation in tadpoles during environmental hypercapnia has not been examined but is of interest in view of the large internal pool of carbonates in the paravertebral lime sacs (Pilkington and Simkiss, 1966) and the skin (Baldwin and Bentley, 1980), which provides tadpoles with the opportunity for mobilising buffer base. The main objectives of the present study were to investigate the acid–base balance in selected body compartments of tadpoles during environmental hypercapnia and to estimate the contribution of body $CaCO_3$ reserves to acid–base compensation.

Materials and methods

Experimental animals

Field-collected tadpoles of *Rana catesbeiana* (Shaw) were air-shipped from North Carolina Biological Supply Company, USA, in July (mass range of individuals 4–13 g, most weighed between 6 and 8 g) and in November (mass range 2.5–4.5 g). All tadpoles were acclimated for at least 7 days at 18–20 °C to aerated tap water. Some of the tadpoles obtained in July were acclimated to Copenhagen tap water with the following ionic composition (mmol l^{-1}): [Ca^{2+}]=3.2, [Na^+]=1.7, [K^+]=0.16, [Cl^-]=3.5 and [HCO_3^-]=4.0. Other tadpoles obtained in July and all tadpoles obtained in November were acclimated to Odense tap water with the same ionic composition except that [Cl^-]=1.7 mmol l^{-1} and [HCO_3^-]=5.4 mmol l^{-1}. Experiments were performed in the same type of water as used for acclimation. Tadpoles were fed boiled spinach until 2 days before experimentation. All tadpoles were in developmental stages XIV–XIX as defined by Taylor and Kollros (1946).

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Experimental protocol

Tadpoles were randomly selected to form six experimental groups. Individual tadpoles were placed in a chamber with 1.5 l of aerated normocapnic tap water at 20 °C. After 4 h, the animals were subjected to different levels of hypercapnia by bubbling humidified CO_2/air gas mixtures, delivered by Wösthoff (Bochum, Germany) gas-mixing pumps, through the water. The six experimental treatments were as follows: (i) 24 h of exposure to normoxic-normocapnic water; (ii) 2 h of exposure to 2 % CO_2; (iii) 24 h at 2 % CO_2; (iv) 2 h at 5 % CO_2; (v) 24 h at 5 % CO_2; and (vi) 48 h at 5 % CO_2 followed by 24 h at 8 % CO_2. At the end of the exposures, the tadpoles were anaesthetised by adding a $NaHCO_3$-buffered (pH 7) solution of MS 222 (final concentration 0.25 g l^{-1}) to the experimental chamber. Following complete anaesthesia (3–8 min), the tail muscle was quickly cut off, freeze-clamped and stored in liquid nitrogen for later analysis of acid–base parameters. To minimise sampling time, no effort was made to remove the tail skin (except for the dorsal and ventral fins, which could be easily removed after freeze-clamping). Because of the small size of the tadpoles, it was difficult to obtain blood by heart puncture. Instead, after cutting the tail, a blood sample was taken from the dorsal aorta into a heparinized capillary tube. To overcome occasional problems with clotting, each capillary was mounted in an ice-cooled cylinder. Approximately 50–125 µl of blood was obtained from tadpoles weighing more than 4 g, and the sampling method allowed tail tissue and blood samples to be collected in under 30 s. The liver was removed from some of the tadpoles subjected to treatments i, ii and iii and freeze-clamped for later measurement of intracellular pH.

In a separate experimental series, tadpoles were exposed for 24 h to normoxic-normocapnic or hypercapnic (5 % CO_2) conditions. Pieces of tail muscle were weighed, placed on a small meshed net mounted in the bottom of a test tube, centrifuged for 4 min at 1000 ***g*** and then reweighed, allowing estimation of extracellular fluid (interstitial fluid + blood) volume according to the simple method developed by Ling and Walton (1975). The spun-off extracellular fluid was collected for determination of calcium concentration. The centrifuged muscle tissue was dried to a constant mass (24 h at 95 °C) and reweighed to evaluate fractional tissue water content (F_{tw}) and fractional extracellular water content (Q). For determination of intracellular Ca^{2+} concentration, the dried muscle samples were digested in 65 % HNO_3, bleached in H_2O_2, and finally redissolved in 0.2 % HNO_3.

