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THE EFFECTS OF HYPOXIA, HYPEROXIA OR HYPERCAPNIA ON THE ACID–BASE DISEQUILIBRIUM IN THE ARTERIAL BLOOD OF RAINBOW TROUT

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Summary

An extracorporeal circulation in combination with a stop–flow technique was used to characterize the acid–base disequilibrium in the arterial blood of rainbow trout *Oncorhynchus mykiss* during environmental hypoxia, hyperoxia or hypercapnia. Arterial blood was routed from the coeliac artery through an external circuit in which pH (pHa), partial pressure of oxygen (P_{aO_2}) and partial pressure of carbon dioxide (P_{aCO_2}) were monitored continuously. The stop–flow condition was imposed by turning off the pump which drove the external loop. Water P_{O_2} or P_{CO_2} was adjusted to give the experimental conditions by bubbling N_2, O_2 or CO_2 through a water equilibration column supplying the fish.

During normoxia, the arterial blood exhibited a positive acid–base disequilibrium of approximately 0.04 pH units; that is, pH increased over the stop–flow period by 0.04 units. The extent of the imbalance was increased significantly by hypoxia (final P_{aO_2}=2.7–3.7 kPa; ΔpH=0.05 units). In fish exposed to hyperoxia (final P_{aO_2}=47–67 kPa), the direction of the disequilibrium was reversed; pHa declined by 0.03 units. During hyperoxia, CO_2 excretion was impaired by 63 % and the P_{CO_2} of postbranchial blood was higher than that of prebranchial blood. It is therefore conceivable that a reversal of the normal, outwardly directed, diffusion gradient for CO_2 accounted for the negative disequilibrium; CO_2 uptake at the gills would drive plasma $CO_2/HCO_3^-/H^+$ reactions towards CO_2 hydration and H^+ formation. During hypercapnia, fish exhibited a twofold increase in the positive pH disequilibrium (ΔpH=0.06 units).

The results of this study confirmed the existence of an acid–base disequilibrium in the arterial blood of rainbow trout and clearly demonstrated that the extent and/or direction of the disequilibrium are influenced by the respiratory status of the fish.

Introduction

An acid–base disequilibrium was recently shown to exist in the arterial blood of the rainbow trout, *Oncorhynchus mykiss* (Gilmour *et al.* 1994). The positive pH imbalance

Key words: *Oncorhynchus mykiss*, hypoxia, hyperoxia, hypercapnia, acid–base disequilibrium, red blood cell, HCO_3^- dehydration, Cl^-/HCO_3^- exchange, gill, ventilation.

observed when the flow of blood through an extracorporeal circulation was stopped (i.e. pH increased during the stop–flow period) was attributed to the relatively slow rate of plasma HCO_3^- dehydration in comparison with branchial CO_2 diffusion. The dehydration reaction, which consumes protons to form CO_2, continues in the postbranchial blood because fish lack functional quantities of gill 'endothelial' carbonic anhydrase (reviewed by Perry and Laurent, 1990). Elimination of the pH disequilibrium by treating fish with carbonic anhydrase (CA) provided verification of the dominant role of plasma HCO_3^- dehydration. The involvement of red blood cell Cl^-/HCO_3^- exchange in the acid–base disequilibrium appeared to be slight under normal conditions; a negative disequilibrium would be anticipated if Cl^-/HCO_3^- exchange did not reach completion during the gill transit time, since this process transfers HCO_3^- from the plasma to the red blood cells (Crandall *et al.* 1981). When red blood cell CO_2 excretion was inhibited by acetazolamide, the persistence of anion exchange in the postbranchial blood contributed to the ensuing negative imbalance (pH decrease).

It is apparent from this model that a number of factors could potentially influence the acid–base imbalance (see also Bidani and Crandall, 1988). In particular, ventilation and the diffusion gradient for CO_2 are likely to play key roles in disequilibrium events through their impact on blood gas variables and CO_2 excretion (Perry, 1986; Perry and Wood, 1989). The involvement of red blood cell Cl^-/HCO_3^- exchange also merits further investigation. The reaction time (T_{67}) for red blood cell Cl^-/HCO_3^- exchange, 0.4 s in rainbow trout (Cameron, 1978), is similar to the gill transit time, 0.5–2.5 s, and any decrease in the latter would therefore elevate the contribution of anion exchange to the net disequilibrium. In the present study, the extent and/or direction of the pH disequilibrium were altered by manipulating the external environment of the fish to elicit predictable changes in ventilation and blood gas variables. The environmental conditions chosen were hypoxia, hyperoxia and hypercapnia. The acid–base disequilibrium in each case was characterized using an extracorporeal preparation in conjunction with a stop–flow technique.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing between 509 and 1202 g (N=28) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). An additional group of fish weighing between 190 and 310 g (N=8) was used in respirometry studies and to provide blood samples. All fish were maintained in the University of Ottawa holding facility on a 12 h:12 h L:D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechlorinated City of Ottawa tap water ($[Na^+]$= 0.12 mmol l^{-1}, $[Cl^-]$=0.15 mmol l^{-1}, $[Ca^{2+}]$=0.35–0.40 mmol l^{-1}, $[K^+]$=0.03 mmol l^{-1}, pH=7.7–8.0, water temperature=7–10 °C). Fish were fed commercial trout pellets daily to satiation; food was withheld for 24 h before experiments were initiated. Trout were acclimated to these conditions for at least 4 weeks prior to experimentation.

