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Mechanisms of acid–base regulation in the African lungfish *Protopterus annectens*

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Summary

African lungfish *Protopterus annectens* utilized both respiratory and metabolic compensation to restore arterial pH to control levels following the imposition of a metabolic acidosis or alkalosis. Acid infusion (3 mmol kg^{-1} NH_4Cl) to lower arterial pH by 0.24 units increased both pulmonary (by 1.8-fold) and branchial (by 1.7-fold) ventilation frequencies significantly, contributing to 4.8-fold and 1.9-fold increases in, respectively, aerial and aquatic CO_2 excretion. This respiratory compensation appeared to be the main mechanism behind the restoration of arterial pH, because even though net acid excretion ($J_{net}H^+$) increased following acid infusion in 7 of 11 fish, the mean increase in net acid excretion, 184.5±118.5 μmol H^+ kg^{-1} h^{-1} (mean ± s.e.m., *N*=11), was not significantly different from zero. Base infusion (3 mmol kg^{-1} $NaHCO_3$) to increase arterial pH by 0.29 units halved branchial ventilation frequency, although pulmonary ventilation frequency was unaffected. Correspondingly, aquatic CO_2 excretion also fell significantly (by 3.7-fold) while aerial CO_2 excretion was unaffected. Metabolic compensation consisting of negative net acid excretion (net base excretion) accompanied this respiratory compensation, with $J_{net}H^+$ decreasing from 88.5±75.6 to –337.9±199.4 μmol H^+ kg^{-1} h^{-1} (*N*=8). Partitioning of net acid excretion into renal and extra-renal (assumed to be branchial and/or cutaneous) components revealed that under control conditions, net acid excretion occurred primarily by extra-renal routes. Finally, several genes that are involved in the exchange of acid–base equivalents between the animal and its environment (carbonic anhydrase, V-type H^+-ATPase and Na^+/HCO_3^- cotransporter) were cloned, and their branchial and renal mRNA expressions were examined prior to and following acid or base infusion. In no case was mRNA expression significantly altered by metabolic acid–base disturbance. These findings suggest that lungfish, like tetrapods, alter ventilation to compensate for metabolic acid–base disturbances, a mechanism that is not employed by water-breathing fish. Like fish and amphibians, however, extra-renal routes play a key role in metabolic compensation.

Key words: acid–base balance, ventilation, lung, gill, kidney, acidosis, alkalosis, African lungfish, *Protopterus annectens*.

Introduction

The regulation of metabolic acid–base disturbances can occur through the control of ventilation and/or the direct transfer of acid–base equivalents between the animal and its external environment. Increased pulmonary ventilation during a metabolic acidosis reduces arterial P_{CO_2}, thereby raising arterial pH, while CO_2 retained *via* hypoventilation can compensate for a metabolic alkalosis. Such respiratory compensation is a mainstay of the strategy used by tetrapods to regulate acid–base disturbances (Heisler, 1986; Swenson, 2000; McNamara and Worthley, 2001). In water-breathing fish, on the other hand, respiratory compensation is of negligible importance and acid–base balance is restored metabolically (for reviews, see Goss et al., 1998; Claiborne et al., 2002; Hirose et al., 2003; Perry et al., 2003b; Evans et al., 2005; Perry and Gilmour, 2006). A systemic acidosis is corrected by the increased output of acidic equivalents (thereby raising the plasma HCO_3^- concentration), whereas the response to alkalosis consists of decreased net efflux of acid (equivalent to increased base efflux). In fish, the gill accounts for 90% or more of such direct transfer of acid–base equivalents (Claiborne et al., 2002). Adjustment of renal acid secretion plays an important complementary role to the gills (Wood et al., 1999; Georgalis et al., 2006a) (for reviews, see Perry and Fryer, 1997; Perry et al., 2003b; Perry and Gilmour, 2006). Tetrapods also employ metabolic mechanisms to compensate for acid–base disturbances, with the kidney being the main regulatory site (Heisler, 1986; Swenson, 2000; McNamara and

Worthley, 2001). Aquatic amphibians, however, parallel fish in utilizing extra-renal transfer of acid–base equivalents, in their case, *via* the skin (Stiffler and Bachoura, 1991; Stiffler, 1991; Talbot and Stiffler, 1992). Regardless of the tissue site, work on a variety of organisms suggests that similar cellular and molecular mechanisms of acid–base equivalent transfer are employed (for reviews, see Claiborne et al., 2002; Kirschner, 2004; Wall, 2005; Perry and Gilmour, 2006).

With this background in mind, the present study focused on characterizing strategies for acid–base regulation in the African lungfish *Protopterus annectens*. Mechanisms of acid–base compensation in lungfish have received little attention to date (Sanchez et al., 2005), but several factors argue compellingly for their investigation. African lungfish (*Protopterus* sp.) are obligate air breathers that possess true lungs and cannot survive without access to air, but gills are also present, permitting bimodal breathing (reviewed by Burggren and Johansen, 1986). The lung is the primary site of O_2 uptake in all four *Protopterus* species, but the majority of CO_2 excretion occurs across the gill (and/or the skin) (Lenfant and Johansen, 1968; Lahiri et al., 1970; McMahon, 1970), except in *P. dolloi*, where the lung appears to be a major route of both O_2 and CO_2 transfer (Perry et al., 2005). Because most O_2 uptake occurs from the air, branchial ventilation in lungfish need not be constrained by convection requirements for O_2 uptake. Consequently, the gill is ventilated by low volumes of water (Jesse et al., 1967) and this factor, coupled with the low surface area and high blood-to-water diffusion distances of the lungfish gill (Sturla et al., 2001), results in arterial P_{CO_2} values that are unusually high for fish (~20–30 mmHg) (Lenfant and Johansen, 1968; Perry et al., 2005). Thus, the respiratory status of lungfish is more suitable for using ventilation to regulate acid–base disturbances than is the case for water-breathing fish. At the same time, however, the gills, although reduced in surface area relative to those of water-breathing fish, are covered by a heterogeneous epithelium containing several cell types, including pavement cells, mitochondria-rich (MR) cells, and sensory cells (Sturla et al., 2001). MR cells are thought to be responsible for the excretion of acid–base equivalents by the gills of freshwater fish (for a review, see Perry and Gilmour, 2006), and the presence of MR cells therefore implies the potential for the lungfish gill to function in metabolic acid–base compensation.

Two hypotheses were tested in the present study. First, the hypothesis that ventilatory adjustments are used by *P. annectens* to regulate acid–base disturbances was tested. It was predicted that lungfish would increase pulmonary and/or branchial ventilation in response to metabolic acidosis, but hypoventilate under conditions of metabolic alkalosis. Second, the transfer of acid–base equivalents across the gill (and/or skin) was hypothesized to play a significant role in recovery from acid–base disturbances. Under these conditions, net acid excretion to the water would be predicted to increase during metabolic acidosis and decrease during metabolic alkalosis. Finally, the molecular mechanisms underlying the transfer of acid–base equivalents at the gill and kidney were investigated by cloning fragments of lungfish V-type H^+-ATPase, Na^+/HCO_3^- cotransporter (NBC) and carbonic anhydrase (CA), and examining the mRNA expression of these genes in the gill and kidney prior to and following acid–base disturbances.

Materials and methods

Experimental animals

Adult specimens of either sex of the African lungfish *Protopterus annectens* Owen (143±6 g; mean mass ± s.e.m.; *N*=46) were imported from central Africa to Singapore through a local fish farm. Lungfish were then shipped to Ottawa by air cargo. During transit, lungfish were placed in partially filled bags of oxygenated water held in insulated containers and upon arrival, the fish were transferred into individual covered plastic aquaria filled with 2–3 l of dechloraminated city of Ottawa tapwater warmed to 25°C. The tank water was changed on alternate days or sooner if obvious fouling of the water occurred. Lungfish were held in a room in which the air temperature was maintained at 25°C, under an artificial photoperiod of 10 h:14 h light:dark. Fish were fed on alternate days with frozen blood worms or pieces of rainbow trout flesh, and were allowed to acclimate to these conditions for at least 1 month prior to experimentation.

To allow periodic blood sampling as well as acid or base infusion, a cannula (Clay-Adams PE50 polyethylene tubing; VWR, Montreal, QC, Canada) was inserted into the dorsal aorta of all fish, as described by Perry et al. (Perry et al., 2005). Surgery was carried out on lungfish that were first anaesthetized by immersion in a solution of MS-222 (ethyl-*p*-aminobenzoate; 0.67 g l^{-1}) adjusted to neutral pH with $NaHCO_3$ (1.3 g l^{-1}); anaesthesia was maintained during surgery by wrapping the fish in paper towels soaked with the anaesthetic solution. A subset of lungfish (*N*=7) was fitted with an external urinary catheter according to the procedure of Curtis and Wood (Curtis and Wood, 1991). The use of an external urinary catheter allows the collection of urine as it is naturally discharged from the urogenital papilla. Following surgery, lungfish were returned to their containers for a 24 h recovery period. Cannulae were flushed daily with Cortland saline (Wolf, 1963).

Experimental protocol

Series 1. Respiratory versus metabolic compensation of acid–base disturbances

Fish (*N*=19) that previously had been fitted with a dorsal aortic cannula were placed into customized respirometry chambers approximately 2 h before an experiment was initiated. The cylindrical chambers were filled with water (~1 l) apart from an adjustable air space (60 ml maximum volume; typically set to ~30 ml) at one end of the respirometer; exact water and air volumes were noted for each fish. Lungfish quickly became aware of the presence of the air space, and normally would begin breathing air at regular intervals, adopting a position in the respirometer where their head was just below the airspace. The occurrence of each air breath was captured by a custom-built device that detected interruptions in

two infrared light beams aimed across the surface of the water. Both compartments were provided with continually flowing media (water or humidified air, at 25°C) except during measurements of the rates of O_2 consumption ($\dot{M}_{O_2}$), CO_2 excretion ($\dot{M}_{CO_2}$), and net acid excretion into the water. During these measurements, the water was re-circulated using a peristaltic pump, and the air chamber was sealed. Fibre-optic O_2 electrodes (Ocean Optics AL300, Dunedin, FL, USA) sealed into the air chamber and into the tubing through which water was re-circulating were used to measure aerial and aquatic P_{O_2}, respectively. Aerial P_{CO_2} was measured using a CO_2 electrode (Analytical Sensors E201, Sugarland, TW, USA) inserted into the air chamber, while measurement of aquatic P_{CO_2} involved using a small peristaltic pump to move water past a CO_2 electrode (Analytical Sensors E201) housed within a thermostatted (25°C) cuvette (Radiometer) before returning it to the respirometer. These respirometers were used in a previous study (Perry et al., 2005) during which the extent of gas transfer across the air–water interface was quantified and found to be negligible.

