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An investigation of the role of carbonic anhydrase in aquatic and aerial gas transfer in the African lungfish *Protopterus dolloi*

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Summary

Experiments were performed on bimodally breathing African lungfish *Protopterus dolloi* to examine the effects of inhibition of extracellular *vs* total (extracellular and intracellular) carbonic anhydrase (CA) activity on pulmonary and branchial/cutaneous gas transfer. In contrast to previous studies on *Protopterus*, which showed that the vast majority of CO_2 is excreted into the water through the gill and/or skin whereas O_2 uptake largely occurs *via* the lung, *P. dolloi* appeared to use the lung for the bulk of both O_2 uptake (91.0±2.9%) and CO_2 excretion (76.0±6.6%). In support of the lung as the more important site of CO_2 transfer, aerial hypercapnia (P_{CO_2}=40 mmHg) caused a significant rise in partial pressure of arterial blood CO_2 (Pa_{CO_2}) whereas a similar degree of aquatic hypercapnia was without effect on Pa_{CO_2}. Intravascular injection of low levels (1.2 mg kg^{-1}) of the slowly permanent CA inhibitor, benzolamide, was without effect on red blood cell CA activity after 30 min, thus confirming its suitability as a short-term selective inhibitor of extracellular CA. Benzolamide treatment did not affect CO_2 excretion, blood acid–base status or any other measured variable within the 30 min measurement period. Injection of the permeant CA inhibitor acetazolamide (30 mg kg^{-1}) resulted in the complete inhibition of red cell CA activity within 10 min. However, CO_2 excretion (measured for 2 h after injection) and arterial blood acid–base status (assessed for 24 h after injection) were unaffected by acetazolamide treatment. Intra-arterial injection of bovine CA (2 mg kg^{-1}) caused a significant increase in overall CO_2 excretion (from 0.41±0.03 to 0.58±0.03 mmol kg^{-1} h^{-1}) and an increase in air breathing frequency (from 19.0±1.3 to 24.7±1.8 breaths min^{-1}) that was accompanied by a slight, but significant, reduction in Pa_{CO_2} (from 21.6±1.6 to 19.6±1.8 mmHg).

The findings of this study are significant because they (i) demonstrate that, unlike in other species of African lungfish that have been examined, the gill/skin is not the major route of CO_2 excretion in *P. dolloi*, and (ii) suggest that CO_2 excretion in *Protopterus* may be less reliant on carbonic anhydrase than in most other fish species.

Key words: carbon dioxide excretion, oxygen uptake, gill, lung, acetazolamide, benzolamide, breathing, lungfish, *Protopterus dolloi*, carbonic anhydrase.

Introduction

Among the numerous species of air-breathing fishes, only three extant genera of lungfish (*Neoceratodus*, *Lepidosiren* and *Protopterus*) exploit true lungs for aerial gas transfer (Graham, 1997). The Australian lungfish *Neoceratodus forsteri* is a facultative air-breather whereas the South American lungfish *Lepidosiren paradoxa* and the African lungfish (genus *Protopterus*) are obligate air breathers that cannot survive without access to air (Burggren and Johansen, 1986; Graham, 1997). The four species of *Protopterus* share, along with their capacity for pulmonary gas transfer, the ability to withstand extended conditions of water deprivation by entering into a state of aestivation (Fishman et al., 1986). Typically, the aestivating state is characterized by formation of a mucus cocoon, metabolic depression, total reliance on aerial gas transfer and an increase in hepatic ureagenesis (Smith, 1930; Fishman et al., 1986; Greenwood, 1986).

Although the lung is the primary site of O_2 uptake in *Protopterus*, the gill (and/or the skin) is believed to be the major route for CO_2 excretion (Lenfant and Johansen, 1968; Lahiri et al., 1970; McMahon, 1970; Babiker, 1979). Thus, even though African lungfish are bimodal breathers, the gill appears to be unimportant in O_2 uptake and the lung relatively unimportant in CO_2 excretion. The reduced reliance on the lung for CO_2 excretion under resting conditions is also a feature of Australian (Lenfant et al., 1966) and South American (Johansen and Lenfant, 1967) lungfish. In comparison to most fish, the gills of lungfish have small surface areas and large blood-to-water diffusion distances, and are ventilated by low

volumes of water (Burggren and Johansen, 1986). Because of the much higher (~30-fold) capacitance of the water for CO_2 relative to O_2, the low ventilation volumes may be adequate to sustain normal rates of CO_2 excretion but inadequate for similar rates of O_2 uptake (Graham, 1997). It was recently demonstrated that the overall contribution of the lung of *Lepidosiren* to CO_2 excretion can increase markedly with increasing metabolic rate (Amin-Naves et al., 2004), indicating that the lungfish lung can effectively excrete CO_2 under the appropriate conditions.

CO_2 transfer across the teleost gill has been demonstrated to behave as a diffusion limited system (Bindon et al., 1994; Greco et al., 1995; Julio et al., 2000) owing to chemical equilibrium limitations (Julio et al., 2000; Desforges et al., 2002). Essentially, the chemical equilibrium limitations reflect the relatively slow rate of conversion of blood HCO_3^- to CO_2 during the brief period of gill transit. Indeed, recent models for branchial gas transfer in teleosts (Perry and Gilmour, 2002) suggest that CO_2 transfer is more apt to be affected by diffusion limitations than is O_2 transfer. Thus, the fact that the lungfish gill is likely to display pronounced diffusion limitations implies that there might be other factors (in addition to low ventilation volumes) to explain why the reduced lungfish gill does not transfer O_2 while excreting CO_2 effectively.

In the present study, two possible mechanisms for the preferential excretion of CO_2 by the gill of African lungfish were investigated. First, we hypothesised that, as in dogfish, extracellular branchial membrane-associated carbonic anhydrase (CA) aids gill CO_2 excretion by allowing the catalysed dehydration of HCO_3^- within the plasma (Gilmour et al., 2001; Gilmour and Perry, 2004). Assuming a high enough buffer capacity in the plasma (Desforges et al., 2001; Gilmour et al., 2004), this would allow CO_2 excretion to occur without a requirement for red blood cell (RBC) Cl^-/HCO_3^- exchange, which is the rate-limiting step in CO_2 excretion (Perry, 1986) and the origin of chemical equilibrium limitations (Gilmour et al., 2004). To test this hypothesis, aerial and aquatic gas transfers were measured before and after inhibition of extracellular CA using a relatively impermeant CA inhibitor, benzolamide (BZ). A second possible explanation for the preferential role of the gill in CO_2 excretion is that the high P_{CO_2} that is characteristic of lungfish blood (Lenfant and Johansen, 1968) obviates the need for catalysed dehydration of plasma HCO_3^-, because blood-to-water P_{CO_2} partial pressure gradients are large enough to sustain CO_2 excretion by diffusion alone. While potentially effective at the gill, a similar scenario might be less likely at the lung owing to a much smaller blood-to-gas diffusion gradient, reflecting the loss of CO_2 at the gill and the mixing of inspired and expired gas within the lung. To test this hypothesis, aerial and aquatic gas transfers were measured before and after inhibition of total CA activity using a permeant CA inhibitor, acetazolamide (AZ). In a final series of experiments, fish were injected with exogenous CA to test the hypothesis that CO_2 excretion in *Protopterus* is constrained by inadequate catalysis of HCO_3^- dehydration. All experiments were performed on *Protopterus dolloi*, a species that, to our knowledge, has not been used previously in studies investigating the mechanisms of gas transfer in African lungfish.

Materials and methods

Experimental animals

Experiments were performed both in Singapore (National University of Singapore) and Ottawa (University of Ottawa) using lungfish derived from a common source. Adult specimens of African lungfish *Protopterus dolloi* Boulenger, weighing 159±9 g [mean ± standard error of the mean (S.E.M.), *N*=25], were imported from Central Africa through a local fish farm in Singapore. Specimens were maintained in plastic aquaria filled with dechlorinated water containing 2.3 mmol l^{-1} Na^+, 0.54 mmol l^{-1} K^+, 0.95 mmol l^{-1} Ca^{2+}, 0.08 mmol l^{-1} Mg^{2+}, 3.4 mmol l^{-1} Cl^- and 0.6 mmol l^{-1} mol l^{-1} HCO_3^-, at pH 7.0 and at 25°C in the laboratory, and the water was changed daily. No attempt was made to separate the sexes. The specimens were acclimated to laboratory conditions for at least 1 month. During the acclimation period, specimens were fed frozen mosquito larvae (blood worms). Food was withdrawn 96 h prior to experiments, which gave sufficient time for the gut to be emptied of all food and waste.