Animal preparation

Following anaesthetization in a neutralized (pH 7.5–8.0) solution of ethyl-*m*-aminobenzoate (0.1 g l^{-1}; MS-222), fish were transferred to an operating table that permitted continuous irrigation of the gills with oxygenated anaesthetic solution. An indwelling cannula of flexible polyethylene tubing (Clay-Adams PE 50) was implanted into the dorsal aorta (Soivio *et al.* 1975) for the measurement of dorsal aortic blood pressure (P_{DA}). Two cannulae (PE 50) were inserted into the coeliac artery in the orthograde and retrograde directions (Thomas and Le Ruz, 1982). To measure ventilation amplitude, a catheter (PE 160) was placed in either the buccal or the opercular cavity. Fish were revived on the operating table by irrigation of the gills with oxygenated water and transferred to the experimental chamber for a 24 h recovery period prior to experimentation.

Extracorporeal circulation and analytical procedures

The extracorporeal stop–flow technique has been described in detail by Gilmour *et al.* (1994). Briefly, the extracorporeal circulation was activated by connecting the coeliac artery cannulae in series with a peristaltic pump which drove blood at 1.2 ml min^{-1} through a circuit containing P_{O_2}, P_{CO_2} and pH electrodes. The transit time of the blood, from the gills to the electrodes, was approximately 30 s. The stop–flow condition was imposed by turning off the peristaltic pump. Approximately 1 ml of blood was contained in the external loop; this represented less than 4 % of the total blood volume of the fish. To avoid clotting, the circuit was pre-rinsed with 10 ml of heparinized (540 i.u. ml^{-1}) Cortland saline (Wolf, 1963).

A Metrohm combination glass pH electrode and Radiometer PHM 73 meter were used to measure pHa. The 50 % response time of the electrode was 25 s for a 0.14 unit pH change in buffer solutions, and the pH change associated with stopping the flow of buffer was 0.002 units. Radiometer P_{O_2} and P_{CO_2} electrodes (E-5046 and E-5036, respectively) and the PHM 73 meter were used to measure $P_{a_{O_2}}$ and $P_{a_{CO_2}}$; the response times of these electrodes were not influenced by stopping the flow of buffer. All three electrodes were housed in thermostatted cuvettes maintained at ambient water temperature and were calibrated by pumping either saline equilibrated with appropriate gas mixtures (supplied by a Wösthoff gas-mixing pump) or buffer solutions through the extracorporeal circuit.

The dorsal aortic and buccal or opercular cannulae were connected to pressure transducers (Bell & Howell, 4-327-I). Dorsal aortic blood pressure was measured directly, while the arithmetic difference between inspiratory and expiratory buccal or opercular pressures was used as a measure of ventilation amplitude. The pressure transducers were calibrated against a static column of water. Breathing frequency was assessed periodically by counting buccal movements. Water P_{O_2} ($P_{W_{O_2}}$) was recorded by siphoning a small volume of water from the experimental chamber through a thermostatted cuvette containing a P_{O_2} electrode (Radiometer, E-5046).

Analogue measurements of $P_{a_{O_2}}$, $P_{a_{CO_2}}$, pHa, $P_{W_{O_2}}$, P_{DA} and ventilation amplitude were transformed into digital output using an analogue to digital interface (Data Translation, Inc.). Mean values for each variable were recorded and stored at 5 s intervals with a PC (software written by P. Thoren; Göteborg, Sweden).

Experimental protocol

Four series of experiments were performed: control (series 1), hypoxia (series 2), hyperoxia (series 3) and hypercapnia (series 4). The experimental protocol in each series consisted of imposing three or four consecutive 8 min stop–flow periods on a fish (N=6 for every series). Ventilation frequency was measured during each stop–flow period.

The initial stop–flow period was identical in all four series: a control stop–flow was performed once the measured ventilatory, cardiovascular and blood respiratory variables had stabilized (generally within 10–30 min of starting the extracorporeal circulation).

Series 1

In the control experiments, four stop–flow periods were imposed on each fish. Care was taken to ensure that the intervals of blood flow between stop–flow periods were similar in length to those in subsequent series (16–30 min). Intra-arterial injections of Cortland saline were performed prior to the final two stop–flow periods, to simulate the CA and acetazolamide treatments.

Series 2

Following the control stop–flow period, fish were subjected to hypoxia by bubbling N_2 through a water equilibration column supplying the experimental chamber. The flow of N_2 was adjusted (final $P\mathrm{w}_{O_2}$=4–12 kPa) according to the $P\mathrm{a}_{O_2}$ of individual fish to yield a final $P\mathrm{a}_{O_2}$ of 2.7–3.7 kPa; this level of hypoxaemia was chosen to avoid the release of catecholamines encountered at slightly lower $P\mathrm{a}_{O_2}$ values (Fievet *et al.* 1987). When the measured variables had re-stabilized, a second stop–flow period was imposed. Fish were then given a bolus injection of bovine CA (200 Wilbur–Anderson units mg^{-1}; 1 Wilbur–Anderson unit causes the pH of 0.012 mol l^{-1} veronal to drop from 8.3 to 6.3 in 1 min at 0 °C; 20 mg dissolved in 1 ml of Cortland saline), and a third stop–flow period was carried out. Finally, the fish were injected intra-arterially with acetazolamide (30 mg kg^{-1}). The high pH of the carrier solution precipitated a transient alkalosis, which was followed by a progressive acidosis (see Gilmour *et al.* 1994). The fourth stop–flow period was imposed during the acidotic phase, as pHa returned to pre-acetazolamide levels.

Series 3

The experimental protocol for series 3 was identical to that for series 2, except that fish were exposed to hyperoxia by bubbling O_2 through the water equilibration column. The goal in this series was to achieve the highest possible $P\mathrm{a}_{O_2}$; at a final $P\mathrm{w}_{O_2}$ of approximately 73 kPa, $P\mathrm{a}_{O_2}$ values of 47–67 kPa were measured.

A number of additional experiments were performed to complement series 3.