In a further experimental series, the net fluxes of acid–base equivalents and Ca^{2+} between the animals and the environment were determined in tadpoles exposed to (i) 28 h of normocapnia and (ii) 4 h of normocapnia followed by 24 h at 5 % CO_2. Transepithelial transfer of acid–base equivalents was measured using a ΔHCO_3^- system (Heisler, 1989). Four tadpoles with a total mass of 13–16 g were placed in the experimental chamber. The total water volume was 550 ml, resulting in a water/animal volume ratio of 33–42, which is higher than the recommended ratio of 6–15 (Heisler, 1989), but was found necessary in order to avoid undue accumulation of ammonia. The water was pumped from the chamber through the ΔHCO_3^- system, consisting of three glass columns in which the water was equilibrated to 1 % CO_2, and then to a pH electrode chamber. Before being returned to the animal chamber, the water passed through a fourth glass column, where it was equilibrated with the same gas mixture as used in the animal chamber. The whole system, including the electrode chamber, was thermostatted at 20±0.5 °C. Changes in water bicarbonate concentration were calculated from P_{CO_2}, the measured pH and values of the solubility coefficient for CO_2 (α_{CO_2}) and pK′ adopted from Maas *et al.* (1984). The reliability of calculated $\Delta[HCO_3^-]$ values was verified by adding known amounts of $NaHCO_3$ to the system. For determination of net fluxes of ammonia and Ca^{2+}, water samples were collected at times −3, 0 (onset of hypercapnia), 3 and 24 h. After having been exposed to hypercapnia for 24 h, the tadpoles were allowed to recover for 1–3 weeks in normocapnic water. The animals were then re-exposed to hypercapnia (5 % CO_2), and the transepithelial fluxes of acid–base equivalents and Ca^{2+} were again measured.

Analytical procedures

Blood pH was measured by sucking blood directly from the sampling capillary tube into the capillary pH electrode of a Radiometer (Copenhagen, Denmark) BMS 3 electrode assembly thermostatted at 20 °C. Total CO_2 (C_{CO_2}) in true plasma and whole blood was determined using the Cameron (1971) method. In some tadpoles, only whole-blood C_{CO_2} measurements were performed. To present all the data in Davenport diagrams, whole-blood C_{CO_2} values were converted to plasma values using the following regression equation: blood C_{CO_2}/plasma C_{CO_2}=–0.0792pH+1.464 (P<0.02, r^2=0.3), which is based on measurements of pH and C_{CO_2} in the whole blood and true plasma of 30 tadpoles. Blood P_{CO_2} and $[HCO_3^-]$ were calculated using the Henderson–Hasselbalch equation. The plasma pK′ and CO_2 solubility coefficient (α_{CO_2}) were calculated using the formulae of Heisler (1989).

Intracellular pH (pHi), C_{CO_2} in tail muscle tissue and pHi in liver were determined according to the method of Pörtner *et al*. (1990). Metabolism was arrested using a medium containing 120 mmol l^{-1} potassium fluoride and 4.6 mmol l^{-1} nitrilotriacetic acid. The homogenate supernatant was analysed for pHi and C_{CO_2} using the same apparatus as for blood. C_{CO_2} in cell water was calculated with the formulae derived by Pörtner *et al.* (1990). These authors used a Donnan factor of 1.05 adopted from mammals but, because of the low plasma protein content of tadpoles (Herner and Frieden, 1960), it is here approximated as 1.03. Intracellular P_{CO_2} and $[HCO_3^-]$ were calculated as described for plasma. The values for cell water pK′ and α_{CO_2} were determined according to the formulae of Heisler (1989).

Calcium concentrations in tissue fluids and ambient water were determined by atomic absorption spectrophotometry

(Perkin-Elmer 2380). Total ammonia content was determined by the phenol–hypochlorite method of Solorzano (1969).