Once fish were breathing air at regular intervals and had been doing so for at least 1 h, baseline ('pre') measurements of $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$, ventilation frequency, net acid excretion and blood acid–base status were carried out over the course of 1 h. The water and air chambers were sealed after stopping the flow of media, and the re-circulating pump was started to provide mixing. Changes in aerial P_{O_2} and P_{CO_2} were monitored for two 30-min periods, between which the air within the chamber was refreshed. Changes in aquatic P_{O_2} and P_{CO_2} were monitored for approximately 30 min or until stable rates of CO_2 accumulation and O_2 depletion were achieved. In addition to the automatic detection of air breaths, water-breathing frequency was determined by visually counting water breaths at periodic intervals. To measure net acid excretion, 20 ml water samples were withdrawn at the beginning and end of the 1–h flux period. A 0.5 ml blood sample was withdrawn at the end of the measurement period. The blood sample was centrifuged and plasma was removed for immediate analysis of pH and total CO_2 content (CCO_2). The remaining red blood cells were re-suspended in heparinized (50 i.u. ml^{-1} ammonium heparin; Sigma, Oakville, ON, Canada) Cortland saline and re-injected into the fish. At the end of this measurement period, the respirometer was returned to a flow-through condition and acid or base infusion was initiated.

Lungfish were infused with acid (0.9 mol l^{-1} NH_4Cl; N=11) or base (0.9 mol l^{-1} $NaHCO_3$; N=8) at a rate of 3 mmol kg^{-1} h^{-1} for 1 h. A syringe pump (SAGE instruments 355) was used to infuse acid or base *via* the dorsal aortic cannula. Once acid or base infusion had been initiated, the water and air chambers were sealed and the series of measurements described above was repeated, culminating with the withdrawal of a blood sample immediately following the infusion period. Blood samples were also withdrawn at 3, 6 and 18 h post-infusion, and were preceded in each case by the same series of measurements of $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$, ventilation frequency and net acid excretion. After withdrawal of the 18 h blood sample, lungfish were anaesthetized *in situ* by intra-arterial injection of 400 mg kg^{-1} of sodium pentobarbital (Somnotol), and then sacrificed by an intra-arterial injection of saturated KCl. A final measurement period for $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$ and net acid excretion was carried out over 45 min to estimate background levels of gas transfer and net acid excretion. These background values were assumed to reflect cutaneous metabolism (which they may underestimate) and/or metabolism arising from microorganisms in the water or on the surface of the fish (Perry et al., 2005). For each fish, the values for $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$ and net acid excretion were corrected for background metabolism by subtracting the rates determined in this final respirometry/flux period.

Series 2. Partitioning of net acid excretion between gills/skin and kidney in base-infused fish

Lungfish (N=7) were placed into the customized respirometry chambers and experiments were not initiated until at least 1 h of breathing air at regular intervals had occurred. Fish used in these experiments were fitted with both a dorsal aortic cannula (for base infusion; blood samples were not collected in these experiments) and an external urinary catheter. Urine was collected continuously throughout the experimental period by allowing the urinary catheter to drain, by gravity, into a vial located outside the respirometer and held approximately 5 cm below water level. The urinary catheter was checked for leaks by raising the catheter 5 cm above water level. Under these conditions, a fall of the urine level in the catheter indicated a leak, and the experiment was terminated. First, a flux period was carried out to determine baseline ('pre') levels of net acid excretion into water and urine, as well as urine flow rate (UFR), pH and CCO_2. Water flow to the respirometer was halted, the chamber was sealed and the re-circulating pump was started to provide mixing. Water samples (20 ml) were collected at the beginning and at 3 h of the 4 h flux period. To ensure that the dead space volume within the urinary catheter was cleared, urine collected during the initial ~60 min of the flux period was discarded. At the end of the flux period, water flow to the respirometer was re-established and base infusion was initiated. Base (0.9 mol l^{-1} $NaHCO_3$) was infused into the dorsal aortic cannula, using a syringe pump, at a rate of 3 mmol kg^{-1} h^{-1} for 1 h. Flux periods were carried out over the initial 4 h following base infusion, and from 17–21 h post base infusion.

Series 3. The impact of acid or base infusion on gene expression

Following recovery from surgery, lungfish fitted with a dorsal aortic cannula were infused intra-arterially with saline (Cortland saline, 0.5 ml h^{-1}; N=3), acid (0.9 mol l^{-1} NH_4Cl; N=8) or base (0.9 mol l^{-1} $NaHCO_3$; N=7) at a rate of 3 mmol kg^{-1} h^{-1} for 1 h using a syringe pump. Lungfish were sacrificed 4 h (N=8) or 8 h (N=7) after the acid or base infusion period, and gill and kidney tissue samples were collected, flash frozen in liquid N_2 and stored at –80°C for later analysis of gene expression. Saline-infused fish were sampled 8 h after the infusion period to provide control data.

Analytical procedures

For respirometry, P_{CO_2} electrodes were connected to a blood gas analyzer (Cameron Instruments BGM200, Port Aransas, TX, USA) that was customized to accept two CO_2 inputs. Output from the blood gas analyzer and the infrared air-breath detector was converted to digital data and stored by interfacing with a data acquisition system (Biopac Systems Inc., Harvard Apparatus Canada, Saint-Laurent, QC, Canada) using Acknowledge™ data acquisition software (sampling rate set at 30 Hz) and a PC. Output from the fibre-optic O_2 electrodes was collected using Ocean Optics software running on the same PC. These data were compiled as text files for later importation into spreadsheet software for storage and analysis. To calibrate the fibre-optic O_2 electrode used for water P_{O_2} measurements, the electrode was immersed in zero solution (2 g l^{-1} sodium sulphite) or air-saturated water until stable readings were recorded. The fibre-optic O_2 electrode used for air P_{O_2} measurements was calibrated *in situ* in the respirometer by flowing humidified N_2 gas (zero) or air continuously through the air chamber. Both CO_2 electrodes were calibrated *in situ* using mixtures of 0.5% and 1.0% CO_2 in air that were provided by a gas mixing flowmeter (Cameron Instruments GF-3/MP). Humidified gas mixtures flowing through the air chamber of the respirometer were used to calibrate the air P_{CO_2} electrode, whereas water equilibrated with the gas mixtures and pumped through the cuvette was used to calibrate the CO_2 electrode used for water P_{CO_2} measurements.

Rates of aerial gas transfer were determined from the slopes of the relationships between inspired gas tensions and time, over the period that the air chamber was sealed, taking into account air chamber volume, fish mass, and the solubility coefficients of O_2 and CO_2 in air at 25°C (Boutilier et al., 1984). Similarly, aquatic gas transfer was calculated using the slopes of the relationships between water gas tensions and time over the interval that the water in the respirometer was re-circulated, taking into account water chamber volume, and fish mass. For O_2, the solubility coefficient in fresh water at 25°C was obtained from Boutilier et al. (Boutilier et al., 1984). For CO_2, the capacitance coefficient in dechloraminated city of Ottawa tap water at 25°C was determined experimentally to be 0.041 μmol l^{-1} $mmHg^{-1}$ by measuring the total CO_2 concentrations (Cameron Instruments Capni-Con 5) of water samples equilibrated to the range of P_{CO_2} values encountered in respirometry trials.

Blood samples were centrifuged (~10 000 ***g*** for 1 min) immediately following withdrawal to yield plasma. Plasma total CO_2 concentration was determined in duplicate on 50 μl samples (Cameron Instruments Capni-Con 5). Plasma pH was measured using a pH electrode and calomel reference (Analytical Sensors E301 glass pH electrode) that were housed in a temperature-controlled, low-volume pH chamber (Cameron Instruments) and connected to a PHM 72 acid–base analyzer (Radiometer). The arterial blood P_{CO_2} (Pa_{CO_2}) and bicarbonate concentration ($[HCO_3^-]$) were then calculated from the Henderson–Hasselbalch equation using appropriate values for αCO_2 and pK′ (Boutilier et al., 1984).

Net acid excretion ($J_{net}H^+$) was determined from measurements of titratable net acid flux ($J_{net}TA$) and the change in ammonia concentration in the water samples collected at the beginning and end of a flux period. Water $J_{net}TA$ and ammonia concentration were assessed within 24 h of sample collection and the remainder of the water sample was frozen for later analysis of ion concentrations. $J_{net}TA$ was determined by titrating (using a Gilmont precision microburet) 5 ml water samples from the beginning and end of each flux period to pH 4.00 with 0.02 mol l^{-1} HCl. Samples were continuously aerated prior to and during titration to ensure mixing and removal of CO_2. A micro-modification of the salicylate–hypochlorite colorimetric assay of Verdouw et al. (Verdouw et al., 1978) was used to measure total ammonia levels in water samples. $J_{net}H^+$ was then calculated as the sum of $J_{net}TA$ and the ammonia flux ($J_{net}NH_3$), signs considered, as described by McDonald and Wood (McDonald and Wood, 1981).

Net renal acid excretion was also determined as the sum of titratable net acid flux and ammonia efflux (Wood and Caldwell, 1978). Urinary $J_{net}TA$ was measured by lowering the pH of a 200 μl aliquot of urine below 5.0 through the addition of a known volume of 0.02 mol l^{-1} HCl. The sample was then aerated for 20 min to remove CO_2. While continuing to aerate, the pH of the urine sample was titrated back to the pH of blood representative of the particular sampling period through the addition of 0.02 mol l^{-1} NaOH using a precision microburet (Gilmont). The titratable component of net renal acid excretion is given by the difference in the quantities of acid and base added to the urine. Urine flow rates were determined gravimetrically. As with water samples, urine $J_{net}TA$ and ammonia concentration were assessed within 24 h of sample collection together with urine pH and CCO_2. Urine ammonia concentration, pH and total CO_2 concentration were assessed using the procedures described above for water or blood. For the calculation of urine $[HCO_3^-]$, constants derived for freshwater were employed (Boutilier et al., 1984).