Fish were shipped by air cargo in partially filled bags of oxygenated water contained within insulated containers to Ottawa. Upon their arrival, fish were placed individually into covered plastic containers containing 2–3 l of dechloraminated city of Ottawa tapwater warmed to 25°C. To maintain this water temperature, the air temperature was held at 25°C. The tank water was changed on alternate days or sooner if there was obvious fouling of the medium. The fish were kept in a sealed room 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. Fish were allowed to acclimate to these conditions for at least 1 month prior to beginning experiments.

Surgical procedures

Fish were anaesthetized in a solution of MS-222 (ethyl-*p*-aminobenzoate; 1.0 g l^{-1}) adjusted to neutral pH with $NaHCO_3$ (2 g l^{-1}). After cessation of breathing movements, the fish were transferred to an operating table where they were draped with paper towels soaked with anaesthetic solution. In this way, they were kept moist and deeply anaesthetized for the duration of the surgery. To allow periodic blood sampling, a cannula (Clay-Adams PE 50 polyethylene tubing) was inserted into the dorsal aorta (DA) according to standard surgical procedures (Axelsson and Fritsche, 1994). Briefly, a lateral incision (~2 cm in length) was made at the level of the vent approximately 3 mm below the lateral line. The DA was exposed and the cannula was inserted *via* a small incision and advanced at least 3 cm in the anterior direction. The incision was sutured using a running stitch and the cannula was then secured to the body wall with silk ligatures, filled with heparinized (100 units ml^{-1}) saline (140 mmol l^{-1} NaCl), and

heat-sealed. Fish were retuned to their containers where they were allowed to recover from surgery for ~24 h.

Experimental protocol

Fish were placed into customized respirometry chambers approximately 2 h prior to beginning an experiment. The chambers were filled with water (1.5 l) except for an adjustable (60 ml maximum volume) air space at one end of the respirometer. Once aware of the presence of the air space, the fish would typically position themselves just underneath it and begin breathing air at regular intervals. Initially, both compartments were provided with continually flowing media (water or air). However, during measurements of rates of O_2 consumption ($\dot{M}_{O_2}$) or CO_2 excretion ($\dot{M}_{CO_2}$), the water was recirculated using a peristaltic pump and the air chamber was sealed. O_2 and CO_2 electrodes inserted into the air chamber were used to measure aerial P_{O_2} and P_{CO_2}. To measure aquatic P_{CO_2}, water was pumped *via* a peristaltic pump into the sample compartment of a CO_2 electrode (Cameron Instruments, Port Aransas, TX, USA) housed within a thermostatted (25°C) cuvette (Radiometer, Copenhagen, Denmark) and returned to the respirometer. To measure aquatic P_{O_2}, an O_2 electrode was inserted into the recirculating water loop.

Series 1. Partitioning of gas transfer between air (lung) and water (gill/skin)

After establishing that the fish was positioned correctly under the air chamber and breathing air at regular intervals, the flows of freshwater and air were stopped, the water and air chambers were sealed, and the water was re-circulated to provide mixing. Changes in aerial and aquatic P_{CO_2} and P_{O_2} as well as air breathing frequency were monitored for approximately 30 min or until stable rates of CO_2 accumulation and O_2 depletion were achieved. Preliminary experiments were performed to determine the extent of gas transfer across the air–water interface. To determine the potential for aerial CO_2 to be transferred to the water and thus be misinterpreted as CO_2 excretion into the water, mixtures of CO_2 and air were added incrementally to the air chamber. After sealing the air chamber, the water P_{CO_2} was monitored without a fish in the respirometer. To determine the potential for aquatic CO_2 to be transferred to the air space and thus be misinterpreted as aerial CO_2 excretion, fish were euthanized by lethal injection while in the respirometer after aerial and aquatic P_{CO_2} had been allowed to rise naturally to high levels. The air-chamber was then flushed with air to lower P_{CO_2} and after re-sealing the chamber, the P_{CO_2} of the air was monitored while the water P_{CO_2} remained at a high level.

Initial experiments revealed substantial $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ in the absence of a fish in the respirometer, or during trials using recently euthanized fish. This background gas transfer presumably reflected cutaneous metabolism or metabolism arising from microorganisms within the water or attached to the fish. Thus, all experiments were terminated by euthanizing the fish (followed by intra-arterial injection of saturated KCl to stop the heart) *in situ* and performing a final respirometry trial for a further 30–60 min. For each fish, the values for $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were corrected for background metabolism by subtracting the rates determined on such trials.

Series 2. Effects of CA inhibition

The standard experimental protocol consisted of monitoring water/air P_{O_2} and P_{CO_2} in addition to air breathing frequency for three consecutive 30 min intervals. The first interval consisted of a control period, after which a blood sample (0.6 ml) was withdrawn from the DA cannula and P_{O_2}, P_{CO_2} and pH immediately analyzed. The remaining blood was centrifuged (1 min × 10 000 ***g***) and the pellet was combined with 0.6 ml of distilled water prior to being flash-frozen in liquid N_2. Red blood cell lysates were stored at –80°C for subsequent analysis of CA activity.

After the control period, the air chamber was flushed with air and re-sealed; the water compartment was occasionally flushed and re-sealed depending upon the extent of CO_2 accumulation during the control run. Once baseline rates of O_2 and CO_2 transfer had been re-established, fish were injected *via* the DA cannula with BZ (1.2 mg kg^{-1} using an injection volume of 1.0 ml kg^{-1}), a CA inhibitor that is relatively slow to cross RBC membranes (Travis et al., 1964). Respirometry was performed for 30 min and blood samples were withdrawn at 10 and 30 min and treated as described above. After flushing and re-sealing, a final 30 min respirometry period ensued following injection of AZ (30 mg kg^{-1} using an injection volume of 1.0 ml kg^{-1}); blood samples were withdrawn at 10 and 30 min. Fish were then euthanized to determine background $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$. In a separate group of fish (N=5), gas transfer and blood gases were monitored for 2 and 24 h, respectively, after AZ injection.

Series 3. Effects of exogenous CA

After a 30 min control period of respirometry and blood sampling, fish were injected *via* the DA cannula with bovine CA (25 mg kg^{-1} using a 0.5 ml injection volume) dissolved in 140 mmol l^{-1} NaCl. A second 30 min period of respirometry was conducted, after which a final blood sample was withdrawn. The blood samples in this series were treated as above except that a RBC lysate was not prepared; instead, the plasma was collected, frozen in liquid N_2 and stored at –80°C for subsequent determination of plasma CA activities.

Series 4. Effects of hypercapnia on blood acid–base status

Aquatic hypercapnia was established by replacing the air supplying a water/gas equilibration column with mixtures of CO_2 and air provided by a gas mixing pump (Wösthoff, Bochum, Germany). The desired water P_{CO_2} of 40 mmHg was pre-set by adjusting the rate of water and/or gas flow through the column. To rapidly achieve aquatic hypercapnia within the respirometer, the bulk of water was removed and replaced with water derived from the water/gas equilibrium column. After 30 min, aquatic normocapnia was re-established and 30 min of aerial hypercapnia was imposed by gassing the air chamber with a mixture of 5% CO_2 and 95% air to yield a final nominal

inspired P_{CO_2} of 37.5 mmHg. Owing to the very high P_{CO_2} gradients between air and water, which resulted in transfer of CO_2 across the air–water interface, no attempts were made to measure $\dot{M}_{CO_2}$ during aquatic or aerial hypercapnia.

Blood samples (0.6 ml) were withdrawn from the DA cannula after 30 min of normocapnia, aquatic hypercapnia and aerial hypercapnia. These samples were analysed for pH and total CO_2 (C_{CO_2}).

Analytical procedures

For experiments conducted in Singapore

In series 1–3 experiments, arterial blood P_{O_2} (Pa_{O_2}) was measured using an Ocean Optics (Dundedin, FL, USA) fibre-optic O_2 sensing system. A fibre-optic O_2 sensor (FOXY AL300; Dundedin, FL, USA) was inserted into a blood-filled syringe. Arterial blood P_{CO_2} (Pa_{CO_2}) was measured by injecting blood into the sample compartment (maintained at 25°C) of a P_{CO_2} (Cameron Instruments) electrode that was connected to a three-channel blood gas analyser (Cameron Instruments). Blood pH (pHa) was determined using a blood gas analyser (Medica, Bedford, MA, USA) that was thermostatted to 37°C. The measured pH was then adjusted for the temperature differential (12°C) between the analyser and fish using a correction factor of –0.018 pH units $°C^{-1}$ (Reeves, 1977).