Tonometry. Blood samples (3 ml) were added to a 5 ml glass tonometer and equilibrated with a humidified gas mixture containing either 0.5 % or 0.1 % CO_2 in air. The blood was pumped from the tonometer through the extracorporeal circuit, allowing continuous measurement of pH, P_{O_2} and P_{CO_2}. An 8 min stop–flow period was carried out once the readings had stabilized. The blood was then made hyperoxic by equilibration with either 0.5 % or 0.1 % CO_2 in O_2, and a second stop–flow was performed.

Prebranchial blood. Cannulae of flexible polyethylene tubing (PE 50) were implanted (500–1200 g rainbow trout, N=4) into the dorsal aorta (DA) and into the afferent branchial artery (ABA) of the second gill arch on the left side. Following a 24 h recovery period, measurements of normoxic prebranchial pH, P_{O_2} and P_{CO_2} were made by withdrawing blood from the ABA cannula through the extracorporeal circuit. Blood was returned to the fish by the DA cannula. Postbranchial pH, P_{O_2} and P_{CO_2} were measured by switching to withdrawing blood from the DA cannula. The fish was then exposed to hyperoxia; postbranchial blood was monitored during this period to ensure that a degree of hyperoxia comparable to that in stop–flow trials was attained. Finally, pre- and postbranchial blood variables were measured under hyperoxic conditions.

Respirometry. Closed-system respirometry experiments were carried out on rainbow trout weighing between 190 and 310 g (N=4). A small volume of water was pumped from an experimental chamber through thermostatted cuvettes containing P_{O_2} and P_{CO_2} electrodes. P_{wO_2}, P_{wCO_2} and total CO_2 (C_{CO_2}) were measured at the beginning and end of a 25 min period during which the flow of water to the chamber was stopped. The fish was then exposed to hyperoxia by gassing the experimental chamber with O_2, and the measurements were repeated. Water samples were assayed for C_{CO_2} using a Capni–con total CO_2 analyzer (Cameron Instrument Company). O_2 consumption ($\dot{M}_{O_2}$) was calculated from P_{O_2} values, while CO_2 excretion ($\dot{M}_{CO_2}$) was determined as the mean of the estimates calculated from P_{CO_2} and C_{CO_2} measurements.

Series 4

Fish in this series were subjected to hypercapnia following the control stop–flow period by bubbling the water equilibration column supplying the experimental chamber with CO_2. The water and CO_2 flows required to achieve a final P_{wCO_2} of 0.67 kPa were established in a preliminary set of experiments. The second stop–flow period was performed once the measured variables had re-stabilized under hypercapnic conditions. The experimental chamber was then rapidly returned to normocapnia by bubbling both it and the water equilibration column with air, and a third stop–flow period was imposed while the blood of the fish remained hypercapnic. CA and acetazolamide trials were not performed in series 4.

Statistical analysis

Data are presented as means ± 1 standard error of the mean (S.E.M.). Statistical differences between control and treatment (hypoxia, hyperoxia or hypercapnia) values were determined by paired t-tests. One-sample t-tests were used to judge whether changes in ventilation amplitude or P_{aCO_2} (see Table 1) were statistically different from zero (Zar, 1984). The fiducial limit of significance in all tests was 5 %.

Results

Mean ventilation frequencies and amplitudes, absolute pHa and P_{aCO_2} levels and pHa and P_{aCO_2} changes during the stop–flow period (ΔpHa and ΔP_{aCO_2}) are shown in Table 1. Data for ventilation amplitude were expressed as percentage changes because of

the high degree of variability observed in resting levels. Figs 1, 2 and 3 illustrate the pHa and $P_{a_{CO_2}}$ data for the hypoxia, hyperoxia and hypercapnia experiments, respectively. Each line in these figures consists of continuous mean values for six fish; S.E.M. values are shown only every 2 min for clarity. $P_{a_{CO_2}}$ and pHa data for individual fish were normalized by subtracting from each point in a response the value at the beginning of the stop–flow period (Table 1). Normalization was necessary because the magnitude of disequilibrium events was small relative to individual variability. A rapid increase in $P_{a_{CO_2}}$ frequently occurred when the pump was re-started following the stop–flow period (Figs 1 and 2). This transient rise was probably an artefact related to the flow-sensitivity of one P_{CO_2} electrode; the effect was absent in series 4 (Fig. 3), where a different P_{CO_2} electrode was used.

The acid–base disequilibrium observed in the arterial blood of rainbow trout under control conditions was similar to that reported previously (Gilmour *et al.* 1994); during

Table 1. *Ventilatory variables during the stopflow periods, magnitudes of pHa (ΔpHa) and* $P_{a_{CO_2}}$ *(*$\Delta P_{a_{CO_2}}$*) changes during the stopflow period, and absolute values of pHa and* $P_{a_{CO_2}}$ *at the beginning of the stopflow period*

	f_V (min^{-1})	ΔVA (%)	pHa	ΔpHa	$P_{a_{CO_2}}$ (kPa)	$\Delta P_{a_{CO_2}}$ (kPa)
Series 1						
Control	56±4	–	7.91±0.08	0.04±0.01	0.16±0.02	0.005±0.003
Control	58±4*	–2±3	7.90±0.09	0.04±0.01	0.15±0.03	0.003±0.005
Saline	56±4	–5±4	7.88±0.10	0.04±0.01	0.16±0.03	0.004±0.003
Saline	57±4	–4±4	7.89±0.09	0.04±0.01	0.17±0.03	0.003±0.004
Series 2						
Control	56±3	–	7.92±0.05	0.04±0.01	0.28±0.03	0.011±0.003†
Hypoxia	66±2*	21±7†	7.98±0.09	0.05±0.01*	0.23±0.04*	0.007±0.003
CA	65±2	15±8	8.04±0.08	–0.01±0.01	0.22±0.04	0.007±0.004
ACTZ	62±2	10±6	8.05±0.07	–0.08±0.01	0.24±0.04	0.069±0.020†
Series 3						
Control	63±3	–	7.99±0.04	0.03±0.01	0.25±0.03	0.015±0.005†
Hyperoxia	40±4*	3±6	7.89±0.02*	–0.03±0.02*	0.34±0.03*	0.019±0.005†*
CA	37±8	5±5	7.91±0.05	–0.05±0.03	0.36±0.05	0.009±0.004
ACTZ	48±2	5±9	7.89±0.05	–0.11±0.02	0.43±0.06	0.091±0.028†
Series 4						
Control	63±4	–	7.93±0.03	0.03±0.00	0.27±0.02	0.020±0.004†
Hypercapnia	74±2*	12±3†	7.65±0.03*	0.06±0.01*	0.61±0.02*	0.020±0.003†
Recovery	70±3	8±3†	7.66±0.02	0.06±0.01	0.62±0.03	–0.009±0.012