Molecular cloning and analysis of lungfish carbonic anhydrase, Na^+/HCO_3^- cotransporters and H^+ V-ATPase

Cloning procedures

Tissues for the cloning of lungfish CA, NBC and H^+ V-ATPase were collected from fish that were terminally anaesthetized by immersion in a solution of MS-222 (see above). Total RNA was isolated from a given tissue using Trizol (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. RNA quantity and quality were verified by spectrophotometry (Eppendorf BioPhotometer) and/or by inspection of an RNA gel. First strand cDNA was synthesized from RNA using Superscript reverse transcriptase (Invitrogen) and an oligo(dT) primer (Sigma). Gene-specific primers (Table 1) were then used to amplify cDNA fragments by PCR. PCR was performed using 1 μl of cDNA template in 25 μl reaction mixture containing 3.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP, 250 nmol l^{-1} each of forward and reverse primers, and 1 i.u. Taq polymerase (New England

Table 1. *Primer sequences used for cloning and amplification of lungfish carbonic anhydrase, Na^+/HCO_3^- cotransporter and H^+ V-ATPase*

Primer pair	Forward primer	Reverse primer
CA	MGNCARWSICCIATHGAYAT	TTCCARTGNACIARRTGIARYTC
CA3R1	TCCACCTTCCAGACCTGAAC	
CA3R2	GAGTGCCCATTGTTGGAAAT	
CA3R3	TCAGCAGTGTGGATGTCAATC	
QCA	TGGACCCCTTGCTGGACACTTCA	ACCAATAACAGCCAGCCCGTCAG
NBC	CATGCAGGGCGTGTTGGAGAGT	AGGGCCAGTGCTGCTAAGAATCG
QNBC	TTTCTGGGGCTGTCTTCTGT	AATGCTGACCACAAGCCAAT
HV1	TGYGARTTYCANGGNGAYAT	TTCATNGCYTGNACRTCRTT
HV2	GARGARATGATHCARCANGG	TTCATNGCYTGNACRTCRTT
HV3R1	TCGGACTTTGAGGAGAATGG	
HV3R2	AAGCACTGCGAGAGGTGTCT	
HV5R1	TGACGGTCCACGTAAATTTGT	
HV5R2	TGTCATCATTTGGCATGGTC	
HV5R3	CGCAGTGCTTCAGCATAAGA	
QHV	CCATGAAGGCGGTAGTCGGTGAG	TTGGGGAAGATTCGCAGCAGC
18S	GCCCTATCAACTTTCGATGG	GGACATCTAAGGGCATCAC
Q18S	GGCGCTCCCTCGATGCTCTTAACT	GTCCCTCTTAATCATGGCCCCAGTTC

All primers are presented in a 5′ to 3′ orientation.
CA, carbonic anhydrase; NBC, Na^+/HCO_3^- cotransporter.

Biolabs, Toronto, ON, Canada) in PCR buffer supplied with the enzyme. All PCR reactions involved an initial denaturation at 94°C for 3 min followed by 39 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 60 s, and ending with a final extension for 10 min at 72°C. PCR products obtained in this manner were gel-purified, cloned into pCR2.0-TOPO vector (TOPO TA cloning kit, Invitrogen) and sequenced. The resulting sequences were compared against those in the GenBank database using BLASTX to identify the gene, and also to design primers (where needed) for 3′- and 5′-rapid amplification of cDNA ends (RACE) to extend the amplified sequence lengths. For 3′-RACE, cDNA was synthesized using a 3′-RACE adapter primer (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Semi-nested PCR was performed on the cDNA using abridged universal amplification primers (AUAP; Invitrogen) and gene-specific 3′-RACE primers (Table 1). For 5′-RACE, cDNA was synthesized using a gene-specific primer (Table 1) and Superscript II reverse transcriptase (Invitrogen), and then purified using a PCR purification kit (Sigma). The newly purified cDNA was tailed with dCTP using a recombinant terminal transferase TdT (Invitrogen) with final reaction conditions; 10 mmol l^{-1} Tris–HCl (pH 8.4), 25 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dCTP, 1 μl cDNA, and 1 μl rTdT. The tailed cDNA was then used for two rounds of PCR using a gene-specific forward primer (Table 1) and a 5′ abridged anchor primer (5AP; Invitrogen) for the first round, and a semi-nested gene-specific forward primer (Table 1) and AUAP (Invitrogen) for the second round. PCR products were cloned into the pCR2.0-TOPO vector using TOPO TA cloning kits (Invitrogen). All RACE product sequences were confirmed by overlap with the initial fragment of cDNA. After repeated bi-directional sequencing of both RACE products, a consensus sequence was created by multiple sequence alignment using DNAMAN (v4.0, Lynnon Biosoft, Vaudreuil, QC, Canada).

Using red blood cell cDNA as template, a 315 base pair (bp) internal segment of a CA coding region (denoted lfCAb) was PCR amplified at an annealing temperature of 46°C using the primer pair CA (Table 1). These degenerate primers were designed based on alignments of vertebrate cytoplasmic CA isoforms exhibiting a high degree of amino acid conservation. The sequence was then extended by three rounds of 3′-RACE using the gene-specific primers CA3R1, CA3R2 and CA3R3.

A 665 bp internal segment of the coding region of NBC was PCR amplified at an annealing temperature of 57°C using cDNA derived from lungfish gill tissue and the primer pair NBC. These primers corresponded to the nucleotides at positions 1492–1512 and 2183–2203 of the tiger salamander (*Ambystoma tigrinum*) kidney NBC (GenBank accession no. AF001958) (Romero et al., 1997). The resultant clone, lfNBC, was deemed of sufficient length for the purposes of the present study and therefore no attempt was made to extend the sequence length using RACE.

Using lungfish gill tissue, an internal segment of the coding region of the B subunit of V-type H^+-ATPase (denoted lfH^+ V-ATPase) was PCR amplified using the primer pair HV1 at an annealing temperature of 50°C. These primers, which were used successfully by Perry et al. (Perry et al., 2000) to clone the rainbow trout V-type H^+-ATPase B subunit, correspond to the nucleotides at positions 441–461 and 1395–1415 of the bovine kidney V-type H^+-ATPase B subunit sequence (GenBank accession no. M88691) (Nelson et al., 1992). A second round of

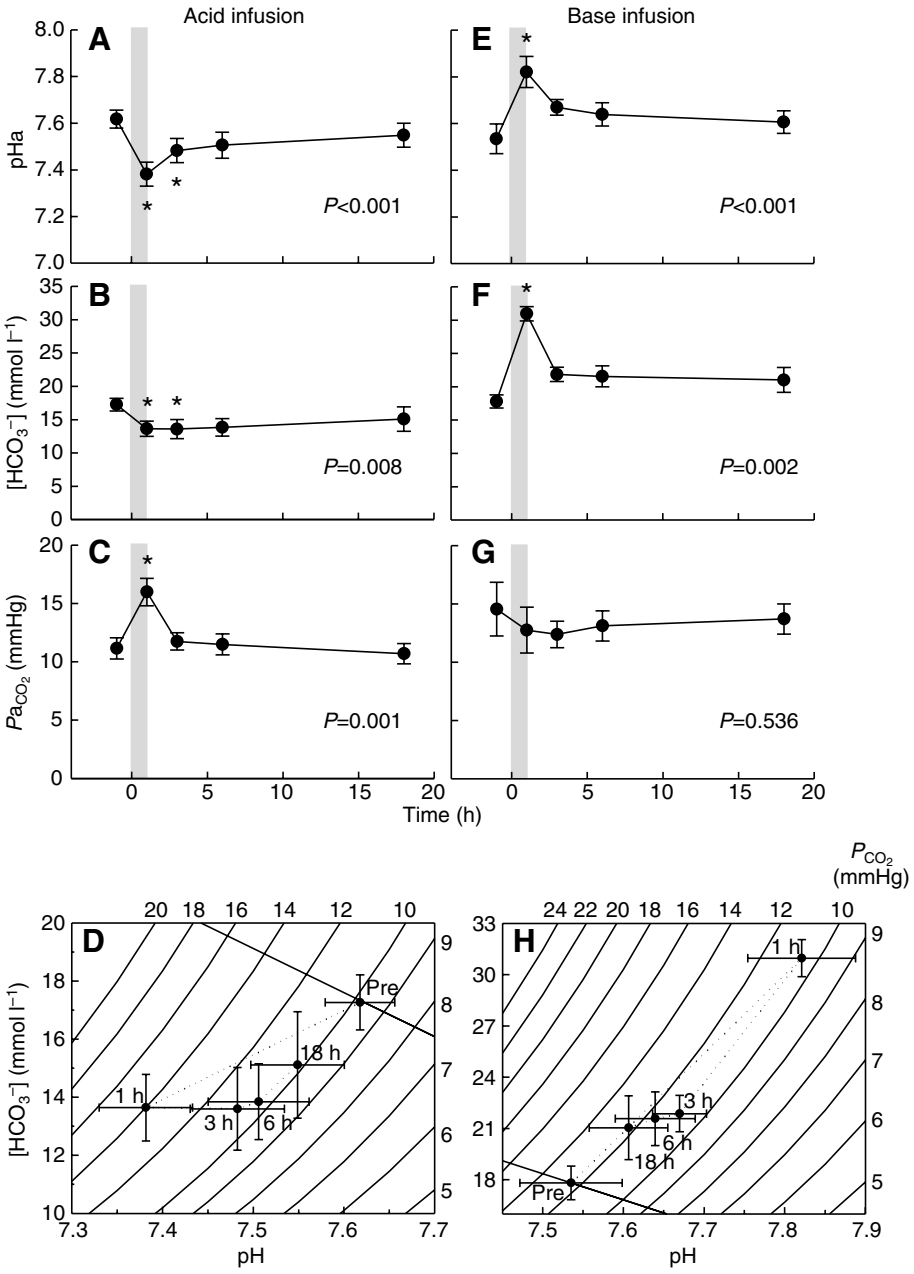

Fig. 1. The effect on the blood acid–base status of African lungfish *Protopterus annectens* of 1 h of (A–D) acid (3 mmol kg^{-1} NH_4Cl) or (E–H) base (3 mmol kg^{-1} $NaHCO_3$) infusion. Acid–base status prior to and following the infusion period (marked by the grey bar) was assessed by determining the pH (A,E), HCO_3^- ion concentration ($[HCO_3^-]$) (B,F) and CO_2 tension (Pa_{CO_2}) (C,G) of arterial blood. (D,H) pH–HCO_3^- diagrams are used to summarize the effect of acid or base infusion on acid–base status. Values are means ± s.e.m.; N=11 for acid-infused fish and N=6–8 for base-infused fish. An asterisk (*) indicates a significant difference from the pre-infusion value (one-way repeated measures ANOVA; P values are indicated on the figure). For pH–HCO_3^- diagrams, the P_{CO_2} for a given combination of pH and $[HCO_3^-]$ was calculated using the Henderson–Hasselbalch equation and the appropriate values for pK′ and Pa_{CO_2} (Boutilier et al., 1984). The buffer line was constructed using data for *Protopterus aethiopicus* (Lenfant and Johansen, 1968). Pre, period prior to acid or base infusion.