For experiments conducted in Ottawa

In series 1–3 experiments, Pa_{O_2}, Pa_{CO_2} and pHa were monitored by injecting blood into the sample compartments (maintained at 25°C) of P_{O_2}, P_{CO_2} (Cameron Instruments) and pH (Metrohm combination 6.0204.100; Herizau, Switzerland) electrodes that were connected in series; the electrodes were linked to a three-channel blood gas analyser (Cameron Instruments). In series 4 experiments, plasma pH was measured using a Cameron pH electrode contained within a temperature controlled chamber and connected to a Radiometer blood gas analyser. Plasma C_{CO_2} was determined in duplicate on 50 μl samples using a Capnicon V total CO_2 analyzer (Cameron Instruments). Plasma P_{CO_2} and [HCO_3^-] were calculated using the Henderson–Hasselbalch equation and physiochemical constants for trout plasma (Boutilier et al., 1984).

In both Singapore and Ottawa, the P_{CO_2} and P_{O_2} within the air compartment were monitored continually by inserting and sealing CO_2 (Cameron Instruments) and fibre-optic O_2 (Ocean Optics AL300) electrodes into the air space of the respirometer. The P_{O_2} of the water (Pw_{O_2}) was monitored continually by inserting a fibre-optic O_2 electrode (Ocean Optics AL300) into the tubing through which water was re-circulating. The Pw_{O_2} was monitored by moving water via a peristaltic pump through the sample compartment of a CO_2 electrode (Cameron Instruments) housed within a thermostatted (25°C) cuvette (Radiometer) before returning it to the respirometer. The P_{CO_2} electrodes were connected to a second three-channel blood gas analyzer (Cameron Instruments) that was customized to accept two CO_2 inputs. Output from the blood gas analyzers was converted to digital data and stored by interfacing with a data acquisition system (Biopac Systems Inc., Goleta, CA, USA) using Acknowledge™ data acquisition software (sampling rate set at 30 Hz) and a Pentium™ PC. Output from the fibre-optic O_2 electrodes was displayed using Ocean Optics software and the data were compiled as text files for later importation into spreadsheet software. The O_2 electrode for water measurements was calibrated by immersing the electrode in a zero solution (2 g l^{-1} sodium sulphite) or air-saturated water, until stable readings were recorded. The O_2 electrode for air measurements was calibrated *in situ* by flowing humidified N_2 gas (zero) or air continuously through the air chamber. The CO_2 electrodes for water and air measurements were calibrated in a similar manner using mixtures of 0.5 and 1.0% CO_2 in air that were provided by a gas mixing flowmeter (GF-3/MP, Cameron Instruments). The pH electrode was calibrated using precision buffers; all electrodes were calibrated prior to each experiment.

Aerial gas transfer was determined from the slopes of the relationships between inspired gas tensions and time, over the period that the air chamber was sealed; the solubility coefficients of O_2 and CO_2 in air at 25°C were obtained from Boutilier et al. (1984). Rates of aquatic gas transfer were determined using the slopes of the relationships between water gas tensions and time over the interval that the respirometer was re-circulating. The solubility coefficient for O_2 in water at 25°C was obtained from Boutilier et al. (1984). The capacitance coefficient of CO_2 in water at 25°C was determined experimentally to be 0.041 μmol l^{-1} $mmHg^{-1}$. This was accomplished by measuring the changes in water total CO_2 concentration (Cameron Instruments Capnicon V total CO_2 analyzer) over the range of P_{CO_2} values encountered in the respirometry trials. Air breathing frequency was assessed visually throughout each experiment.

The CA activity of plasma or RBC lysates was measured using the electrometric ΔpH method of Henry (1981). In brief, samples containing CA activity were added to 6 ml of reaction buffer (in mmol l^{-1}, 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.40 with 30% phosphoric acid) held in a temperature-controlled (4°C) vessel, and the reaction was initiated by the addition of 200 μl of CO_2-saturated water. The rate of change of pH was measured over a fall of approximately 0.15 pH units. To obtain the true catalyzed rate, the uncatalysed rate (addition of CO_2-saturated water in the absence of any CA source) was subtracted from the observed rate. A pH electrode (Radiometer GK2401C or Metrohm 6.0204.100) and PHM 84 pH meter (Radiometer) linked to a data acquisition system (Biopac Systems Inc.) were used to measure pH.

In general, 50 μl samples of plasma or diluted RBC lysate were used to assay CA activity. CA activity in RBC lysates was also titrated with increasing volumes of 5 μmol l^{-1} acetazolamide (AZ). The resulting data were plotted according to the presentation of Easson and Stedman (1937) to yield an inhibition constant (K_i) for lungfish RBC CA against acetazolamide.

To determine levels of CA inhibitors in RBCs, lysates were thawed and then heated to 100°C for 10 min to denature endogenous CA. After heating, the samples were spun at 1000 ***g*** for 15 min and the supernatant was assayed for inhibitor content using the micro-method of Maren (1960), in which a fixed amount of human RBC CA is used to test the inhibitory activity of an unknown sample. Known concentrations of the appropriate inhibitor were used to generate a standard curve of catalysis time *vs* inhibitor concentration and this was used to back-calculate the RBC inhibitor concentration *in vivo*, taking into account appropriate dilution factors.

Statistical analysis

The data are reported as means ± 1 standard error of the mean (S.E.M.). Treatments were compared using one-way repeated measures analysis of variance (RM ANOVA) or paired Student's *t*-tests (when two means were compared). If significant differences ($P<0.05$) were found for multiple comparisons, a *post-hoc* multiple comparisons test (Bonferroni *t*-test) was applied.

Results

Series 1. Partitioning of gas transfer between air (lung) and water (gill/skin)

Preliminary experiments were performed to ensure that the measured rates of aerial and aquatic gas transfer were not influenced by artefacts arising from diffusion of gases across the air–water interface of the respirometer; representative data are depicted in Fig. 1. Increasing aerial P_{CO_2} in discrete steps to mimic the breath-by-breath addition of CO_2 was without effect on water P_{CO_2} during a 30 min period of simulated respirometry (Fig. 1A). These data demonstrated that there was likely to be negligible CO_2 transfer from air to water. Fig. 1B presents the results of a typical experiment designed to assess the potential for contamination of the air with CO_2 derived from the water. While the fish was alive there were linear increases in both air and water P_{CO_2}. After euthanasia and flushing of the air chamber, the water P_{CO_2} continued to rise yet air P_{CO_2} remained constant, indicating insignificant transfer of CO_2 from water to air. The continued rise in water P_{CO_2} after the death of the fish indicated that factors other than fish metabolism *per se* were contributing to aquatic CO_2 accumulation during respirometry trials. Fig. 1C illustrates that a similar situation applied to O_2 depletion from the water. While the fish was alive, the rates of P_{O_2} decline in water and air were linear and roughly equivalent. After its death, the P_{O_2} of the water continued to decrease whereas the air P_{O_2} was stable.