Values are means ± 1 S.E.M.; N=6 for each treatment. f_V, ventilation frequency; ΔVA, percentage change from control in ventilation amplitude.

* indicates a significant difference (paired t-test, P<0.05) between the treatment (control, hypoxia, hyperoxia or hypercapnia) value and its associated control, and † indicates that ΔVA or $\Delta P_{a_{CO_2}}$ is significantly different (one sample t-test, P<0.05) from 0.

CA, carbonic anhydrase; ACTZ, acetazolamide.

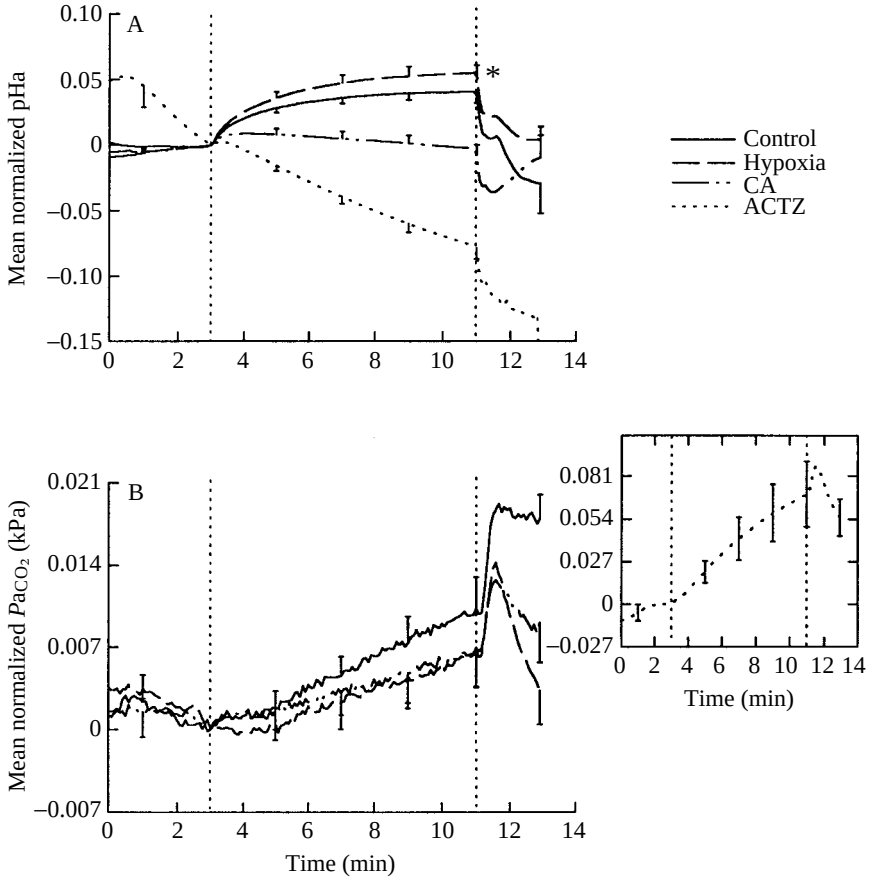

Fig. 1. Mean normalized (see text) values for (A) pHa and (B) P_{aCO_2} during blood flow and the four consecutive stop–flow periods (marked by the dotted lines) of series 2 (N=6): control, hypoxia, after CA injection and after acetazolamide infusion. Owing to the difference in the magnitude of P_{aCO_2} changes under control, hypoxic and CA treatments *versus* acetazolamide treatment, the results for acetazolamide have been displayed separately (inset in B). Error bars represent S.E.M. and are shown only every 2 min for clarity. An asterisk indicates a significant difference (paired t-test, P<0.05) at 11 min between the control and hypoxic values. CA, carbonic anhydrase; ACTZ, acetazolamide.

the stop–flow period, pHa increased by 0.03–0.04 units and P_{aCO_2} by 0.003–0.02 kPa (Table 1).

Series 2

Exposure of fish to environmental hypoxia resulting in a P_{aO_2} of 2.7–3.7 kPa induced a hyperventilation encompassing increases in both frequency and amplitude, and a decrease in P_{aCO_2}; the increase in pHa was not significant (Table 1). The positive pH disequilibrium was significantly increased (paired t-test, P<0.05) over the control value (Table 1; Fig. 1). The imbalance was abolished in the presence of exogenous CA