PCR was required to obtain an 810 bp fragment. This PCR round used the primer pair HV2 at an annealing temperature of 49°C. The new forward primer corresponded to the nucleotides at positions 606–626 of the bovine kidney H^+ V-ATPase. The sequence length was then further extended by two rounds of 3′-RACE using gene-specific primers HV3R1 and HV3R2, and by 5′-RACE. For 5′-RACE, the gene-specific primer HV5R1 was used for cDNA synthesis. Two rounds of 5′-RACE were then carried out using gene-specific primers HV5R2 and HV5R3.

Phylogenetic and sequence analyses

Lungfish deduced amino acid sequences were aligned with GenBank sequences with which high identity was exhibited (determined by BLASTX searching of GenBank databases) using ClustalX version 1.83 (Thompson et al., 1997) with penalties for gap opening and gap extension set to 30 and 0.75, respectively, for pairwise alignments, and 15 and 0.3, respectively, for multiple alignments. Note that full-length sequences from other vertebrates were truncated appropriately so as to compare only regions overlapping with the lungfish sequences. The PHYLIP package was then used to carry out neighbour-joining phylogenetic analysis (Saitou and Nei, 1987) on a matrix of mean character distances, with a bootstrapping resampling option to assess the support for nodes (100 pseudoreplicates). In general, default parameters were used except that the outgroup was specified (as *Drosophila*) and the input order of species was randomized where possible. The accession numbers for sequences used in alignments or phylogenetic analysis are presented in the appropriate figure legends.

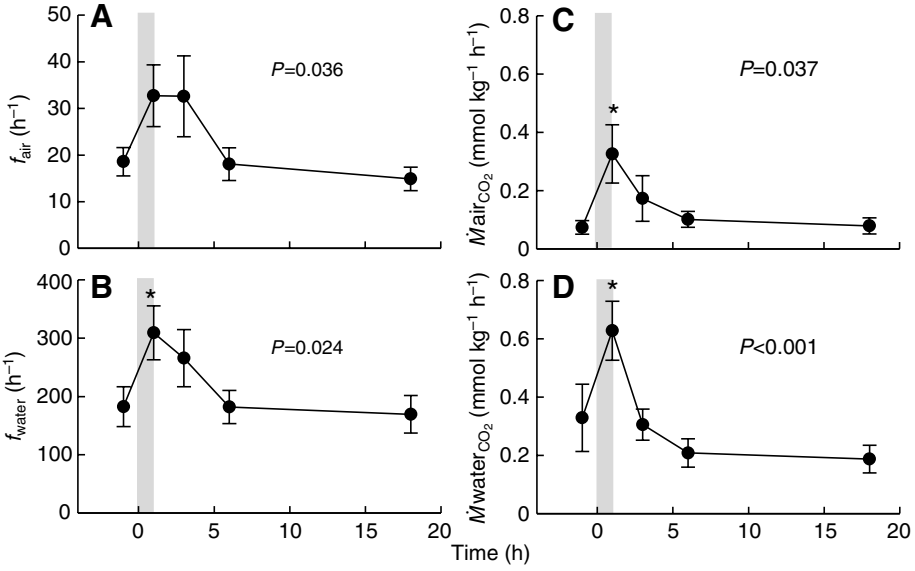

Fig. 2. The effect on (A,B) the frequency of breathing air (f_{air}; A) and water (f_{water}; B), and (C,D) CO_2 excretion into air ($\dot{M}air_{CO_2}$; C) and water ($\dot{M}water_{CO_2}$; D), of a 1 h acid (3 mmol kg^{-1} NH_4Cl) infusion (marked by the grey bar) in African lungfish *Protopterus annectens*. Values are means ± s.e.m. for the period prior to acid infusion ('pre'), and for measurement periods 0–1, 2–3, 5–6 and 17–18 h post-infusion; N=9–11 for breathing frequencies and $\dot{M}water_{CO_2}$, and 8 for $\dot{M}air_{CO_2}$. An asterisk (*) indicates a significant increase from the pre-infusion value (one-way RM-ANOVA; P values are indicated on the figure); note that in A, *post hoc* multiple comparisons tests were unable to detect the origin of the significant difference indicated by the P value.

Real-time PCR

Real-time PCR was used to assess the expression of the various lungfish mRNAs in gill and kidney tissue under control conditions and following acid or base infusion. Total RNA was extracted using Trizol (Invitrogen) from tissues that were homogenized to powder under liquid N_2 with a mortar and pestle, and then passed through a needle using a syringe. The extracted total RNA was treated with amplification grade DNase I (RNase free; Invitrogen) according to the manufacturer's instructions for 15 min at room temperature, followed by 10 min at 70°C, to remove any remaining genomic DNA. The quality of the RNA was verified by spectrophotometry (Molecular Devices, Sunnyvale, CA, USA) and cDNA was then synthesized from 2 μg of RNA using random hexamer primers (IDT DNA) and RevertAid M-MuLV H-minus (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. A Brilliant SYBR Green QPCR Master Mix kit (Stratagene, Cedarlane Laboratories, Hornby, ON, Canada) and Stratagene MX-4000 multiplex quantitative PCR system were then used to carry out real-time PCR, with ROX (Stratagene) as the reference dye. The PCR conditions (final reaction volume 25 μl) were as follows: 1 μl cDNA template (note that for 18S, the cDNA was diluted 1000 fold), 100 nmol l^{-1} forward and reverse primers, 12.5 μl of 2× Master Mix, 0.375 μl of 1:1500 ROX final dilution. The annealing and extension temperatures over 40 cycles were, respectively, 58°C (30 s) and 72°C (30 s). Primer pairs for each lungfish gene and lungfish 18S were designed using Primer3 software and are listed in Table 1 (pairs designated 'Q' were used for real-time PCR). A 1150 bp segment of lungfish 18S was cloned from gill tissue using the methods described above and the primer pair 18S (Table 1; annealing temperature of

Table 2. *The effect of acid infusion or base infusion on O_2 uptake and respiratory exchange ratios in African lungfish Protopterus annectens*

		Time post-infusion (h)				
	Pre	0–1	2–3	5–6	17–18	P
Acid infusion						
$\dot{M}air_{O_2}$ (mmol kg^{-1} h^{-1})	0.61±0.11 (6)	0.76±0.15 (7)	0.59±0.06 (7)	0.61±0.14 (8)	0.49±0.12 (8)	0.652
$\dot{M}water_{O_2}$ (mmol kg^{-1} h^{-1})	0.014±0.009 (10)	0.034±0.018 (10)	0.027±0.018 (10)	0.006±0.005 (10)	0.025±0.015 (10)	0.628
RER	0.76±0.23 (6)	1.48±0.29 (7)	0.91±0.17 (7)	0.57±0.12 (8)	0.98±0.35 (8)	0.116
Base infusion						
$\dot{M}air_{O_2}$ (mmol kg^{-1} h^{-1})	0.67±0.07 (6)	0.54±0.07 (7)	0.56±0.05 (7)	0.47±0.05 (7)	0.68±0.08 (7)	0.066
$\dot{M}water_{O_2}$ (mmol kg^{-1} h^{-1})	0.18±0.06 (7)	0.18±0.05 (7)	0.13±0.03 (7)	0.12±0.05 (7)	0.10±0.01 (7)	0.276
RER	0.69±0.04 (6)	0.69±0.09 (7)	0.52±0.06 (7)	0.56±0.09 (7)	0.27±0.04* (7)	0.002

$\dot{M}air_{O_2}$, aerial O_2 uptake; $\dot{M}water_{O_2}$, aquatic O_2 uptake; RER, respiratory exchange ratios.

Values are means ± s.e.m. for the period prior to infusion (Pre), and for measurement periods 0–1, 2–3, 5–6 and 17–18 h post-infusion; Number of samples are indicated in parentheses.

One-way RM-ANOVA was used to assess the statistical significance of differences between pre- and post-infusion values; P values are indicated for each ANOVA and an asterisk indicates a post-infusion value that is significantly different from the corresponding pre-infusion value.

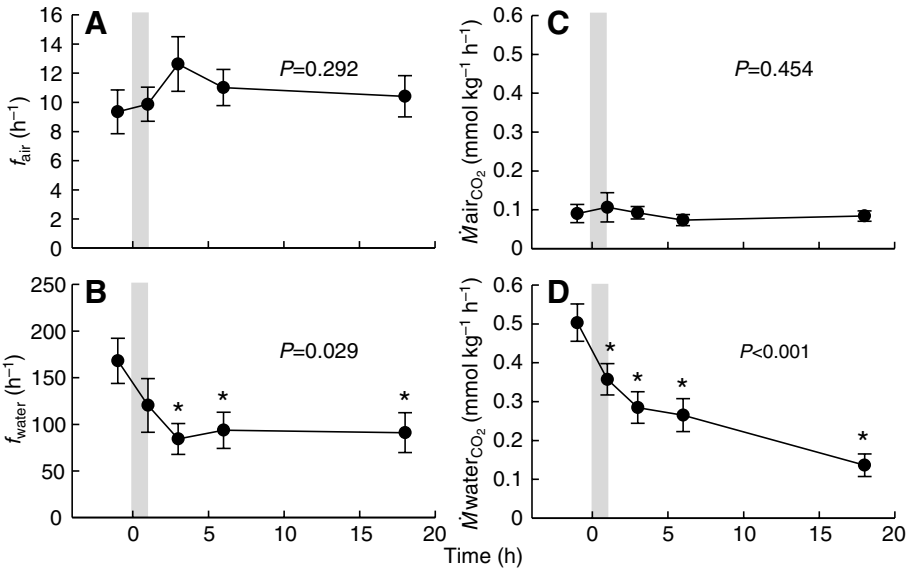

Fig. 3. The effect on (A,B) the frequency of breathing air (f_{air}; A) and water (f_{water}; B), and (C,D) on CO_2 excretion into air ($\dot{M}air_{CO_2}$; C) and water ($\dot{M}water_{CO_2}$; D), of a 1 h base (3 mmol kg^{-1} $NaCO_3$) infusion (marked by the grey bar) in African lungfish *Protopterus annectens*. Values are means ± s.e.m. for the period prior to base infusion ('pre'), and for measurement periods 0–1, 2–3, 5–6 and 17–18 h post-infusion; N=8 throughout. An asterisk (*) indicates a significant difference from the pre-infusion value (one-way RM-ANOVA; P values are indicated on the figure).