The results are presented as means ± S.E.M. Differences between groups were assessed using two-way analysis of variance (ANOVA) followed by the Tukey test. Differences were accepted to be significant at $P<0.05$.

Results

Acid–base parameters in tadpoles

Exposure to 2 % or 5 % environmental CO_2 for 2 h caused a rise in blood P_{CO_2} from 3.3 to 18.1 and 35.7 mmHg, respectively, and decreased plasma pH from 7.85 to 7.35 and 7.19, respectively (all changes significant) (Fig. 1). Between 2 h and 24 h, plasma $[HCO_3^-]$ increased significantly at constant P_{CO_2}, which resulted in an extracellular pH compensation of 85 % and 65 % (compared with the pH corresponding to the normocapnic bicarbonate concentration and hypercapnic P_{CO_2}; see Claiborne and Heisler, 1986) in the 2 % and 5 % CO_2 groups, respectively (Fig. 1). During progressive hypercapnia (48 h at 5 % CO_2 followed by 24 h at 8 % CO_2), plasma $[HCO_3^-]$ increased to 46 mmol l^{-1}, effecting a 65 % pH compensation (Fig. 1).

Intracellular pH in tail muscle decreased significantly from 7.18 to 7.07 after 2 h of exposure to 2 % CO_2 (Fig. 2). Exposure to 5 % CO_2 caused a similar decrease in pHi despite the higher P_{CO_2}. This was due to a larger increase in intracellular $[HCO_3^-]$ in the 5 % group than in the 2 % group (Fig. 2). After 24 h, muscle pHi was completely restored to control values in both groups. In the liver, pHi fell significantly from 7.00±0.02 (N=8) to 6.86±0.02 (N=7) after 2 h of exposure to 2 % CO_2. After 24 h, liver pHi had returned to control values (7.03±0.03, N=7).

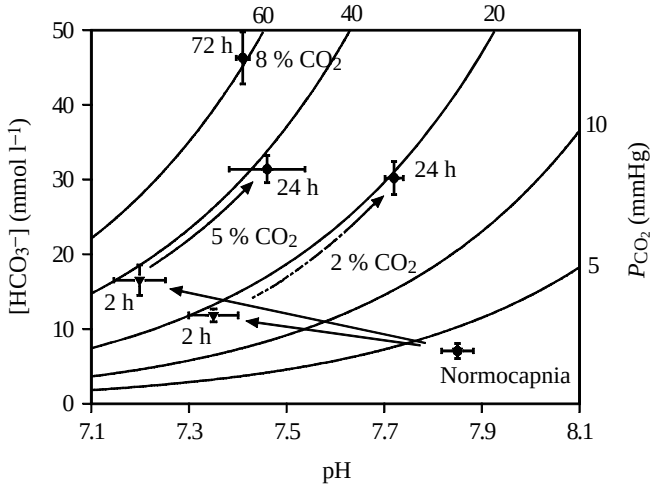

Fig. 1. Davenport diagram with calculated P_{CO_2} isopleths, showing changes in extracellular acid–base parameters in tadpoles following exposure to 2 % and 5 % CO_2 for 2 and 24 h, and progressive hypercapnia to a final level of 8 % CO_2 after 72 h. Arrows indicate expected *in vivo* changes. Means ± S.E.M., N=5–8 for individual points. 1 mmHg=0.1333 kPa.

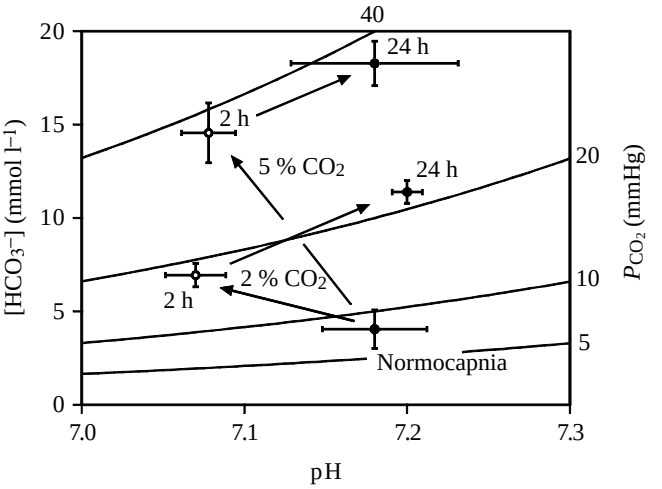

Fig. 2. Davenport diagram with calculated P_{CO_2} isopleths, showing changes in intracellular acid–base parameters in tail muscle from tadpoles subjected to different levels of environmental hypercapnia. Arrows indicate expected *in vivo* changes. Means ± S.E.M., N=5–10. 1 mmHg=0.1333 kPa.