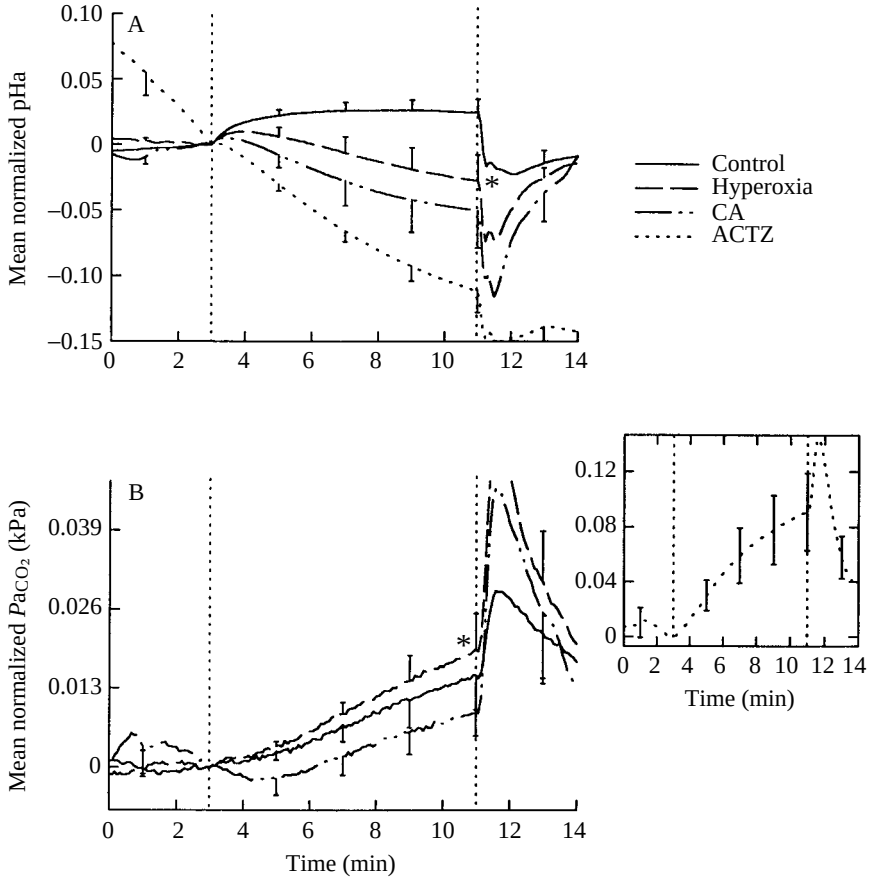

Fig. 2. Mean normalized values for (A) pHa and (B) Pa_{CO_2} during blood flow and the four consecutive stop–flow periods of series 3 (N=6): control, hyperoxia, after CA injection and after acetazolamide injection. The results for Pa_{CO_2} under the acetazolamide treatment are displayed in the inset of B. Error bars represent S.E.M. Symbols and abbreviations are as in Fig. 1.

(Table 1; Fig. 1); comparable results were obtained with CA under normoxia (Gilmour *et al.* 1994). Similarly, the effects of acetazolamide injection resembled those reported previously (Gilmour *et al.* 1994); a negative pH disequilibrium accompanied by a large, significant increase in Pa_{CO_2} were observed (Table 1; Fig. 1).

Series 3

Decreased ventilation frequency, pHa and elevated Pa_{CO_2} were detected in fish subjected to environmental hyperoxia (final Pw_{O_2}=73 kPa) (Table 1). Arterial blood P_{O_2} increased from 14.7±1.3 kPa to 61.8±5.0 kPa, whereas venous (prebranchial) P_{O_2} increased from 4.1±0.1 kPa to 11.3±1.3 kPa (Table 2). Closed-system respirometry experiments indicated that $\dot{M}_{CO_2}$ during hyperoxia (0.70±0.12 mmol kg^{-1}, N=4) was significantly reduced (paired t-test, P<0.05) in comparison with normoxia

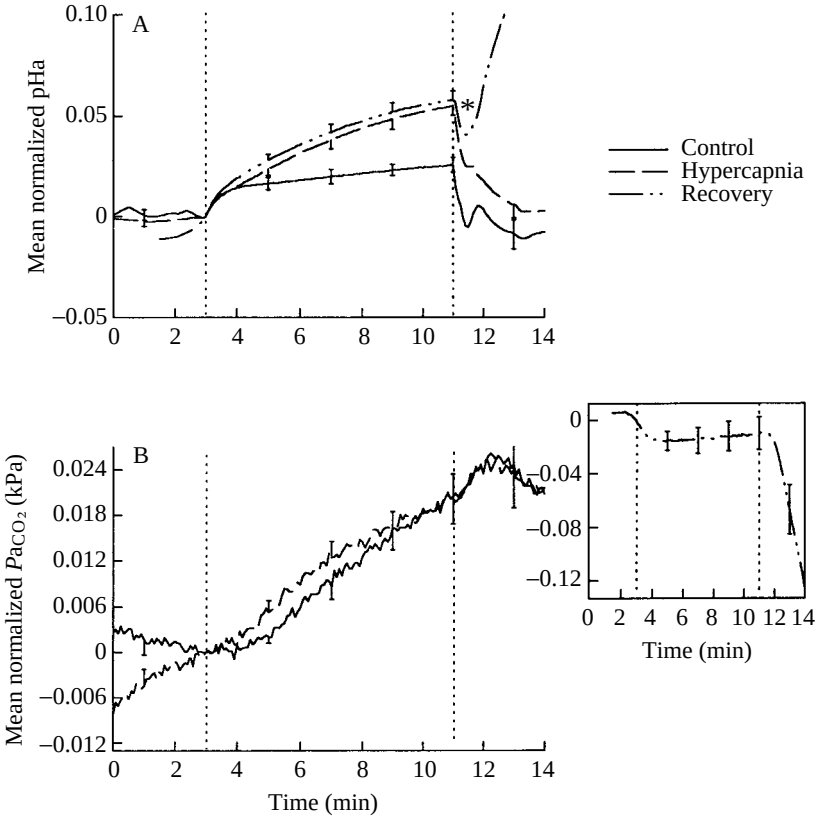

Fig. 3. Mean normalized values for (B) pHa and (B) Pa_{CO_2} during blood flow and the three consecutive stop–flow periods of series 4 (N=6): control, hypercapnia and recovery. The recovery stop–flow was performed after the water had been returned to normocapnia, but while the fish remained hypercapnic. The results for Pa_{CO_2} during recovery are displayed in the inset of B. Error bars represent S.E.M. Symbols are as in Fig. 1.