52°C), which consisted of primers designed against regions of high amino acid conservation identified by the examination of an alignment of 18S sequences from a range of vertebrates. The sequence was then used to design the real-time PCR 18S primers listed in Table 1. The specificity of each primer pair was verified by the cloning (TOPO TA cloning kit; Invitrogen) and sequencing of the amplified product. SYBR green dissociation curves were constructed after the completion of 40 PCR cycles and revealed the presence of single amplicons for each primer pair. The omission of reverse transcriptase during cDNA synthesis was used as a control to ensure that residual genomic DNA was not being amplified. For comparisons among treatments (control, acid infused, base infused), mRNA expression relative to the control group was calculated using the modified delta-delta Ct method (Pfaffl, 2001) with lungfish 18S mRNA expression as a normalizing factor.

Statistical analyses

Data are reported as mean values ± 1 s.e.m. The statistical significance of treatment effects was assessed by one-way repeated measures analysis of variance (RM-ANOVA). Where one-way RM-ANOVA indicated that significant differences existed, a *post hoc* multiple comparisons test (Holm–Sidak method) was applied. Where assumptions of normality or equal variance were violated, equivalent non-parametric tests were used. Statistical analyses were carried out using SPSS SigmaStat v3.0 (Systat Software) software with a fiducial limit of significance in all tests of 0.05.

Results

In acid-infused lungfish, arterial blood pH (pHa) fell by 0.24 units but had recovered to a level not significantly different from the control by 6 h (Fig. 1). The fall in pHa was accompanied by a significant 20% reduction in the plasma HCO_3^- concentration ($[HCO_3^-]$) and a more transient 1.4-fold elevation of arterial CO_2 tension (Pa_{CO_2}). Base infusion caused a metabolic alkalosis in the arterial blood, characterized by a 0.29 unit increase in pHa associated with a 1.7-fold increase in plasma $[HCO_3^-]$ and unchanged $PaCO_2$. By 3 h, blood acid–base status was not significantly different from the control situation (Fig. 1).

Rapid correction of the acid–base disturbances was accomplished, at least in part, through adjustment of pulmonary and branchial ventilation frequencies (Figs 2 and 3). Acid-infused lungfish (Fig. 2) approximately doubled both air (1.8-fold) and water (1.7-fold) breathing frequencies, thereby increasing total CO_2 excretion ($\dot{M}_{CO_2}$) 2.4-fold. This increase in total $\dot{M}_{CO_2}$ was accomplished through a doubling of CO_2 transfer to water together with a 4.8-fold increase in aerial CO_2 excretion, illustrating the fact that *P. annectens* is capable of effective CO_2 excretion *via* the lungs, even though under control conditions aquatic CO_2 transfer accounted for 78.2±4.6% (N=18) of total $\dot{M}_{CO_2}$. By contrast with the enhancement of CO_2 excretion, O_2 uptake ($\dot{M}_{O_2}$) was unaffected by acid infusion, causing the respiratory exchange ratio (RER) to increase, although not significantly (Table 2). Air-breathing frequency and consequently aerial CO_2 transfer were unaffected by the infusion of a base load (Fig. 3), whereas water-breathing frequency in base-infused lungfish was halved (Fig. 3), accounting for the 2.6-fold decrease in total $\dot{M}_{CO_2}$ and driving the RER value down significantly (Table 2).

Adjustment of the net excretion of acidic equivalents into the water ($J_{net}H^+$) was also important in correcting acid–base disturbances, particularly following the infusion of a base load (Fig. 4). Acid infusion resulted in a significant 5.7-fold increase in net ammonia excretion ($J_{net}NH_3$). However, owing to more variable titratable acid net flux ($J_{net}TA$) data in which only seven of 11 fish increased $J_{net}TA$ in response to acid infusion, there was no significant increase in the net excretion of acidic equivalents. By contrast, both $J_{net}H^+$ and $J_{net}TA$ values were strongly negative following base infusion, indicating that net excretion of base to the water occurred; net ammonia excretion was unaffected by base infusion.

To further investigate metabolic compensation of an infused

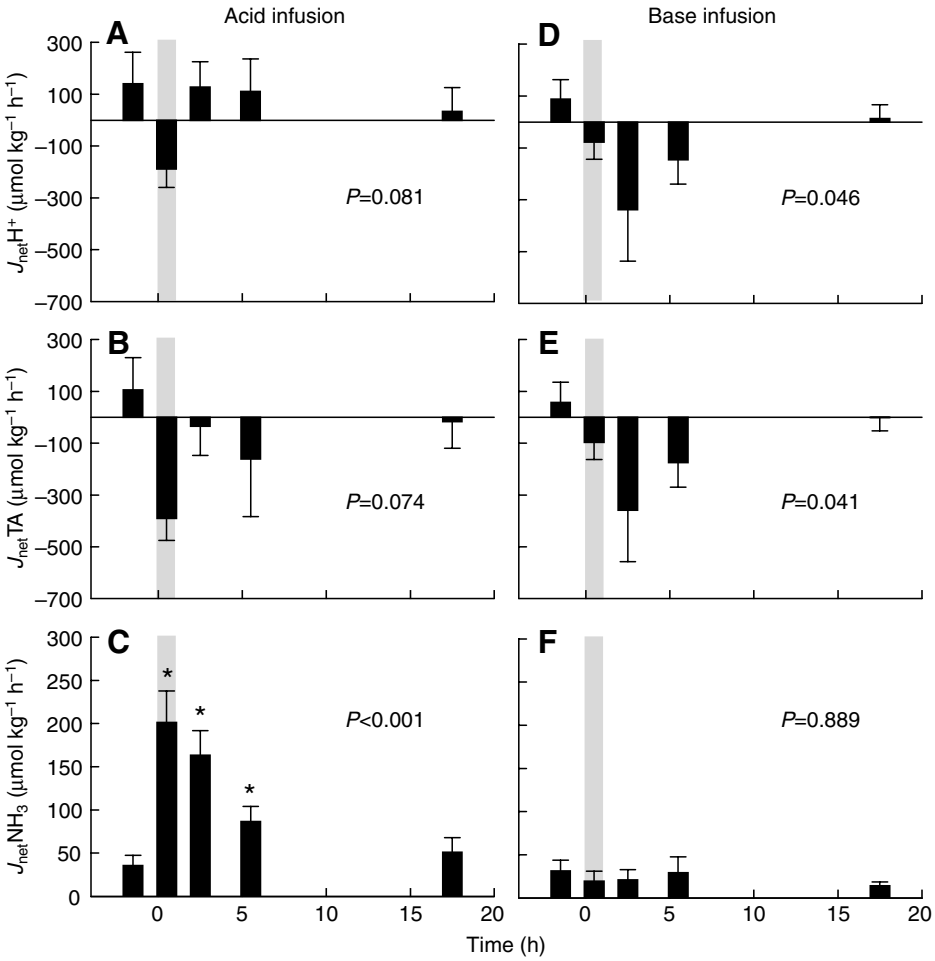

Fig. 4. The effect in African lungfish *Protopterus annectens* of 1 h of (A–C) acid (3 mmol kg^{-1} NH_4Cl) or (D–F) base (3 mmol kg^{-1} $NaHCO_3$) infusion (marked by the grey bar) on net excretion of acidic equivalents into the water ($J_{net}H^+$; A,D), titratable net acid flux ($J_{net}TA$; B,E), and net ammonia excretion ($J_{net}NH_3$; C,F). $J_{net}H^+$ was calculated as the sum of $J_{net}TA$ and $J_{net}NH_3$, signs considered. Values are means ± s.e.m. for the period prior to acid or base infusion ('pre'), and for measurement periods 0–1, 2–3, 5–6 and 17–18 h post-infusion; *N*=11 and 8 for, respectively, acid- and base-infused lungfish. An asterisk (*) indicates a significant difference from the pre-infusion value (one-way RM-ANOVA; *P* values are indicated on the figure); note that in E and F, *post hoc* multiple comparisons tests were unable to detect the origin of the significant differences indicated by the *P* values.

base load in African lungfish, a separate group of base-infused lungfish was fitted with urinary catheters, allowing the contribution of the kidneys (basic equivalents appearing in urine) to net base excretion to be distinguished from that of the gills and/or skin (basic equivalents appearing in water). Responses of total (i.e. the sum of branchial and renal) $J_{net}H^+$, $J_{net}TA$ and $J_{net}NH_3$ of these fish to base infusion were qualitatively and quantitatively comparable to those of the lungfish not fitted with urinary catheters (Fig. 5; Table 3). Moreover, branchial and renal $J_{net}H^+$, $J_{net}TA$ and $J_{net}NH_3$ responses to base infusion were

Table 3. *The effect of base infusion on the renal versus branchial excretion of acidic equivalents in African lungfish Protopterus annectens*

		Time post-infusion (h)		
	Pre	0–4	17–21	*P*
$J_{net}TA$ (μmol h^{-1} kg^{-1})				
Total	–36.7±17.1 (6)	–90.1±28.6 (6)	–177.5±6.7* (5)	0.002
Branchial	–1.78±21.5 (7)	–55.9±28.7* (7)	–75.7±18.6* (6)	0.015
Renal	–16.9±5.0 (6)	–11.8±2.6 (6)	–75.4±8.3* (5)	<0.001
$J_{net}NH_3$ (μmol h^{-1} kg^{-1})				
Total	38.2±13.1 (6)	35.2±6.7 (6)	53.8±17.8 (5)	0.454
Branchial	12.4±7.6 (7)	9.5±3.4 (7)	14.7±7.1 (6)	0.956
Renal	24.1±6.2 (6)	24.1±4.8 (6)	36.1±14.1 (5)	0.592

$J_{net}TA$, titratable acid net flux; $J_{net}NH_3$, net ammonia excretion; 'branchial' refers to excretion to the water, 'renal' refers to excretion to the urine, and 'total' represents the sum of branchial and renal values.