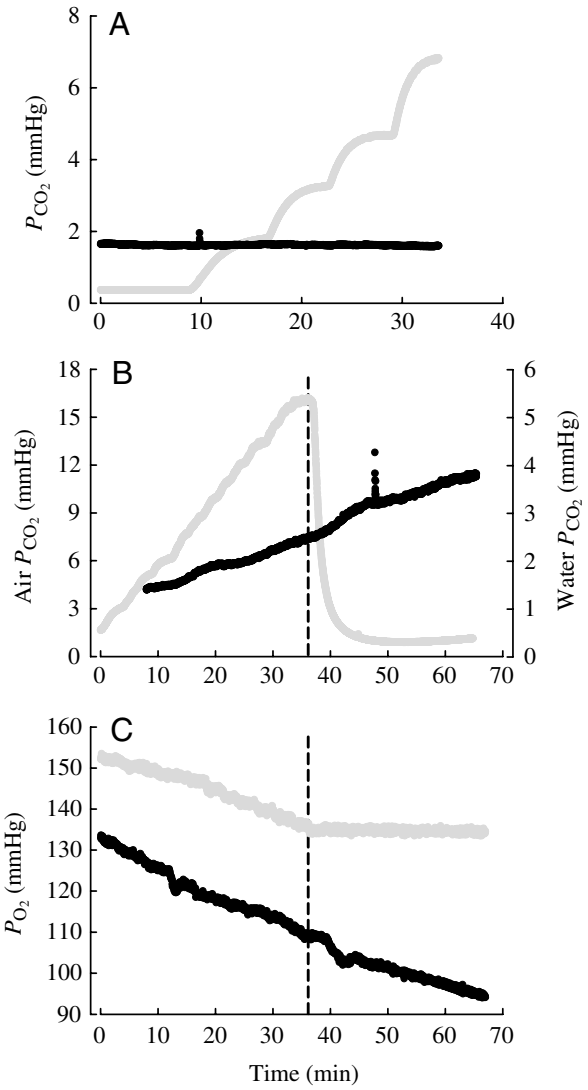

Fig. 1. Representative original data recordings illustrating the negligible effects of gas transfer across the air–water interface during respirometry experiments and the contribution of non-piscine metabolism to aquatic O_2 and CO_2 changes. (A) The effects of incremental increases in aerial P_{CO_2} (grey line) on aquatic P_{CO_2} (black line) during ~35 min of simulated respirometry. (B) The changes in aerial P_{CO_2} (grey line) and aquatic P_{CO_2} (black line) immediately before and after euthanizing (denoted by the broken line) a fish during a respirometry experiment. (C) The P_{CO_2} of the air chamber was lowered after euthanasia by flushing the chamber with air. The changes in aerial P_{O_2} (grey line) and aquatic P_{O_2} (black line) immediately before and after euthanizing (denoted by the broken line) a fish during a respirometry experiment.

The data shown in Fig. 2 summarise the partitioning of gas transfer between the air (*via* the lung) and water (*via* the gills and/or skin). The lung was responsible for 91.0±2.9% of $\dot{M}_{O_2}$ and 76.0±6.6% of $\dot{M}_{CO_2}$ (N=11). Mean respiratory exchange ratios RER ($\dot{M}_{CO_2}/\dot{M}_{O_2}$) for total gas transfer (sum of air plus water) and aerial gas transfer were 0.56±0.03 and 0.45±0.02, respectively. It was not possible to calculate RERs for aquatic gas transfer using data from individual fish because in many cases, there was no gas transfer. However, using the mean data (Fig. 2A), the RER for aquatic gas transfer was 1.63.

Casual observation suggested that air-breathing frequency tended to be higher in fish held in respirometers compared to their holding aquaria. Furthermore, it was apparent that in some fish, air-breathing frequency varied greatly during a single experiment. Several approaches were used to determine

whether the partitioning of CO_2 transfer between water and air was influenced by breathing frequency. First, regression analyses revealed that the relative proportion of CO_2 excreted *via* the gills/skin was unrelated to pulmonary breathing frequency between 6 and 48 breaths min^{-1} (r^2=0.03; P>0.05; N=19). Second, we were able to exploit several instances where fish dramatically changed their pulmonary breathing frequency. Fig. 3 illustrates two such cases where air-breathing frequency was suddenly decreased or increased. A sudden decline in air breathing frequency (Fig. 3A) was accompanied by a marked reduction in aerial CO_2 excretion, yet the rate of CO_2 accumulation in the water was unchanged. Conversely, a sudden increase in air breathing frequency (Fig. 3B) was accompanied by an obvious increase in aerial CO_2 excretion without any change in aquatic CO_2 accumulation.

Series 2. Effects of CA inhibition

Analysis of RBC lysates demonstrated that the levels of BZ in RBCs were undetectable (detection limit=45 nmol l^{-1}) in BZ-injected fish (Table 1). In agreement, RBC CA activity was unaffected within 30 min (Table 1). The low sample sizes for these analyses (N=2–3) reflect the loss of some samples that thawed during shipment between labs. Blood acid–base status, air breathing frequency and the rates of aerial and aquatic gas transfer were unaffected by BZ treatment (Table 2; Fig. 4). The measured levels of AZ in RBCs were 0.4–1.5 mmol l^{-1} 10–30 min after injection of AZ (Table 1). These values are several orders of magnitude greater than the K_i that was determined for *P. dolloi* RBC CA (2.1±0.2 nmol l^{-1}; N=3). Consistent with these data, the injection of AZ resulted in essentially complete inhibition of RBC CA activity within 10 min (Table 1). Blood acid–base status, air-breathing frequency and the rates of aerial and aquatic gas transfer were unaffected for 30 min after AZ treatment (Table 2; Fig. 4). In a separate group of fish (N=5), $\dot{M}_{CO_2}$ was monitored for 2 h after AZ treatment; total (air plus water) $\dot{M}_{CO_2}$ was 0.37±0.12 and 0.35±0.06 mmol kg^{-1} h^{-1} before and 2 h after AZ, respectively.

Series 3. Effects of exogenous CA

Injection of bovine CA caused a marked increase in plasma CA activity at 30 min from 39±36 to 9642±2179 μmol CO_2 ml^{-1} min^{-1} (N=5). The mean CA activity in the plasma of pre-injected fish reflected an unusually

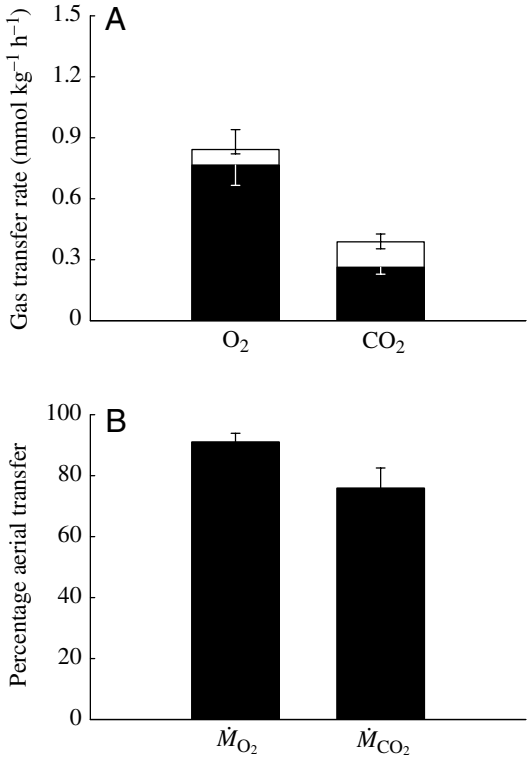

Fig. 2. Partitioning of gas transfer between the air (lungs) and water (gills/skin) in *P. dolloi* (N=11). (A) Absolute rates of O_2 uptake ($\dot{M}_{O_2}$) and CO_2 excretion ($\dot{M}_{CO_2}$) derived from the air (filled component of bar) and water (unfilled component of bar). The S.E.M. for total (sum of air plus water) gas transfer is denoted in the upward direction whereas those for aerial or aquatic gas transfer are denoted in the downward direction. (B) The relative proportions of aerial $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$.

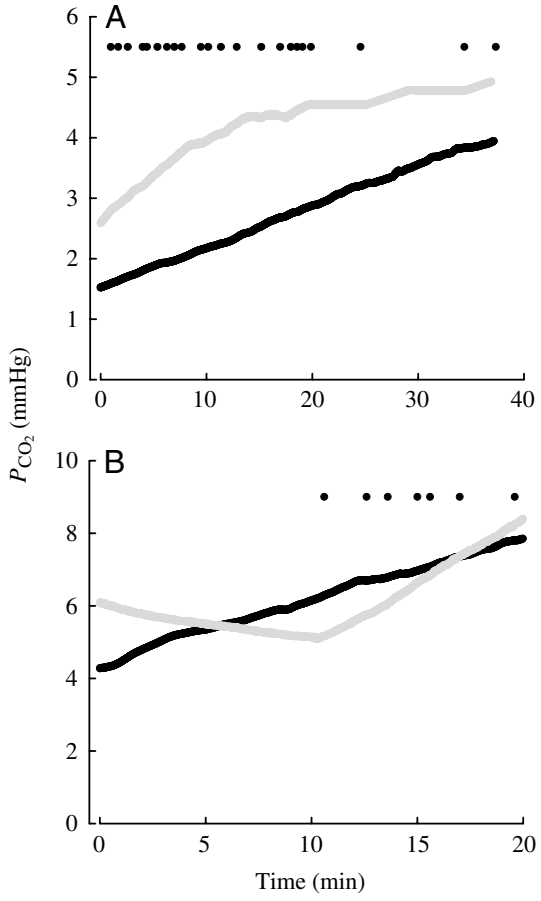

Fig. 3. (A,B) Representative traces from two different fish depicting the effects of changes in breathing frequency (individual breaths are denoted by solid circles) on aerial (grey lines) and aquatic (black lines) CO_2 accumulation. The decrease in aerial P_{CO_2} during the period of apnoea (first 10 min) in B represents a slight leak within the air chamber in this particular experiment.