(1.89±0.18 mmol kg^{-1}), while $\dot{M}_{O_2}$ was constant (normoxic $\dot{M}_{O_2}$=2.56±0.36 mmol kg^{-1}; hyperoxic $\dot{M}_{O_2}$=2.44±0.42 mmol kg^{-1}). Further, the 63 % decrease in $\dot{M}_{CO_2}$ was coupled to a reduction in, or reversal of, the P_{CO_2} difference between post- and prebranchial blood (Table 2; Fig. 4). Red blood cell CO_2 excretion *in vitro*, measured using the assay of Wood and Perry (1991), was not affected by hyperoxia (normoxic HCO_3^- dehydration rate=11.0±1.1 μmol ml^{-1} h^{-1}, N=4; hyperoxic rate=12.7±0.4 μmol ml^{-1} h^{-1}, N=4).

The direction of the hyperoxic pHa disequilibrium was opposite to that found in normoxic conditions; pHa decreased by 0.03 units (Table 1; Fig. 2). The mean pH disequilibrium of normoxic blood in the corresponding tonometry experiments was 0.007±0.003 units (N=6), but in hyperoxic blood a slightly negative imbalance (−0.009±0.007 units) was detected. While taken individually, neither imbalance was significantly different from zero (one-sample t-tests), the difference between normoxia and hyperoxia was significant (paired t-test, P<0.05). The pHa disequilibrium in fish

Table 2. *The effects of environmental hyperoxia on the* P_{O_2} *of pre- (ABA) and postbranchial (DA) blood and on the* P_{CO_2} *difference (DA value minus ABA value) between arterial and prebranchial blood for four rainbow trout*

Fish number	Normoxic ABA P_{O_2} (kPa)	Hyperoxic ABA P_{O_2} (kPa)	Normoxic DA P_{O_2} (kPa)	Hyperoxic DA P_{O_2} (kPa)	Normoxic ΔP_{CO_2} (kPa)	Hyperoxic ΔP_{CO_2} (kPa)
1	4.3	11.5	17.7	72.7	–0.07	–0.05
2	3.9	12.0	13.2	66.9	–0.06	0.05
3	4.1	7.6	15.9	57.3	–0.13	0.10
4	4.2	13.9	12.2	50.1	0.00	0.09
Mean ± S.E.M.	4.1±0.1	11.3±1.3	14.7±1.3	61.8±5.0	–0.06±0.03	0.05±0.03

remained negative following the injection of CA (Table 1; Fig. 2) and, as in previous experiments, a negative imbalance was measured following acetazolamide injection (Table 1; Fig. 2).

Series 4

Ventilation variables were significantly elevated, P_{aCO_2} was increased approximately twofold and an acidosis was present in fish exposed to environmental hypercapnia (final P_{wCO_2}=0.67 kPa) (Table 1). The positive hypercapnic pHa disequilibrium was double that in normoxia (Table 1; Fig. 3). A 'recovery' stop–flow period was imposed while the fish still exhibited respiratory acidosis, but as P_{wCO_2} returned to normocapnia. The 'recovery' pHa imbalance resembled that in hypercapnia (Table 1; Fig. 3).

Discussion

The results of the present study confirm that an acid–base disequilibrium exists in the postbranchial arterial blood of rainbow trout. In addition, the environmental conditions experienced by the fish and its resultant respiratory status have been clearly demonstrated to affect the extent and/or direction of the pH disequilibrium. Manipulation of the fish's environment to produce predictable changes in ventilation and blood gas variables allowed factors influencing the imbalance to be examined. Although the disequilibrium amounts to only a few per cent of P_{aCO_2} and the mechanisms underlying the variations in the disequilibrium remain speculative, as will be discussed below, the results of this study are nevertheless of practical value, because they imply that caution must be exercised in interpreting data collected from blood samples. That is, the acid–base status of an arterial blood sample will change after it is acquired, and the magnitude and direction of the changes will depend on the respiratory status of the fish at the time of sampling.

The disequilibrium during hypoxia (series 2)

A significantly larger positive disequilibrium was measured in hypoxic than in normoxic fish (Table 1; Fig. 1). Hypoxia also increased the magnitude of the pH rise in stop–flow experiments on mammals treated with benzolamide to inhibit plasma CA activity (Crandall *et al.* 1977). The intensity of hypoxia used here, P_{aO_2}=2.7–3.7 kPa,