Tissue water and Ca^{2+} distribution

Fractional tissue water content (F_{tw}) was approximately 90 % of total tissue mass, and 33 % of F_{tw} was extracellular fluid (Q). Neither total tissue water content nor the distribution of water between the extra- and intracellular compartments was affected by exposure to hypercapnia. Hypercapnia had no significant effects on body fluid $[Ca^{2+}]$. Extracellular $[Ca^{2+}]$ was 1.07±0.13 mmol l^{-1} (N=6) in normocapnic tadpoles and 1.44±0.26 mmol l^{-1} (N=9) in hypercapnic (5 % CO_2) animals, and the corresponding intracellular Ca^{2+} concentrations were 0.66±0.04 mmol l^{-1} cell water (N=8) and 0.44±0.10 mmol l^{-1} cell water (N=9) respectively.

Transepithelial fluxes of acid–base equivalents

Normocapnic tadpoles had a low steady-state H^+-equivalent excretion rate of 271±73 μmol h^{-1} kg^{-1} (slope of dotted line in Fig. 3), composed of a total ammonia efflux rate of 232±31 μmol h^{-1} kg^{-1} and an apparent bicarbonate uptake rate of 39±72 μmol h^{-1} kg^{-1}. A similar net rate of H^+ excretion was seen in the control period of the hypercapnic experiments (Fig. 3, filled line before time zero). Upon exposure to 5 % CO_2, the small excretion of H^+ equivalents changed significantly to a large excretion of HCO_3^- equivalents, rising to 13 mmol kg^{-1} after 4–6 h. The ammonia excretion rate was not affected by hypercapnia. Net base excretion reached a maximal rate of 9151 μmol h^{-1} kg^{-1} between 0.5 and 1 h of hypercapnia (Fig. 3, filled line, slope between 0.5 and 1 h). After some 4–6 h, the net excretion of HCO_3^- equivalents changed to a slow net release of H^+ equivalents, which was not significantly different from the normocapnic H^+ excretion rate.

Re-exposure of the same tadpoles to hypercapnia after 1–3 weeks of recovery under normocapnic conditions resulted in much smaller changes in transepithelial fluxes of acid–base

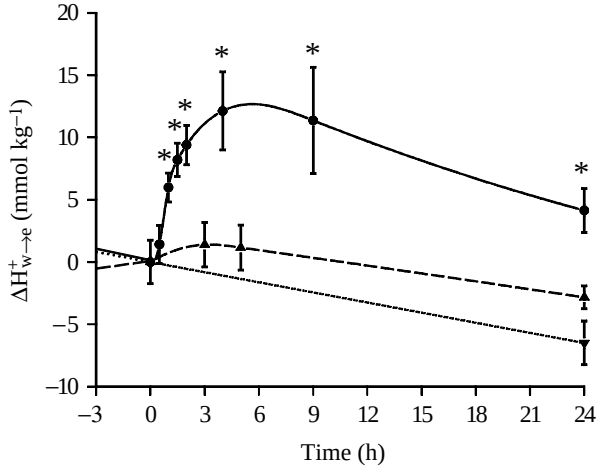

Fig. 3. Cumulative net transfer of H^+ equivalents from the water to the extracellular space of tadpoles ($\Delta H^{+}_{w\rightarrow e}$, a positive value being equivalent to net acid uptake or net base excretion), calculated as the difference between $\Delta[HCO_3^-]_{water}$ and $\Delta[NH_3{+}NH_4^+]_{water}$. The dotted line is for normocapnic control tadpoles, the solid line is for the first exposure of tadpoles to 5% CO_2 (time 0 indicates the onset of hypercapnia) and the dashed line is for the second exposure of tadpoles to 5% CO_2 after 1–3 weeks of recovery in normocapnic water. Means ± S.E.M. (the numbers of experiments were 6, 5 and 4 for normocapnic, first and second exposures to hypercapnia, respectively, and four tadpoles participated in each experiment). Asterisks indicate a significant difference from the cumulative normocapnic net transfer of H^+ equivalent at a given time (P<0.05).