Table 1. *The effects of intravascular injection of the carbonic anhydrase inhibitors benzolamide or acetazolamide on the red blood cell levels of each inhibitor and red blood cell CA activity*

			Benzolamide		Acetazolamide	
	N	Pre	10 min	30 min	10 min	30 min
[Inhibitor] (mmol l^{-1})	3		ND	ND	1.5±0.2	0.4±0.1
CA activity (μmol CO_2 min^{-1} ml^{-1})	2	110.6±14.5	232.4±123.1	219.9±157.7	2.6±1.9	5.5±1.4

Pre, pre-injection value; CA, carbonic anhydrase.
ND, non detectable (detection limit=45 nmol l^{-1}).

Table 2. *The effects of intravascular injection of the carbonic anhydrase inhibitors benzolamide or acetazolamide on arterial blood respiratory variables and breathing frequency in* P. dolloi

			Benzolamide		Acetazolamide			
	N	Pre	10 min	30 min	10 min	30 min	2 h	24 h
Pa_{CO_2} (mmHg)	6	16.9±0.6	17.1±0.6	17.6±0.5	13.3±1.2	16.6±0.9	15.5±0.8	17.1±3.8
Pa_{O_2} (mmHg)	6	68.0±5.6	84.7±12.6	86.2±12.4	80.0±15.5	92.2±13.3	–	–
pHa	6	7.57±0.02	7.54±0.02	7.54±0.02	7.73±0.05	7.56±0.02	7.49±0.04	7.47±0.10
fv (h^{-1})	10*	21.2±1.0	–	24.7±1.5	–	23.9±2.5	–	–

Pre, pre-injection value; fv, breathing frequency.
*A separate group of 5 fish were used to examine the longer term (2–24 h) effects of acetazolamide on blood gases.

high value in a single sample (184 μmol CO_2 ml^{-1} min^{-1}); in three of five samples, CA activity was undetectable. The rise in plasma CA activity was associated with a slight, but statistically significant, decrease in Pa_{CO_2} and an increase in breathing frequency (Table 3). Although $\dot{M}_{O_2}$ was unaffected, CA treatment was associated with a significant increase in $\dot{M}_{CO_2}$ that largely resulted from increased CO_2 excretion by gills/skin (Fig. 5). The apparent rise in $\dot{M}_{CO_2}$ at the lung was not statistically significant (P=0.641).

Series 4. Effects of hypercapnia on blood acid–base status

Aerial hypercapnia (37.5 mmHg) caused a marked respiratory acidosis within 30 min. Pa_{CO_2} increased from 12.1±0.5 to 20.9±1.7 mmHg (N=6) and pHa decreased from 7.57±0.02 to 7.41±0.03. Aquatic hypercapnia (40 mmHg) did not affect arterial blood acid–base status.

Discussion

Accepted models of gas transfer partitioning in lungfish (e.g. Burggren and Johanssen, 1986) identify the gill/skin as the major route of CO_2 excretion and the lung as the principal route for O_2 uptake. This general consensus is based on the results of several prior studies that examined the three genera of extant lungfish including the African genus, *Protopterus.* To our knowledge, however, only a single species of *Protopterus* (*P. aethiopicus*) has been studied. The upper estimates of aerial CO_2 excretion in *P. aethiopicus* are that approximately 30% of total CO_2 transfer occurs *via* this route (reviewed by Burggren and Johansen, 1986). Recently, however, Amin-Naves et al. (2004) demonstrated that the lung of the South American lungfish *Lepidosiren paradoxa*, while excreting only a small fraction of the total CO_2 under normal conditions, became the major site of CO_2 excretion when animals were acclimatised to a 20°C increase in temperature. Thus, under conditions of increased metabolic rate, the lungfish lung may play a more significant role in overall CO_2 excretion.

Although the principal aim of the present study was to investigate the potential roles of CA in aerial and aquatic CO_2 transfer, it quickly became apparent that gas transfer partitioning in *P. dolloi* was markedly different than in other lungfish because the lung appeared to be the major site of both O_2 and CO_2 transfers. In this respect, *P. dolloi* resembled *Lepidosiren* acclimated to elevated ambient temperatures (Amin-Naves et al., 2004). Owing to the pronounced differences between this and previous studies on African lungfish, a series of experiments was performed to validate the basic respirometry protocol.

Table 3. *The effects of intravascular injection of bovine carbonic anhydrase on arterial blood respiratory variables and breathing frequency in* P. dolloi

	N	Pre	30 min CA
Pa_{CO_2} (mmHg)	9	21.6±1.6	19.6±1.8*
Pa_{O_2} (mmHg)	9	76.3±10.9	87.7±8.3
pHa	9	7.48±0.04	7.53±0.03
fv (h^{-1})	10	19.0±1.3	24.7±1.8*

CA, carbonic anhydrase; Pre, pre-injection value; fv, breathing frequency.
*Significant differences (P<0.05) from Pre.

Potentials for error during respirometry

Several comprehensive reviews have been published that discuss some common problems associated with performing respirometry on aquatic animals (Steffensen, 1989; Kaufmann and Forstner, 1989; Cech, 1990). Arguably, the most serious problem is the potential for modification of water P_{O_2} and P_{CO_2} by bacteria or other extraneous sources. The potential for bacterial metabolism to contribute to the measured changes in gas partial pressures is greatest in recirculating respirometers at high water temperatures (Kaufmann and Forstner, 1989). Because antibiotics are not recommended to eliminate bacterial metabolism during respirometry trials (Kaufmann and Forstner, 1989), the protocol employed in the present study was to subtract background metabolism during blank runs conducted using dead fish. Thus, we were unable to distinguish microbial metabolism in the water, *per se*, from microbial metabolism occurring on the surface of the fish. Clearly, however, bacterial metabolism was a major source of O_2 depletion and CO_2 accumulation in the water during closed system respirometry. Indeed in many cases, there were no differences in the rates of water gas changes after euthanasia. Because all blank runs were performed with a fish in the respirometer, it is conceivable that cutaneous metabolism, in

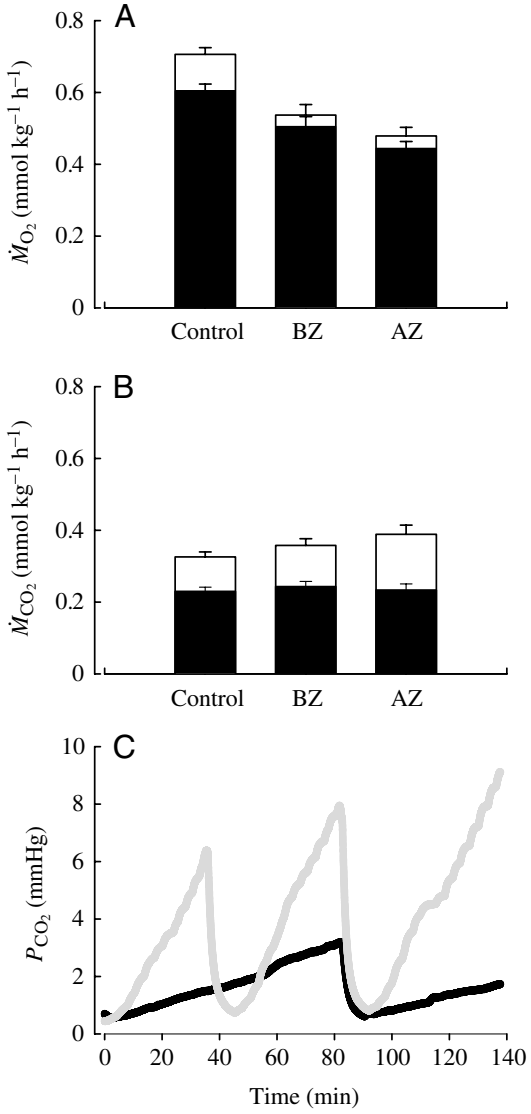

Fig. 4. The effects of injection of the carbonic anhydrase (CA) inhibitors benzolamide (BZ; N=11) or acetazolamide (AZ; N=10) on (A) O_2 uptake ($\dot{M}_{O_2}$) and (B) CO_2 excretion ($\dot{M}_{CO_2}$) in *P. dolloi*. Aerial gas transfer is depicted by the filled portions of each bar and aquatic gas transfer by the unfilled portions. (C) Representative recordings of aerial CO_2 transfer (grey line) and aquatic CO_2 transfer (black line) from a single fish. Whereas the air chamber was flushed after each period of respirometry, the water was refreshed only after the BZ trial.