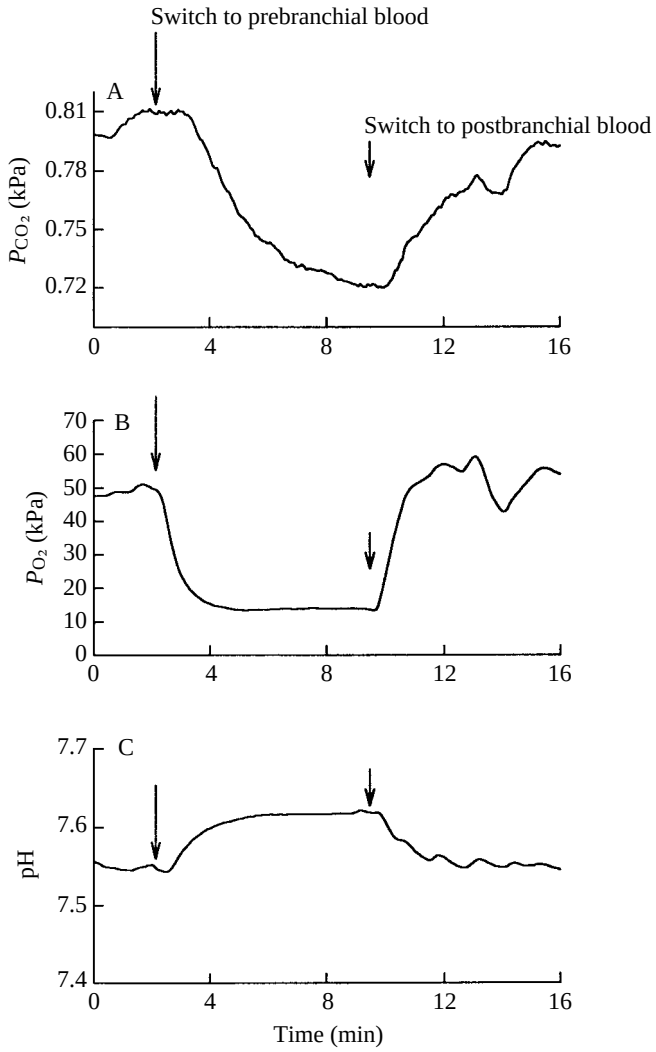

Fig. 4. Representative continuous and simultaneous recordings of (A) P_{CO_2}, (B) P_{O_2} and (C) pH, illustrating the changes in these variables associated with measurements of pre- and postbranchial blood during hyperoxia. In the initial and final periods of the traces, blood was withdrawn from the dorsal aorta, permitting postbranchial blood to be examined. Prebranchial blood variables were measured in the centre portion of the recordings; at this time, blood input to the circuit was from the afferent branchial artery.

typically elicits increases in ventilation accompanied by a hypocapnic alkalosis in rainbow trout (Thomas *et al.* 1988); series 2 fish exhibited these responses with the exception of the alkalosis, which was not significant (Table 1). The elevated flow of water over the gills will reduce the thickness of the water and mucus boundary layer adjacent to the gill epithelium (Piiper *et al.* 1986). This fluid layer (see Fig. 1 in Wright

et al. 1989) between the freely circulating water and the epithelial cells forms a gill microenvironment in which chemical and physical conditions can be different from those prevailing in the bulk water flow (Playle and Wood, 1989; Wright *et al.* 1986, 1989; Randall *et al.* 1991; Shephard, 1992). CO_2, as well as HCO_3^- and H^+ formed through the catalyzed (by boundary layer CA) hydration of excreted CO_2, must diffuse across the unstirred layer (Wright *et al.* 1986). A thinner boundary layer may therefore decrease the barrier to CO_2 diffusion, facilitating CO_2 excretion and resulting in a larger positive disequilibrium.

A reduction, in hypoxic fish, of red blood cell Cl^-/HCO_3^- exchange involvement in the disequilibrium may also have contributed to the larger positive imbalance. The residence time of blood in the gills is 0.5–2.5 s under normal conditions (Cameron and Polhemus, 1974) and the reaction time (T_{67}) for Cl^-/HCO_3^- exchange is 0.4 s (Cameron, 1978), so most anion exchange should be completed during the gill transit time (see also Gilmour *et al.* 1994). Any Cl^-/HCO_3^- exchange that persists in postbranchial blood will decrease the amplitude of the positive disequilibrium. The duration of anion exchange should be shortened in hypoxic fish for two reasons. First, some experimental evidence indicates that the operation of the exchanger is faster in deoxygenated blood. Perry and Gilmour (1993) reported that CO_2 excretion *in vitro* was enhanced in deoxygenated blood samples and suggested that Cl^-/HCO_3^- exchange could be linked to oxygenation through changes in red blood cell intracellular pH. Similar results were obtained by Wood and Simmons (1993), who proposed that haemoglobin acted as a transducer, influencing the rate of operation of the band 3 protein according to its degree of oxygenation. Second, the total Cl^-/HCO_3^- exchange should be reduced in hypoxic fish. Cl^-/HCO_3^- exchange is driven by the diffusive loss of CO_2 from the red blood cell and by the Bohr protons derived from the oxygenation of haemoglobin (reviewed by Klocke, 1988; see also Perry and Gilmour, 1993). Owing to the reduced oxygenation of haemoglobin during hypoxia, fewer Bohr protons will be available and it can therefore be assumed that the amount of Cl^-/HCO_3^- exchange will be lower.

The disequilibrium during hyperoxia (series 3)

The direction of the acid–base disequilibrium was reversed under hyperoxic conditions (Table 1; Fig. 2). The negative disequilibrium is unlikely to have resulted from an increase in the involvement of Cl^-/HCO_3^- exchange. The P_{O_2} of prebranchial blood in hyperoxic fish was about 11 kPa (Table 2), a level which corresponds to approximately 95 % O_2 saturation (Perry and Reid, 1992). Thus, little oxygenation would take place during the gill transit time and, as discussed above, Cl^-/HCO_3^- exchange should be completed more quickly. The negative pH imbalance may have been due in part to hyperoxia-specific metabolic effects. While the flow of blood is stopped, the electrodes have access to only a small pool of blood in a closed system, and levels of blood gases may be altered by red blood cell metabolism. For example, red blood cell metabolism is probably one source of the continuing P_{aCO_2} rise during the stop–flow period, even after a plateau has been reached for pHa (Fig. 1). The non-significant pH rise which occurred in stop–flow experiments on blood equilibrated to normoxia in a tonometer flask may have

been an artefact arising from the sensitivity of the electrode to the flow of viscous blood (a 0.002 unit change was measured when the flow of buffer solution was stopped). The corresponding hyperoxic pH change was −0.009 units, a decrease of 0.016 units from normoxia. While the cause of this reversal is uncertain, it accounts for approximately 30 % of the *in vivo* difference between normoxia and hyperoxia.