equivalents, and the net transfer of H^+ was not significantly different from the steady-state excretion (Fig. 3, dashed line).

During normocapnia there was a non-significant Ca^{2+} uptake of 39±96 μmol h^{-1} kg^{-1} (N=6, evaluated from the 24 h normocapnic experiments). Exposure to 5% CO_2 significantly changed the flux to a large Ca^{2+} efflux of 393±102 μmol h^{-1} kg^{-1} (N=5, averaged over the 24 h first-time exposure to hypercapnia).

Discussion

Extra- and intracellular acid–base parameters

The acid–base status of normocapnic tadpoles of *Rana catesbeiana* at 20 °C was close to the values reported by Just *et al.* (1973) at 23 °C. Tadpoles have three areas for gas exchange (skin, gills and lungs), but the blood P_{CO_2} of 3.3 mmHg (Fig. 1) is low, as in exclusively water-breathing animals, reflecting the fact that CO_2 exchange with air across the lung epithelium is not important in unstressed animals. This is in line with the finding of Burggren and West (1982) that less than 5% of metabolically produced CO_2 is eliminated across the lungs of *Rana catesbeiana* tadpoles. The normocapnic extracellular pH of 7.85 (Fig. 1) and the intracellular pH in muscle of 7.18 (Fig. 2) are similar to values in adult frogs at the same experimental temperature (Wood *et al.* 1989), reflecting complete pH compensation of the internal hypercapnia that accompanies the increased dependency on lung gas exchange during the climax of metamorphosis (Burggren and West, 1982).

After the onset of hypercapnia, there was an initial (0–2 h) extracellular respiratory acidosis followed by a compensatory accumulation of bicarbonate at constant P_{CO_2} (2–24 h) (Fig. 1). Tadpoles subjected to 2% CO_2 or 5% CO_2 for 24 h elevated their plasma $[HCO_3^-]$ to the same level of approximately 30 mmol l^{-1} (Fig. 1). The rise in $[HCO_3^-]$ resulted in a higher degree of pH compensation (85 and 65% at 2% and 5% CO_2, respectively) than is observed in most adult aquatic amphibians (0–30%) (Boutilier *et al.* 1992). Tadpoles exposed to progressive hypercapnia to a final level of 8% CO_2 increased their plasma $[HCO_3^-]$ to 46 mmol l^{-1} (Fig. 1), which is above the proposed upper bicarbonate level in amphibians of 22–33 mmol l^{-1} (Heisler, 1986) but similar to the value in adult *Rana catesbeiana* at 8% CO_2 (Toews and Stiffler, 1990).

During environmental hypercapnia, intracellular pH compensation was more complete than extracellular compensation. Full pH normalisation was recorded in tail muscle tissue (Fig. 2) and liver tissue at all levels of CO_2, reflecting the priority for intracellular pH restoration (Boutilier *et al.* 1992). Intracellular pH compensation lasted longer than 2 h, even though mobilisation of internal buffer base reserves, in principle, could have compensated the acidosis within 2 h by being transferred to the intracellular space rather than to the environment (see below).