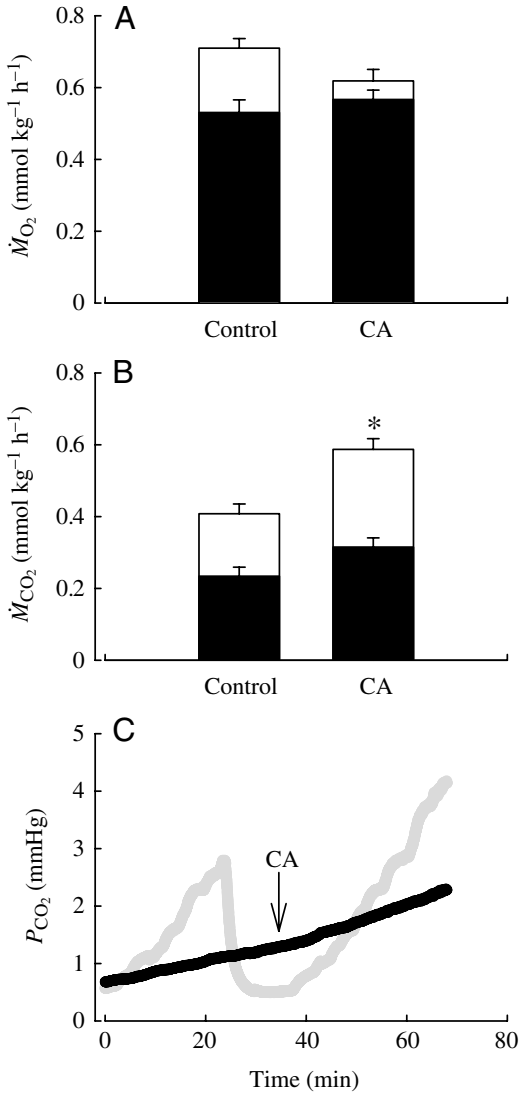

Fig. 5. The effects of injecting bovine carbonic anhydrase (CA; N=9) on (A) O_2 uptake ($\dot{M}_{O_2}$) and (B) CO_2 excretion ($\dot{M}_{CO_2}$) in *P. dolloi*. Aerial gas transfer is depicted by the filled portions of each bar and aquatic gas transfer by the unfilled portions. (C) Representative recordings of aerial CO_2 transfer (grey line) and aquatic CO_2 transfer (black line) from a single fish. Note that in this particular experiment, only the air chamber was flushed after the control period of respirometry. A statistical difference from the control group is denoted by an asterisk (P<0.05).

addition to microbial activity, may have contributed to the background metabolism. Regardless of its source, the true rates of aquatic $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ could only be determined after taking into account these significant background components. It is unclear whether previous respirometry studies on *P. aethiopicus* utilised corrections for background metabolism.

Closed system respirometry on bimodal breathers is particularly challenging because of the potential for gases to diffuse across the air–water interface of the respirometer (Graham, 1997). For example, the entry of exhaled CO_2 from the air chamber into the water, if occurring, could mistakenly be attributed to CO_2 excretion across the gills/skin. Because of the high capacitance of water for CO_2, the potential for contamination of the water with exhaled CO_2 is likely to be significantly greater than that for O_2 entry from water into the air. In the present study, we were unable to detect any transfer of gas across the air–water interface. Regardless, to minimize diffusion gradients between the air and water, the chambers were flushed at regular intervals.

Partitioning of gas transfer in P. dolloi

The results obtained in this study clearly implicate the lung as the primary route of both O_2 and CO_2 transfer in *P. dolloi*, but also demonstrate that the gill/skin is more suited to CO_2 excretion than to O_2 uptake. The former finding is in marked contrast to conclusions derived from previous studies on African lungfish (Lenfant and Johansen, 1968; McMahon, 1969; Lahiri et al., 1970). Because the data demonstrating the lung as the principal site of CO_2 excretion were unexpected and in opposition to accepted models, we considered it important to corroborate the respirometry data using independent methods. This was accomplished by assessing the impact of aerial *vs* aquatic hypercapnia on blood acid–base status. In support of the respirometry data, only aerial hypercapnia elicited an arterial blood respiratory acidosis. This result plainly indicated that pulmonary CO_2 excretion was being inhibited and that the gill was ineffective at completely clearing the accumulating CO_2. However, the fact that P_{aCO_2} remained lower than inspired P_{CO_2} may have reflected an increased involvement of branchial CO_2 excretion. Alternatively, the arterial blood may not have yet reached equilibrium with inspired P_{CO_2}. The most likely explanation for the absence of an effect of aquatic hypercapnia on blood acid–base status is that inhibition of the small component of CO_2 excretion occurring at the gills/skin or any CO_2 entry from the water was simply compensated for by increased pulmonary CO_2 excretion.

As reported in other studies (e.g. Johansen and Lenfant, 1968), pulmonary breathing frequency was highly variable within, and between, individuals. Moreover, while not quantified, it appeared that the average breathing frequency (~20 min^{-1}) during respirometry trials was higher than that in fish being held in aquaria. Given previous reports that pulmonary hyperventilation in *P. aethiopicus* is associated with a lowering of P_{aCO_2} (Lahiri et al., 1970) or increased pulmonary $\dot{M}_{CO_2}$ (McMahon, 1970), we considered the possibility that CO_2 excretion into the water was artificially low in the present experiments because of pulmonary hyperventilation. This is unlikely, however, based on the absence of any correlation in this study between breathing frequency and aquatic CO_2 excretion over a wide range (6–48 breaths min^{-1}). Moreover, there were no obvious changes in the rates of CO_2 accumulation in the water during periods of pulmonary apnoea.

Although CO_2 transfer into the water comprised only a minor component of overall CO_2 excretion, the relative proportion of aquatic CO_2 transfer (24% of overall CO_2 excretion) was greater than for aquatic O_2 transfer (9% of overall O_2 uptake). These data confirm previous conclusions that the gill/skin of lungfish is more suited to CO_2 excretion than O_2 uptake (see reviews by Burggren and Johansen, 1986; Graham, 1997). The possibility that extracellular catalysis of plasma HCO_3^- dehydration underlies this phenomenon is discussed below.

It would be presumptuous to assume that previous conclusions concerning gas transfer partitioning in lungfish are incorrect. Discrepancies between the present, and other studies, may simply reflect species differences. Why *P. dolloi* should differ from other species of *Protopterus*, however, is unclear. Alternatively, by analogy to *Lepidosiren* (Amin-Naves et al., 2004), it is possible that the fish used in the present study had increased metabolic rates (compared to fish used in previous studies) and hence were more reliant on the lung as a route for CO_2 excretion. Interestingly, we (S. F. Perry, R. Euvermann, S. F. Chew, Y. K. Ip and K. M. Gilmour; unpublished data) have recently completed a series of experiments on *P. annectens* and found that, as in previous reports, the gill was the predominant site of CO_2 excretion whereas the lung was the major site of O_2 uptake.

Carbonic anhydrase and CO_2 excretion

Initially, the present study was conceived to test the idea that the reputed preferential excretion of CO_2 by the lungfish gill (compared to the lung) might reflect the participation of extracellular branchial CA. As is the case in dogfish (Wood et al., 1994; Henry et al., 1997; Gilmour et al., 2001; Gilmour and Perry, 2004; Perry and Gilmour, 2002), it was hypothesized that a membrane-associated plasma-facing CA isoform could participate in the dehydration of plasma HCO_3^-, thereby aiding CO_2 excretion. Such a scheme might be particularly beneficial to *Protopterus* owing to its low rate constant for RBC Cl^-/HCO_3^- exchange (Jensen et al., 2003), the step normally considered to be rate limiting in CO_2 excretion (Perry, 1986; Tufts and Perry, 1998). In light of the results of the initial respirometry experiments, which showed the gill to be a minor route for CO_2 elimination, clearly the original hypothesis was no longer appropriate. Thus, the hypothesis was modified *post hoc* to test instead the idea that excretion of CO_2 at either site of gas exchange (gill/skin or lung) is aided by extracellular CA.