Values are means ± s.e.m. for the period prior to infusion (Pre), and for measurement periods 0–4 h and 17–21 h post-infusion; Number of samples are indicated in parentheses.

One-way RM-ANOVA was used to assess the statistical significance of differences among measurements periods; *P* values are indicated for each comparison and an asterisk indicates a value that was significantly different from the corresponding pre-infusion value.

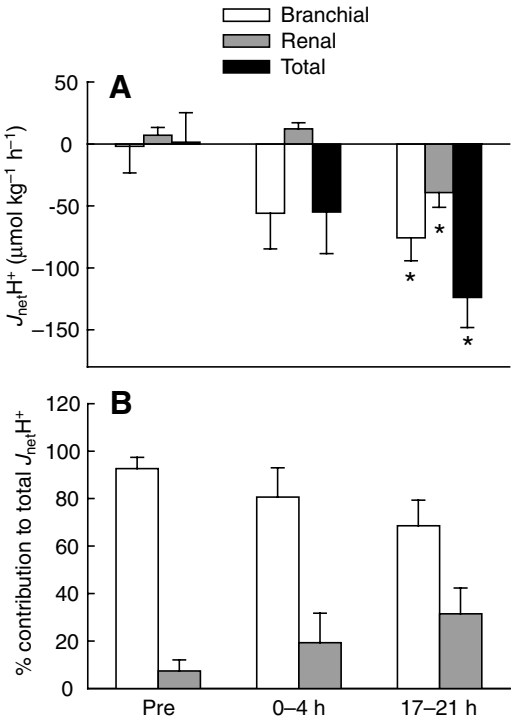

Fig. 5. The effect of 1 h of base (3 mmol kg^{-1} $NaHCO_3$) infusion (A) on net excretion of acidic equivalents ($J_{net}H^+$) into the water (branchial) or urine (kidney), as well as total excretion, and (B) on the relative contributions of branchial and renal excretion to total net excretion of acidic equivalents in African lungfish *Protopterus annectens*. Values are means ± s.e.m. for the period prior to base infusion ('pre'), and for measurement periods immediately following base infusion (0–4 h) and ~18 h post-infusion (17–21 h); *N*=5–7. In A, an asterisk (*) indicates a significant difference from the pre-infusion value (one-way RM-ANOVA with *P* values of 0.03, 0.003 and 0.008 for branchial, renal and total $J_{net}H^+$, respectively). Changes in the relative contributions of branchial and renal excretion to total $J_{net}H^+$ were not statistically significant in B.

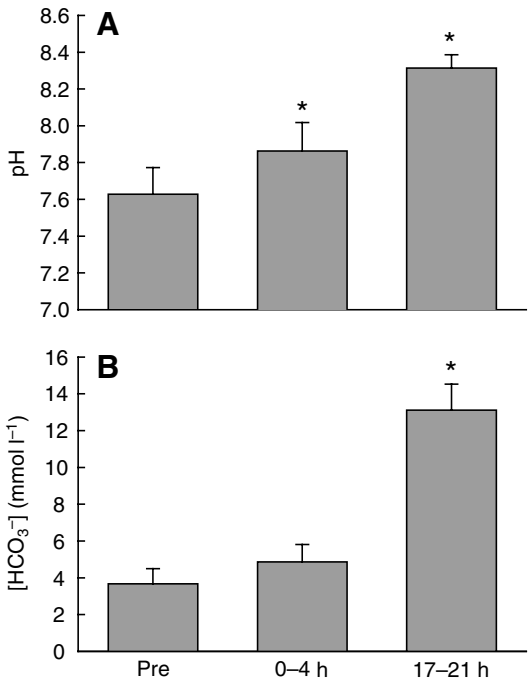

Fig. 6. The effect of a 1 h base (3 mmol kg^{-1} $NaHCO_3$) infusion in African lungfish *Protopterus annectens* on urine (A) pH and (B) HCO_3^- ion concentration ($[HCO_3^-]$). Values are means ± s.e.m. for the period prior to base infusion ('pre'), and for measurement periods immediately following base infusion (0–4 h) and ~18 h post-infusion (17–21 h); *N*=5 or 6. An asterisk (*) indicates a significant difference from the pre-infusion value [one-way RM-ANOVA with *P* values of 0.002 (A) and 0.001 (B)].

qualitatively similar, with net ammonia excretion into water and urine remaining constant and the transition to negative values of branchial and renal $J_{net}H^+$ being driven by increasingly negative $J_{net}TA$ values. Consistent with the loss of basic equivalents in the urine, urine $[HCO_3^-]$ increased 3.6-fold and urine pH rose by 0.68 units in response to base infusion (Fig. 6); urine flow rate over the experimental period did not differ significantly from the control value of 5.9±0.7 ml kg^{-1} h^{-1}(*N*=6). Quantitatively, however, $J_{net}H^+$ was dominated by branchial (and/or cutaneous) contributions to the net excretion of acidic equivalents, which accounted for >90% of $J_{net}H^+$ under control conditions (Fig. 5B). During metabolic compensation of an acid–base disturbance, the renal contribution to the transfer of acid–base equivalents appeared to increase, although the effect was not statistically significant.

Because the responses of lungfish to acid–base disturbances included changes in the net excretion of acid–base equivalents *via* the gills and kidneys, the expression of mRNA of three genes thought to be involved in both renal and branchial mechanisms of acid–base excretion was assessed. Using homology cloning strategies, a 683 base pair cDNA was assembled from lungfish gill tissue. This cDNA included a deduced protein fragment of 226 amino acid residues. A BLAST search of the GenBank protein database determined that this lungfish gene shared high sequence identity with several vertebrate NBCs, and in particular, NBC1. Protein alignment revealed that the lungfish NBC fragment (dubbed lfNBC) corresponded to residues 498–723 of the human NBC (GenBank acc. no. AAG47773), and was 73–77% identical to amphibian and mammalian NBC1s, and 65–67% identical to NBCs from other fish (Fig. 7A). A second cDNA, of 921 base pairs, was assembled from lungfish gill tissue and included a deduced protein fragment of 307 amino acid residues that was found to share high sequence identity with the B subunit, particularly the type 2 B subunit, of the V-type H^+-ATPase from several vertebrates. Using protein alignment, the lungfish H^+ V-ATPase (lfVATPase) corresponded to residues 195–511 of the human B2 subunit (GenBank acc. no. CAA44721) and was ⩾95% identical and ⩾98% similar to B subunits from a range of vertebrates including fish, amphibians, birds and mammals (Fig. 7B). Finally, degenerate primers were used to obtain a 663 base pair cDNA from lungfish blood (Fig. 8). This

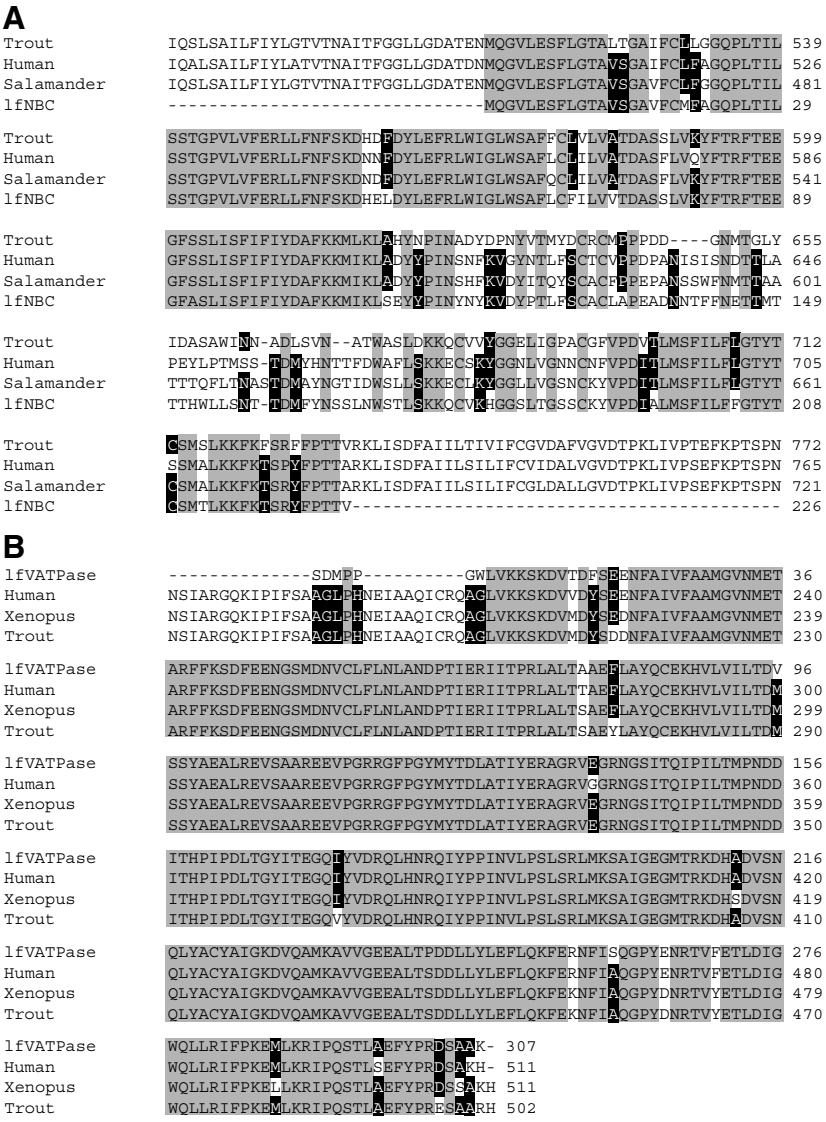

Fig. 7. The deduced amino acid sequences of the cloned fragments of African lungfish *Protopterus annectens* (A) Na^+/HCO_3^- cotransporter (NBC) and (B) V-type H^+-ATPase aligned with corresponding regions of NBC and H^+ V-ATPase sequences (from GenBank databases) for selected vertebrates. Grey shading indicates amino acid residues that are conserved across all four species, whereas black shading indicates residues that are conserved across three species. lf, lungfish. GenBank protein accession numbers for the sequences used were as follows: (A) NBC: rainbow trout AAN52239, human AAC39840, salamander (*Ambystoma tigrinum*) AAB61339; (B) H^+ V-ATPase: rainbow trout AAD33861, human AAH30640, *Xenopus* AAH46738.

cDNA encoded a deduced protein fragment of 221 amino acid residues. The collective evidence of a BLAST search of the GenBank protein database, protein alignment and phylogenetic analysis suggests that this lungfish gene is a cytoplasmic carbonic anhydrase (CA) that, based on its tissue of origin, was labelled lfCAb. Phylogenetic analysis grouped lfCAb with fish cytoplasmic (blood-specific and general cytoplasmic) isoforms as well as mammalian CA I, II, III and XIII. Similarly, lfCAb was found to be 61–65% identical and 77% similar to blood CA isoforms from zebrafish, trout, carp and gar (GenBank protein acc. no. NP_571185, AAP73748, AAZ83748 and AAM94169, respectively) as well as 60% identical and 78% similar to mouse and rat CA XIII (NP_078771 and XP_574890, respectively). Examination of the mRNA expression for these three lungfish genes, lfNBC, lfVATPase and lfCAb, in gill and kidney tissue 4 h and 8 h following a 1 h infusion of acid or base did not reveal any statistically significant changes (Fig. 9).