Transepithelial transfer of acid–base equivalents

Exposure to hypercapnia changed a small net release of H^+ equivalents in normocapnic tadpoles to a large release of HCO_3^- (uptake of H^+ equivalents) (Fig. 3) and Ca^{2+}, indicating dissolution of calcium stores in the paravertebral lime sacs and/or the skin. A 1:2 molar relationship between the summed changes in Ca^{2+} levels and base excess in the body fluids and environmental water would support this hypothesis. The measurements of calcium levels in extracellular fluid and muscle cell water suggested that the change in total body fluid Ca^{2+} was marginal; the amount of Ca^{2+} liberated from $CaCO_3$ deposits during 24 h of exposure to 5% CO_2 approximates the 9.4 mmol kg^{-1} released to the water. In the same period, there was a release of 10.6 mmol kg^{-1} HCO_3^- equivalents to the water (corrected for the normocapnic excretion of H^+ equivalents). Assuming that the complete pH restoration in liver and tail muscle applies to all intracellular compartments, information on intracellular non-bicarbonate buffer values are not needed to calculate the change in base excess of the body fluids. It is, however, necessary to make assumptions about the size of body compartments. A total body water content of 90% (determined for *Rana catesbeiana* tadpoles by Cecil and Just, 1979) and the blood volume of 5.4% for adult *Rana catesbeiana* (Thorson, 1964) were adopted. Tissue water distribution was approximated using values for tail muscle. An estimate of the intracellular change in base excess was

Table 1. *Calculated changes in body fluid base excess and net transfer of bicarbonate equivalents from the animal to the environment in response to the transition from normocapnia to hypercapnia (5 % CO_2) for 24 h*

Variable	Method of calculation	Change ($mmol\,kg^{-1}$)
Blood	$V_{blood}\times(\Delta[HCO_3^-]_{plasma}+\beta_{NC,plasma}\times\Delta pH_{plasma}\times F_{CO_2})$	0.7
Interstitial fluid	$V_{inter}\times1.03\times\Delta[HCO_3^-]_{plasma}$	5.8
Intracellular fluid	$V_{intra}\times\Delta[HCO_3^-]_{tail\ muscle}$	8.0
Release to environment	$\Delta H^+_{w\rightarrow e}$(hypercapnia)– $\Delta H^+_{w\rightarrow e}$(normocapnia)	10.6
Total change in base excess (body fluids and environment)		25.1

F_{CO_2} is the ratio between total CO_2 contents in blood and plasma; V_{blood}, V_{inter} and V_{intra} are the blood, interstitial fluid and intracellular fluid volumes, respectively; $\beta_{NC,plasma}$ is the true plasma non-bicarbonate buffer value; $\Delta H^+_{w\rightarrow e}$ is the net transfer of H^+ equivalents from water to the extracellular space.

obtained by assuming that the tail muscle tissue is representative of the total intracellular space (Fig. 2). The true plasma buffer value was adopted from Just *et al.* (1973), and the buffer value in interstitial fluid was assumed to be negligible. The calculated changes in base excess in body compartments and ambient water are shown in Table 1.

In view of the many assumptions involved, the summed change in base excess of $25.1\,mmol\,kg^{-1}$ was fairly close to the $18.8\,mmol\,kg^{-1}$ bicarbonate equivalents created by dissolution of $9.4\,mmol\,kg^{-1}$ $CaCO_3$, supporting the hypothesis that dissolution of internal $CaCO_3$ buffer stores is a major event during exposure to hypercapnia.

Tadpoles that were re-exposed to hypercapnia after 1–3 weeks of recovery in normocapnic water only showed a very small loss of bicarbonate (Fig. 3), suggesting that the labile $CaCO_3$ buffer reserves had been almost fully depleted during the first exposure to hypercapnia and that the $CaCO_3$ reserves are refilled slowly, if at all.

It is likely that the high P_{CO_2} mediates the dissolution of carbonates by increasing $[H^+]$ at the $CaCO_3$ crystal surfaces (Arends *et al.* 1982). A respiratory acidosis is easily transferred to the crystal surfaces in the lime sacs/skin by CO_2 diffusion, even if they are covered by cell membranes (e.g. osteoclasts). This makes calcium reserves especially susceptible during a respiratory acidosis. Tadpoles experience a large rise in internal P_{CO_2} during the climax of metamorphosis, but the hypercapnia develops slowly, and bicarbonate mobilised from $CaCO_3$ stores can probably be retained in the body fluids by appropriate changes in regulatory mechanisms.

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