It is likely that all air-breathing animals possess membrane-associated endothelial pulmonary CA that is oriented toward the vascular lumen and thus able to catalyze extracellular reactions (Stabenau and Heming, 2003). However, its role in CO_2

excretion is constrained by the low buffering capacity of plasma that limits the supply of substrate (H^+) for the HCO_3^- dehydration reaction (Bidani and Heming, 1991). It is generally accepted that less than 10% of overall excreted CO_2 is derived from the extracellular catalysis of HCO_3^- (Bidani, 1991; Cardenas et al., 1998; Henry and Swenson, 2000). Based on the present results obtained using the functionally impermeant CA inhibitor, BZ, pulmonary CA (if present in lungfish), as in other air-breathers, is clearly not playing a significant role in aerial CO_2 excretion. Although the use of BZ as an impermeant CA inhibitor has been challenged (Supuran and Scozzafava, 2004), results of this and previous studies (Gilmour et al., 2001) clearly demonstrate that low doses of BZ do not sufficiently penetrate RBCs of lungfish, trout or dogfish within 30 min to significantly influence HCO_3^- dehydration. Currently, studies are underway to determine if a membrane-associated CA isoform does actually exist in the lung of *Protopterus*.

CO_2 excretion into the water also was unaffected by BZ treatment, and thus the apparent preferential transfer of CO_2 by the gill/skin in comparison to O_2 presumably does not reflect extracellular catalysis of HCO_3^-. To date, the only fish for which extracellular CA has been shown to significantly contribute to CO_2 excretion is *Squalus acanthias* (Gilmour et al., 2001; Gilmour and Perry, 2004), a species known to possess high plasma buffering capacity (Lenfant and Johansen, 1966; Gilmour et al., 2002).

Results obtained using the permeant CA inhibitor, AZ, demonstrate that CO_2 excretion in *P. dolloi* can occur normally in the absence of RBC CA activity. The absence of any effect of AZ on CO_2 excretion or blood acid–base status could not be attributed to insufficient inhibition of CA, based on independent measures of RBC CA activity, and AZ concentrations in treated fish compared with the K_i for lungfish RBC CA against AZ; this K_i value for lungfish was also comparable to the corresponding value for rainbow trout (Henry et al., 1993), where a similar dose of AZ elicits a profound respiratory acidosis (e.g. Hoffert and Fromm, 1973). Moreover, the AZ solution that was used in Singapore was brought back to Canada and injected into two rainbow trout *Oncorhynchus mykiss*; arterial P_{CO_2} increased over 30 min by approximately 2.72 and 2.22 mmHg in the two trout (S. F. Perry and K. M. Gilmour, unpublished observations). To our knowledge, *P. dolloi* is the only fish species examined to date that is unaffected by AZ treatment despite total inhibition of RBC CA activity. We must emphasise, however, that this result does not necessarily exclude a role for RBC CA in CO_2 excretion under normal conditions. The results simply show that RBC CA activity is not a prerequisite for normal rates of CO_2 excretion. Examples of air breathing species for which AZ was found to inhibit CO_2 excretion or raise arterial P_{CO_2} include *Xiphister atropurpureus* (Daxboeck and Hemimg, 1982), *Trichogaster trichopterus* (Burggren and Haswell, 1979), *Blennius pholis* (Pelster et al., 1988) and *Lepisosteus oculatus* (Smatresk and Cameron, 1982). The likeliest explanation for the absence of any effect of CA inhibition on CO_2 excretion or blood acid–base status in *P. dolloi* is that CO_2 excretion, at both the gills/skin and lung, is occurring in the absence of appreciable catalyzed HCO_3^- dehydration during gill/skin or lung transit. As discussed by Brauner and Val (1996) to explain CO_2 excretion in *Arapaima gigas*, the high P_{CO_2} values that are characteristic of lungfish blood, coupled with low rates of metabolism, may negate the requirement for catalysed HCO_3^- dehydration during the passage of blood through the respiratory organs. Nevertheless, it is puzzling that, given the presence of RBC CA activity and the likelihood of RBC Cl^-/HCO_3^- exchange (based on its presence in the related *P. aethiopicus*; Jensen et al., 2003), there would appear to be no obligate requirement for the catalysed dehydration of plasma HCO_3^- within the gills or lungs. One possible explanation is that the RBC CA in lungfish may be a slow-turnover isoform and thus unable to keep pace with the rate of substrate delivery by the Cl^-/HCO_3^- exchanger (Tufts et al., 2003). In support of this idea, we (L. Kenney, S. F. Perry, S. F. Chew, Y. K. Ip and K. M. Gilmour, unpublished data) have recently cloned and sequenced two CA isoforms from *Protopterus* blood, both of which are most similar to CA XIII, an enzyme with only moderate catalytic activity (Lehtonen et al., 2004). Because injection of bovine CA increased gill/skin CO_2 excretion (without increasing O_2 uptake), it would indeed appear that the extent of catalysed HCO_3^- dehydration is a constraint on aquatic CO_2 transfer in *P. dolloi*.

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References

Amin-Naves, J., Giusti, H. and Glass, M. L. (2004). Effects of acute temperature changes on aerial and aquatic gas exchange, pulmonary ventilation and blood gas status in the South American lungfish, *Lepidosiren paradoxa*. *Comp. Biochem. Physiol.* **138A**, 133-139.

Axelsson, M. and Fritsche, R. (1994). Cannulation techniques. In *Analytical Techniques* (ed. P. W. Hochachka and T. P. Mommsen), pp. 17-36. Amsterdam: Elsevier.

Babiker, M. M. (1979). Respiratory behaviour, oxygen consumption and relative dependence on aerial respiration in the African lungfish (*Protopterus annectens*, Owen) and an air-breathing teleost (*Clarias lazera*, C.). *Hydrobiologia* **65**, 177-187.

Bidani, A. (1991). Analysis of abnormalities of capillary CO_2 exchange *in vivo*. *J. Appl. Physiol.* **70**, 1686-1699.

Bidani, A. and Heming, T. A. (1991). Effects of perfusate buffer capacity on capillary CO_2-HCO_3^--H^+ reactions: theory. *J. Appl. Physiol.* **71**, 1460-1468.

Bindon, S. F., Fenwick, J. C. and Perry, S. F. (1994). Branchial chloride cell proliferation in the rainbow trout, *Oncorhynchus mykiss*: Implications for gas transfer. *Can. J. Zool.* **72**, 1395-1402.

Boutilier, R. G., Heming, T. A. and Iwama, G. K. (1984). Physiochemical parameters for use in fish respiratory physiology. In *Fish Physiology*. Vol. XA (ed. W. S. Hoar and D. J. Randall), pp. 403-430. New York: Academic Press.

Brauner, C. J. and Val, A. L. (1996). The interaction between O_2 and CO_2

exchange in the obligate air breather, *Arapaima gigas*, and the facultative air breather, *Lipossarcus paradalis*. In *Physiology and Biochemistry of the Fishes of the Amazon* (ed. A. L. Val, V. M. Almeida-Val and D. J. Randall), pp. 101-110. Manaus: INPA.

Burggren, W. and Haswell, S. (1979). Aerial CO_2 excretion in the obligate air breathing fish *Trichogaster trichopterus* – role for carbonic anhydrase. *J. Exp. Biol.* **82**, 215-225.

Burggren, W. W. and Johansen, K. (1986). Circulation and respiration in lungfishes (Dipnoi). In *Biology and Evolution of Lungfishes* (ed. W. E. Bemis, W. W. Burggren, and N. E. Kemp), pp. 217-236. New York: Alan R. Liss.

Cardenas, V., Jr, Heming, T. A. and Bidani, A. (1998). Kinetics of CO_2 excretion and intravascular pH disequilibria during carbonic anhydrase inhibition. *J. Appl. Physiol.* **84**, 683-694.

Cech, J. J. (1990). Respirometry. In *Methods for Fish Biology* (ed. C. B. Schreck and P. B. Moyle), pp. 335-362. Bethesda: American Fisheries Society.

Daxboeck, C. and Hemimg, T. A. (1982). Bimodal respiration in the intertidal fish, *Xiphister atropurpureus* (Kittlitz). *Mar. Behav. Physiol.* **9**, 23-33.

Desforges, P., Gilmour, K. M. and Perry, S. F. (2001). The effects of exogenous extracellular carbonic anhydrase on CO_2 excretion in rainbow trout (*Oncorhynchus mykiss*): Role of plasma buffering capacity. *J. Comp. Physiol.* **171**, 465-473.

Desforges, P. R., Harman, S. S., Gilmour, K. M. and Perry, S. F. (2002). The sensitivity of CO_2 excretion to changes in blood flow in rainbow trout is determined by carbonic anhydrase availability. *Am. J. Physiol.* **282**, R501-R508.

Easson, L. H. and Stedman, E. (1937). The absolute activity of choline esterase. *Proc. R. Soc. Lond. B* **121**, 142-164.