Discussion

The results of the present study demonstrate that the African lungfish *P. annectens* utilizes both respiratory and metabolic compensation to recover from metabolic acid–base disturbances. In modulating ventilation to meet the demands of acid–base regulation, *P. annectens* adopts what is considered to be a primarily tetrapod strategy (for a review, see Perry and Gilmour, 2006). By contrast, metabolic compensation of pH imbalances in *P. annectens* relies predominantly on the exchange of acid–base equivalents across the gills (and/or skin, although with a possible role for the intestine) rather than on urinary excretion of acid–base equivalents, a strategy that is distinctly fish-like (for a review, see Perry and Gilmour, 2006). Because CO_2 excretion and net H^+ excretion were not monitored continuously following acid or base infusion, it was not possible to accurately quantify the relative contributions of respiratory and metabolic mechanisms to acid–base compensation. However, estimates can be obtained by examination of the 1 h periods of greatest excretion ('peak' values), providing at least a snapshot of the relative contributions of the different mechanisms. Lungfish received an acid or base load of 3 mmol kg^{-1}. Following acid infusion, CO_2 excretion into air and water peaked at 0.62 mmol kg^{-1} above the pre-infusion value, while net H^+ excretion peaked at 0.18 mmol kg^{-1} in excess of the pre-infusion value. Thus, peak CO_2 and H^+ excretion (for a 1 h period) amounted to a loss of 27% of the total acid load, with respiratory compensation accounting for 77% of this value, and metabolic compensation, 23%. Respiratory and metabolic contributions were more balanced following a base load, accounting for 46% and 54%, respectively, of the 0.91 mmol kg^{-1} of the base load that was lost during the 1 h period of greatest excretion (30% of the total base load).

The compensatory strategies of *P. annectens* reflect the unique opportunities available to bimodally breathing animals that inhabit an aquatic environment. Yet despite the opportunity afforded by such animals to understand acid–base

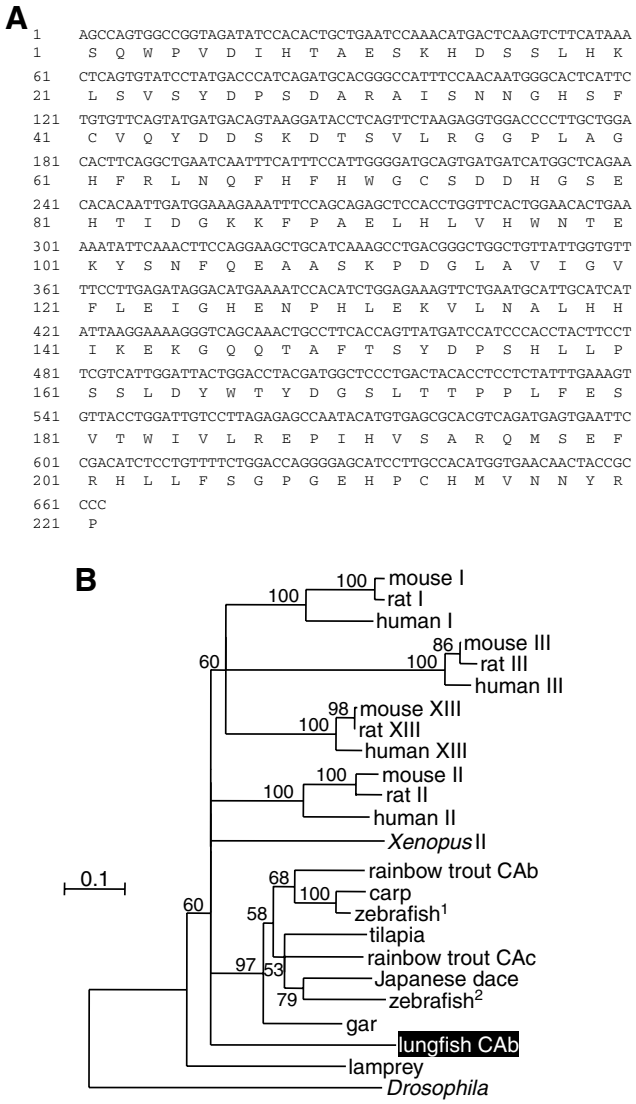

Fig. 8. (A) The nucleotide and deduced amino acid sequences of the carbonic anhydrase (CA) fragment cloned from African lungfish *Protopterus annectens* blood, together with (B) a phylogenetic tree to illustrate the relationship between this lungfish CA (highlighted in black) and selected vertebrate cytoplasmic CA isoforms. The phylogenetic tree was constructed using neighbour-joining analysis (see Materials and methods for more detail), and was ordered using *Drosophila* CA (GenBank protein accession AAY56645) as a monophyletic outgroup. Horizontal branch lengths are scaled to represent the relative number of amino acid substitutions occurring along a branch, and support values at the nodes are indicated as a percentage from bootstrap analysis using 100 pseudoreplicates. GenBank protein accession numbers for the sequences used in the tree were as follows. CA I: mouse AAH11223, rat XP_226922, human AAH27890; CA II: mouse AAA37357, rat CAA41227, human AAA51909, *Xenopus* CAJ83242; CA III: mouse NP_031632, rat AAA40846, human AAA52293, CA XIII: mouse NP_078771, rat XP_222295, human NP_940986; fish cytoplasmic CAs: lamprey AAZ83742, gar AAM94169, tilapia AAQ89896, rainbow trout blood isoform (CAb) AAP73748, rainbow trout cytoplasmic isoform (CAc) AAR99329, zebrafish[1] NP_571185, zebrafish[2] NP_954685, Japanese dace BAB83090, and carp AAZ83743. Sequences obtained from GenBank were truncated appropriately so as to use only the region that overlapped with the lungfish CAb sequence.

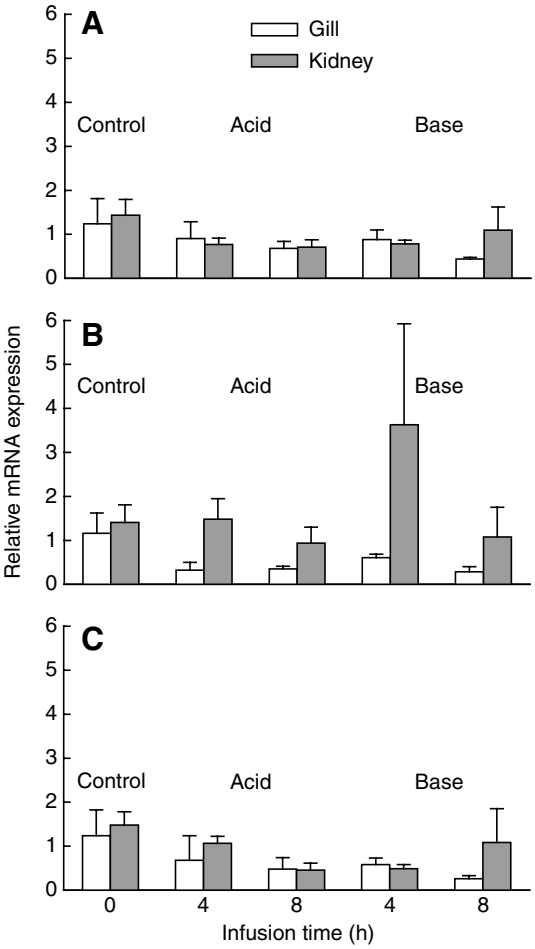

Fig. 9. Relative mRNA expression as determined by real-time PCR for (A) Na^+/HCO_3^- cotransporter (NBC), (B) V-type H^+-ATPase and (C) lungfish carbonic anhydrase b isoform (CAb) in gill and kidney tissue sampled from lungfish infused with saline (control), acid (3 mmol kg^{-1} NH_4Cl) or base (3 mmol kg^{-1} $NaHCO_3$) for 1 h and sampled 4 h or 8 h post-infusion (control fish were sampled only at 8 h). Values are means ± 1 s.e.m.; N=3 or 4. No statistically significant differences in the expression of a gene within a tissue were detected as a result of acid or base infusion (one-way ANOVA, P>0.05 in all cases).

strategies, few studies have investigated, and none appear to have attempted to quantify, the relative involvement of respiratory *versus* metabolic compensatory mechanisms in bimodal breathers (reviewed by Truchot, 1987). The involvement of air breathing in the recovery from acid–base disturbances elicited by exhaustive exercise was assessed for two fish species that are facultative air-breathers, bowfin *Amia calva* and spotted gar *Lepisosteus oculatus* (Burleson et al., 1998; Gonzalez et al., 2001). Both studies concluded that air breathing delayed compensation of the post-exercise acid–base disturbance because branchial ventilation was not increased to the extent that occurs in unimodal water breathers, thereby hindering branchial exchange of acid–base equivalents and CO_2 elimination, processes critical for the restoration of

acid–base balance (Burleson et al., 1998; Gonzalez et al., 2001). Neither study, however, assessed metabolic compensation post-exercise. The blood acid–base responses of lungfish in the present study were very comparable to those of channel catfish, a unimodal water-breather, subjected to virtually the same infusion protocol (Cameron and Kormanik, 1982), implying that air-breathing in lungfish did not appear to delay recovery. Indeed, CO_2 loss *via* the lungs of *P. annectens* should have aided recovery from an infused acid load, a difference from bowfin and gar that may reflect the obligate nature of pulmonary gas transfer in lungfish *versus* the facultative role it plays in bowfin and gar.