The remainder of the negative pH disequilibrium in hyperoxic fish appears to be caused by a reversal of the diffusion gradient for CO_2 (i.e. the mean blood-to-water P_{CO_2} difference). In agreement with previous studies (Wood and Jackson, 1980; see review by Perry and Wood, 1989), environmental hyperoxia induced hypoventilation, an increase in Pa_{CO_2} and an associated fall in pHa (Table 1). Respirometry experiments indicated that, while O_2 consumption was not affected by hyperoxia, CO_2 excretion was halved. $\dot{M}_{CO_2}$ *in vivo* was probably not inhibited by the red blood cell HCO_3^- dehydration rate, since this was not influenced by hyperoxia *in vitro*. Postbranchial P_{CO_2} values were higher than prebranchial levels in three of four hyperoxic fish examined and, even in the fourth, the P_{CO_2} difference was decreased by hyperoxia (Table 2; Fig. 4). This increase in P_{CO_2} could conceivably be explained by a reversal of the CO_2 diffusion gradient. The decreased flow of convective water (Haswell *et al.* 1978; Wood and Jackson, 1980) might not be sufficient to remove CO_2 from the boundary layer at the same rate as CO_2 delivery to the boundary layer *via* excretion from the blood and titration of boundary layer HCO_3^- by acid equivalents excreted across the gills. Thus, boundary-layer P_{CO_2} could increase substantially, conceivably even exceeding arterial P_{CO_2}. Two gradients would be established: one for CO_2 movement from the boundary layer to the water, accounting for the CO_2 excretion (albeit reduced) detected in respirometry experiments; and the other a gradient for CO_2 diffusion from the boundary layer to the blood, resulting in the reversal of the normal P_{CO_2} difference between post- and prebranchial blood. In contrast to normoxia, in which HCO_3^- dehydration predominates, the inverted CO_2 diffusion gradient in hyperoxia would drive plasma $CO_2/HCO_3^-/H^+$ reactions towards CO_2 hydration (at the uncatalyzed rate), thereby creating a negative pH disequilibrium.

The principal argument against the reversed CO_2 gradient hypothesis lies in the CA data for hyperoxia. If the negative pH imbalance were indeed caused by the slow rate of uncatalyzed plasma CO_2 hydration, then treatment with CA should reduce the extent of the disequilibrium. However, CA-treated hyperoxic fish exhibited a negative disequilibrium similar in magnitude to that measured prior to CA injection (Table 1; Fig. 2). A satisfactory explanation for this discrepancy has not been found, although one conjecture is that the hyperoxia-related metabolic effects were enhanced by treatment with CA.

Surprisingly, the negative pH disequilibrium was accompanied by a rise in Pa_{CO_2} (Table 1; Fig. 2), rather than by the decrease predicted by the reversed CO_2 gradient theory. This anomaly was one of several inconsistencies between ΔpHa and ΔPa_{CO_2} values (Table 1). For example, the increase in Pa_{CO_2} anticipated during control stop–flow periods was not always significantly different from zero (Table 1; Gilmour *et al.* 1994). While the reliability of the pHa data is not in question, the slow response time of the P_{CO_2}

electrode, particularly for small changes in P_{CO_2} and under conditions in which the direction of P_{CO_2} readings changed, may account for the discrepancies.

The disequilibrium during hypercapnia (series 4)

The magnitude of the positive acid–base disequilibrium was doubled by environmental hypercapnia (Table 1; Fig. 3). Ventilation rate and Pa_{CO_2} were increased, while pHa was decreased (Table 1); similar responses have been reported previously (Janssen and Randall, 1975; Thomas and Le Ruz, 1982; Kinkead and Perry, 1991). The high P_{CO_2} of the inspired water initially constrains P_{CO_2} to increase during hypercapnia but, once equilibrium has been established, CO_2 excretion is probably raised over the normoxic level by the hyperventilation. The elevated convective flow of water will, as in fish exposed to hypoxia, reduce the boundary layer and facilitate CO_2 excretion. In hypercapnic fish, the greater CO_2 loss is coupled with an increase in proton concentration to yield a larger positive disequilibrium. The significance of proton availability was similarly demonstrated by Bidani and Crandall (1978) using acid- or base-infused dogs and cats treated with acetazolamide.

A stop–flow trial was performed following the return to normocapnic water ('recovery' stop–flow; Table 1; Fig. 3). Ventilation rate and P_{CO_2} remained elevated and pHa remained depressed (Table 1) but, in comparison with the hypercapnic stop–flow, the CO_2 diffusion gradient should have been enlarged owing to the low inspired water P_{CO_2}. However, the magnitude of the disequilibrium was not altered (Table 1; Fig. 3), suggesting that the CO_2 diffusion gradient can enhance the positive pH imbalance only to a limited extent. An alternative possibility is that the immediate hypoventilation in the recovery phase (Table 1) countered the low inspired P_{CO_2}, such that the blood-to-water P_{CO_2} difference was not modified with respect to the hypercapnic situation.

In conclusion, this study has identified and examined several variables that could potentially influence the magnitude and/or direction of the acid–base disequilibrium in the arterial blood of rainbow trout. These variables included: the duration of Cl^-/HCO_3^- exchange, the CO_2 diffusion gradient, the boundary layer thickness and the pH of the blood. Cl^-/HCO_3^- exchange contributes little to the net acid–base disequilibrium under normal conditions; the extent of the positive imbalance was only slightly increased when the duration of the exchange process was reduced (hypoxia). The CO_2 diffusion gradient appears to be a key factor in generating the disequilibrium, since a reversal of the normal gradient between the boundary layer and the blood effected a reversal of the direction of the disequilibrium (hyperoxia). However, the capacity of this factor to increase the extent of a positive disequilibrium may be limited (hypercapnic 'recovery'). A decrease in the thickness of the boundary layer appeared to be involved in the larger positive disequilibrium measured in hyperventilating fish (hypoxia, hypercapnia). Finally, the extent of the acid–base disequilibrium was sensitive to plasma pH, in that an increase in proton concentration was associated with a larger positive disequilibrium.

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