Fishman, A. P., Pack, A. I., Delaney, R. G. and Galante, R. J. (1986). Estivation in *Protopterus*. In *Biology and Evolution of Lungfishes* (ed. W. E. Bemis, W. W. Burggren and N. E. Kemp), pp. 237-248. New York: Alan R. Liss.

Gilmour, K. M. and Perry, S. F. (2004). The role of extracellular carbonic anhydrase in CO_2 excretion in dogfish (*Squalus acanthias*) as revealed by severe experimental anaemia and selective carbonic anhydrase inhibitors. *Am. J. Physiol.* **286**, R1138-R1148.

Gilmour, K. M., Perry, S. F., Bernier, N. J., Henry, R. P. and Wood, C. M. (2001). Extracellular carbonic anhydrase in the dogfish, *Squalus acanthias*: A role in CO_2 excretion. *Physiol. Biochem. Zool.* **74**, 477-492.

Gilmour, K. M., Shah, B. and Szebedinszky, C. (2002). An investigation of carbonic anhydrase activity in the gills and blood plasma of brown bullhead (*Ameiurus nebulosus*), longnose skate (*Raja rhina*), and spotted raffish (*Hydrolagus colliei*). *J. Comp. Physiol. B* **172**, 77-86.

Gilmour, K. M., Desforges, P. R. and Perry, S. F. (2004). Buffering limits plasma HCO_3^- dehydration when red blood cell anion exchange is inhibited. *Respir. Physiol. Neurobiol.* **140**, 173-187.

Graham, J. B. (1997). *Air-Breathing Fishes: Evolution, Diversity and Adaptation*. San Diego: Academic Press.

Greco, A. M., Gilmour, K. M., Fenwick, J. C. and Perry, S. F. (1995). The effects of soft-water acclimation on respiratory gas transfer in the rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol.* **198**, 2557-2567.

Greenwood, P. H. (1986). The natural history of African lungfishes. *J. Morphol.* **Suppl. 1**, 163-179.

Henry, R. P. (1981). Techniques for measuring carbonic anhydrase activity *in vitro*: the electrometric delta pH and pH stat assays. In *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (ed. S. J. Dodgson, R. E. Tashian, G. Gros and N. Carter), pp. 119-126. New York: Plenum.

Henry, R. P. and Swenson, E. R. (2000). The distribution and physiological significance of carbonic anhydrase in vertebrate gas exchange organs. *Respir. Physiol.* **121**, 1-12.

Henry, R. P., Tufts, B. L. and Boutilier, R. G. (1993). The distribution of carbonic anhydrase type-I and type-II isozymes in lamprey and trout – possible co-evolution with erythrocyte chloride bicarbonate exchange. *J. Comp. Physiol. B* **163**, 380-388.

Henry, R. P., Gilmour, K. M., Wood, C. M. and Perry, S. F. (1997). Extracellular carbonic anhydrase activity and carbonic anhydrase inhibitors in the circulatory system of fish. *Physiol. Zool.* **70**, 650-659.

Hoffert, J. R. and Fromm, P. O. (1973). Effect of acetazolamide on some hematological parameters and ocular oxygen concentration in rainbow trout. *Comp. Biochem. Physiol.* **45A**, 371-378.

Jensen, F. B., Brahm, J., Koldkjaer, P., Wang, T., Mckenzie, D. J. and Taylor, E. W. (2003). Anion exchange in the giant erythrocytes of African lungfish. *J. Fish Biol.* **62**, 1044-1052.

Johansen, K. and Lenfant, C. (1967). Respiratory function in the South American lungfish, *Lepidosiren paradoxa* (Fitz). *J. Exp. Biol.* **46**, 205-218.

Julio, A. E., Desforges, P. and Perry, S. F. (2000). Apparent diffusion limitations for carbon dioxide excretion in rainbow trout (*Oncorhynchus mykiss*) are relieved by intravascular injections of carbonic anhydrase. *Respir. Physiol.* **121**, 53-64.

Kaufmann, R. and Forstner, H. W. W. (1989). Respirometry – Methods and approaches. In *Techniques in Comparative Respiratory Physiology*, *SEB Seminar Series,* vol. 37 (ed. C. R. Bridges and P. J. Butler), pp. 51-76. Cambridge: Cambridge University Press.

Lahiri, S., Szidon, J. P. and Fishman, A. P. (1970). Potential respiratory and circulatory adjustments to hypoxia in the African lungfish. *Fed. Proc.* **29**, 1141-1148.

Lehtonen, J., Shen, B., Vihinen, M., Casini, A., Scozzafava, A., Supuran, C. T., Parkkila, A. K., Saarnio, J., Kivela, A. J., Waheed, A. et al. (2004). Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. *J. Biol. Chem.* **279**, 2719-2727.

Lenfant, C. and Johansen, K. (1966). Respiratory function in the elasmobranch *Squalus suckleyi* G. *Respir. Physiol.* **1**, 13-29.

Lenfant, C. and Johansen, K. (1968). Respiration in the African lungfish *Protopterus aethiopicus*. *J. Exp. Biol.* **49**, 437-452.

Lenfant, C., Johansen, K. and Grigg, G. C. (1966). Respiratory properties of blood and pattern of gas exchange in the lungfish *Neoceratodus forsteri* (Krefft). *Respir. Physiol.* **2**, 1-21.

Maren, T. H. (1960). A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. *J. Pharmacol. Exp. Ther.* **130**, 26-29.

McMahon, B. R. (1969). A functional analysis of the aquatic and aerial respiratory movements of an African lungfish, *Protopterus aethiopicus*, with reference to the evolution of the lung-ventilation mechanism in vertebrates. *J. Exp. Biol.* **51**, 407-430.

McMahon, B. R. (1970). The relative efficiency of gaseous exchange across the lungs and gills of an African lungfish *Protopterus aethiopicus*. *J. Exp. Biol.* **52**, 1-15.

Pelster, B., Bridges, C. R. and Grieshaber, M. K. (1988). Physiological adaptations of the intertidal rockpool teleost *Blennius pholis* L. to aerial exposure. *Respir. Physiol.* **71**, 355-374.

Perry, S. F. (1986). Carbon dioxide excretion in fish. *Can. J. Zool.* **64**, 565-572.

Perry, S. F. and Gilmour, K. M. (2002). Sensing and transfer of respiratory gases at the fish gill. *J. Exp. Zool.* **293**, 249-263.

Reeves, R. B. (1977). The interaction of body temperature and acid–base balance in ectothermic vertebrates. *Annu. Rev. Physiol.* **39**, 559-584.

Smatresk, N. J. and Cameron, J. N. (1982). Respiration and acid–base physiology of the spotted gar, a bimodal breather. III. Response to a transfer from fresh water to 50% sea water and control of ventilation. *J. Exp. Biol.* **96**, 295-306.

Smith, H. W. (1930). Metabolism of the lungfish, *Protopterus aethiopicus*. *J. Biol. Chem.* **88**, 97-130.

Stabenau, E. K. and Heming, T. A. (2003). Pulmonary carbonic anhydrase in vertebrate gas exchange organs. *Comp. Biochem. Physiol.* **136A**, 271-279.

Steffensen, J. F. (1989). Some errors in respirometry of aquatic breathers: how to avoid and correct for them. *Fish Physiol. Biochem.* **6**, 49-59.

Supuran, C. T. and Scozzafava, A. (2004). Benzolamide is not a membrane-impermeant carbonic anhydrase inhibitor. *J. Enzyme Inhib. Med. Chem.* **19**, 269-273.

Travis, D. M., Wiley, C., Nechay, B. R. and Maren, T. H. (1964). Selective renal carbonic anhydrase inhibition without respiratory effect: pharmacology of 2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide (CL 11,366). *J. Pharmacol. Exp. Ther.* **143**, 383-394.

Tufts, B. L. and Perry, S. F. (1998). Carbon dioxide transport and excretion. In *Fish Respiration* (ed. S. F. Perry and B. L. Tufts), pp. 229-281. New York: Academic Press.

Tufts, B. L., Esbaugh, A. and Lund, S. G. (2003). Comparative physiology and molecular evolution of carbonic anhydrase in the erythrocytes of early vertebrates. *Comp. Biochem. Physiol.* **136A**, 259-269.

Wood, C. M., Perry, S. F., Walsh, P. J. and Thomas, S. (1994). HCO_3^- dehydration by the blood of an elasmobranch in the absence of a Haldane effect. *Respir. Physiol.* **98**, 319-337.