The best-studied bimodal breathers are amphibians, among which responses to acid–base disturbances reflect the degree of terrestrialization of the species (reviewed by Toews and Boutilier, 1986; Truchot, 1987; Toews and Stiffler, 1989). Amphibians that are predominantly aquatic rely on the cutaneous exchange of acid–base equivalents to adjust extracellular pH (reviewed by Stiffler, 1989). By contrast, semi-terrestrial amphibians with well-developed lung ventilation, such as adult anurans, exhibit respiratory compensation supported to a variable extent by the exchange of acid–base equivalents *via* the kidney/bladder or skin (e.g. Boutilier et al., 1979; Tufts and Toews, 1985; Boutilier and Heisler, 1988; Stiffler, 1991).

The use of ventilatory adjustments to compensate for acid–base disturbances by semi-terrestrial but not predominantly aquatic amphibians parallels the situation in fish – water-breathing fish do not appear to modulate ventilation to correct pH imbalances (for a review, see Perry and Gilmour, 2006), whereas *P. annectens* clearly utilized respiratory compensation (Figs 2 and 3). The implication of this finding is that *P. annectens*, like tetrapod vertebrates, possesses CO_2 and/or pH-sensitive chemoreceptors that monitor the status of the body fluids. In tetrapods, central and peripheral CO_2/pH-sensitive chemoreceptors initiate ventilatory adjustments for acid–base regulation (reviewed by Gonzalez et al., 1994; Milsom, 1995; Milsom, 2002; Taylor et al., 1999). By contrast, the balance of evidence suggests that strictly water-breathing fish lack internally oriented CO_2/pH chemoreceptors – central chemoreceptors appear to be absent, and ventilatory chemoreflexes are dominated by branchial chemoreceptors that respond primarily to changes in water CO_2 (reviewed by Gilmour and Perry, 2007). Although the locations of CO_2/pH chemoreceptors in *P. annectens* remain to be determined, work on the South American lungfish *Lepidosiren paradoxa* suggests the existence of both central and peripheral CO_2 and/or pH chemoreceptors (Sanchez et al., 2001; Amin-Naves et al., 2007). In *Lepidosiren*, pulmonary ventilation increased as cerebrospinal fluid (CSF) pH was lowered by perfusing the fourth cerebral ventricle with mock CSF, providing evidence of central CO_2/pH chemosensitivity (Sanchez et al., 2001). Similar manipulation of CSF pH moderated but did not eliminate pulmonary ventilation responses to (combined) aerial and aquatic hypercapnia, a result that supports the existence of peripheral CO_2/pH chemoreceptors in *Lepidosiren* but without providing information on their orientation (water/air *versus* blood) (Amin-Naves et al., 2007). Interestingly, the data for *P. annectens* indicate that branchial as well as pulmonary ventilation is sensitive to internal acid–base status, a possibility that was not examined in the studies on *Lepidosiren*. Similarities (e.g. in acid–infused lungfish; Fig. 2) and differences (e.g. in base-infused lungfish; Fig. 3) between pulmonary and branchial ventilatory responses to acid–base disturbances suggest that both shared and independent control systems are present.

Although the modulation of ventilation to correct acid–base disturbances, and the underlying implication that internal CO_2/pH sensors must be present evokes a tetrapod scenario of acid–base regulation for *P. annectens*, the primary reliance on branchial (and/or cutaneous) rather than renal routes for the exchange of acid–base equivalents is typical of fish and predominantly aquatic amphibians. In *P. annectens*, partitioning of the peak excretion of a base load into branchial/cutaneous *versus* renal contributions revealed that 62% was eliminated into the water, and 38% into urine. Urine is similarly a minor route of net acid efflux during acid–base disturbances in fish (Kobayashi and Wood, 1980; Wood and Jackson, 1980; Cameron and Kormanik, 1982; Wood, 1991; Curtis and Wood, 1992) and aquatic amphibians (Stiffler and Bachoura, 1991; Stiffler, 1991; Talbot and Stiffler, 1992), with extra-renal excretion of acid–base equivalents playing a dominant role. In fish, up to 90% or more of acid–base movements occur across the branchial epithelium (Claiborne et al., 2002; Evans et al., 2005), whereas cutaneous acid–base excretion appears to predominate in aquatic amphibians (Stiffler, 1989). These widely accepted views are based primarily on morphological considerations rather than on explicit attempts to partition extra-renal net acid efflux into branchial *versus* cutaneous components. For example, the role of the gill in correcting pH imbalances in fish is strongly supported by the abundance of ion-transporting MR cells in the branchial epithelium coupled with remodelling of this epithelium in response to acid–base disturbances (reviewed by Goss et al., 1992; Goss et al., 1994; Goss et al., 1995; Laurent and Perry, 1995; Perry and Gilmour, 2006). MR cells are found in both the branchial epithelium and skin of *P. annectens* (Sturla et al., 2001), indicating that either surface could contribute to the movement of acid–base equivalents, but the gills may be more likely to play a larger role. Whereas the skin is covered by a protective coating of scales, the gills are actively ventilated and involved in CO_2 excretion, as evidenced by the relationship between ventilation frequency and $\dot{M}_{CO_2}$ (Figs 2 and 3), and the hydration of CO_2 provides H^+ and HCO_3^- for exchange with the environment (see below). However, whether the branchial epithelium of *P. annectens* is a more important contributor to metabolic acid–base regulation than the skin requires empirical confirmation.

The molecular mechanisms involved in the movement of acid–base equivalents across the gills of freshwater fish remain a subject of debate. Recent models (reviewed by Perry and Gilmour, 2006) suggest that acid secretion reflects the

combined actions of apical membrane H^+ efflux, probably *via* a V-type H^+-ATPase in freshwater fish, and basolateral membrane HCO_3^- efflux by means of Cl^-/HCO_3^- exchange and/or NBC. Mechanisms for base excretion are probably located in a different cell type, and involve apical membrane HCO_3^- efflux *via* Cl^-/HCO_3^- exchange coupled to basolateral H^+ efflux by a V-type H^+-ATPase. In both cases, the hydration of molecular CO_2 catalyzed by a high activity cytosolic CA provides the necessary protons and HCO_3^- ions. Several components of these mechanisms, specifically NBC, V-type H^+-ATPase, and cytosolic CA, were cloned from lungfish tissues (Figs 7 and 8), but no evidence of changes in mRNA expression was detected in the gills following acid or base infusion (Fig. 9). This result was somewhat surprisingly given the considerable support for transcriptional regulation of branchial proteins during acid–base disturbances in other fish (Galvez et al., 2002; Hirata et al., 2003; Perry et al., 2003a; Perry et al., 2003b; Georgalis et al., 2006b). It is possible that the acid–base challenges were not sufficient to activate transcriptional regulation, that isolation of discrete cell types (acid *versus* base excreting) is necessary to detect such changes, or that post-translational mechanisms are involved, as recently reported for dogfish (Tresguerres et al., 2005; Tresguerres et al., 2006). Alternatively, the sampling times (4 and 8 h post infusion) and/or animal numbers may not have been appropriate or sufficient to detect changes that did occur; unfortunately, these were constrained by animal availability.

The kidney of *P. annectens*, although quantitatively less important than the gill/skin, nevertheless played a significant role in eliminating an infused base load, a finding that is true for other freshwater fish (Cameron and Kormanik, 1982; Curtis and Wood, 1992; Wood et al., 1999). Moreover, the freshwater teleost kidney exhibits a flexibility of response that is comparable to that of the mammalian kidney (Wood et al., 1999), a statement that arguably can also be applied to the lungfish. Urine flow rate in *P. annectens* in the present study, at ~6 ml kg^{-1} h^{-1}, was within the range of values reported previously for *Protopterus* sp., ~2–6 ml kg^{-1} h^{-1} (Sawyer, 1965; Sawyer, 1970; Babiker and Rankin, 1979), although somewhat higher than values for rainbow trout (*Oncorhynchus mykiss*) or channel catfish (*Ictalurus punctatus*), ~2–4 ml kg^{-1} h^{-1} (Cameron and Kormanik, 1982; Curtis and Wood, 1992; Wood et al., 1999). This result is surprising in view of the reduced gill surface area of lungfish, and may indicate a degree of stress-induced diuresis (Babiker and Rankin, 1979). A high control value may also have masked any tendency for metabolic alkalosis to increase urine flow rate, as observed previously in trout and catfish (Cameron and Kormanik, 1982; Curtis and Wood, 1992; Wood et al., 1999). As in other freshwater fish as well as mammals (Cameron and Kormanik, 1982; Curtis and Wood, 1992; Wood et al., 1999), the renal response of *P. annectens* to a metabolic alkalosis was characterized by greatly elevated urinary HCO_3^- loss (Fig. 6), presumably reflecting increased HCO_3^- filtration that was not matched by a corresponding increase in tubular HCO_3^- reabsorption.

The mechanisms involved in renal HCO_3^- reabsorption appear to be similar in freshwater fish and mammals (Perry et al., 2003b; Perry and Gilmour, 2006; Georgalis et al., 2006a). In mammals, filtered HCO_3^- ions combine with protons secreted *via* apically localized Na^+/H^+ exchangers (NHE3) and/or V-type H^+-ATPase. Molecular CO_2 formation is catalyzed by membrane-associated CA IV; CO_2 diffuses into the tubule cell and is hydrated in the presence of cytosolic CA. While protons are recycled into the tubule lumen, HCO_3^- ions move across the basolateral membrane *via* NBC1, resulting in a net transfer of HCO_3^- ions from filtrate to blood. In *P. annectens*, mRNA expression of NBC1, V-type H^+-ATPase and cytosolic CA was detected in kidney tissue, suggesting that these components of the HCO_3^- reabsorption mechanism are present, but expression was not affected by acid or base loading (Fig. 9). As discussed above for the branchial mRNA expression of acid–base relevant proteins, the apparent lack of transcriptional regulation in lungfish was surprising in view of responses in both fish and mammals (Tsuruoka et al., 1998; Amlal et al., 2001; Perry et al., 2003a; Perry et al., 2003b; Georgalis et al., 2006a), and may reflect the selection of inappropriate sampling times, insufficient sample number, that increased activities of these proteins are not required to respond to the acid–base disturbance, or the involvement of posttranslational modifications, as reported for mammalian renal tubules (Amlal et al., 2001; Nakhoul and Hamm, 2002).

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