# THE EFFECT OF VARYING WATER pH ON THE ACIDIFICATION OF EXPIRED WATER IN RAINBOW TROUT

By HONG LIN and D. J. RANDALL

*Zoology Department, University of British Columbia,*
*6270 University Boulevard, Vancouver, BC, V6T 2A9, Canada*

## Summary

Acidification of expired water was studied in rainbow trout exposed to water of pH 9.91, 6.80 (control) and 3.88. For the high-pH and the control exposures, the water flowing over the gills was acidified because of the dominant effect of $CO_2$ hydration. During the low-pH treatment, the water was alkalized because of ammonium ion formation and perhaps bicarbonate dehydration. Carbon dioxide excretion was not significantly affected by the high-pH and low-pH treatments but ammonia accumulated in the plasma in both cases.

## Introduction

Fish excrete molecular $CO_2$ and $NH_3$ into the water passing over the gills. The excretion rates are an order of magnitude greater for $CO_2$ than for $NH_3$. Some carbon dioxide is excreted as bicarbonate in exchange for chloride, and some ammonia as ammonium ion in exchange for sodium. The former represents about 10 % of the total resting carbon dioxide excretion and the latter up to 50 % of the total ammonia excretion (see review by Randall and Wright, 1989). Because molecular $CO_2$ is the dominant excretory product in the gills, the water should be acidified because of $CO_2$ hydration catalyzed by carbonic anhydrase in the gill mucus and water boundary layer (Wright *et al.* 1986) and on the apical surface of the gill epithelium (Rahim *et al.* 1988). In neutral and alkaline waters most of the excreted $CO_2$ should be converted to bicarbonate, but under acid conditions only a small fraction should be hydrated. Thus, the extent of acidification of water as it passes over the gills should decrease with water pH. The opposite should be true for the $NH_3/NH_4^+$ reaction. Under acid conditions almost all the $NH_3$ should be converted to $NH_4^+$ but under alkaline conditions only a fraction of the excreted $NH_3$ should form $NH_4^+$ in the gill water. The following experiments were designed to investigate the effect of varying the pH of inspired water on the acidification of water as it passes over the gills.

## Materials and methods

### Animals and preparation

Rainbow trout (*Salmo gairdneri*, Richardson), weighing 324–494 g, were obtained from the West Creek Trout Farm (Aldergrove, BC) and housed in outdoor fiberglass tanks supplied with flowing dechlorinated Vancouver tap water (pH 6.5–6.8; temperature, 8–12°C; hardness, 12 p.p.m. $CaCO_3$). Fish were fed with commercial trout pellets and feeding was suspended at least 2 days before experimentation.

Fish were prepared with a dorsal aortic cannula for sampling blood, an opercular cannula for sampling expired water and a van Dam mask for measurement of ventilation, as described by Wright *et al.* (1986). After the surgical procedure, fish were left to recover for 20–40 h in the van Dam apparatus (Fig. 1) supplied with a flowing aerated test solution of 40 mmol l$^{-1}$ NaCl and 0.5 mmol l$^{-1}$ $CaCl_2$ in dechlorinated tap water (9.5–11.2°C). The test solution had an ionic
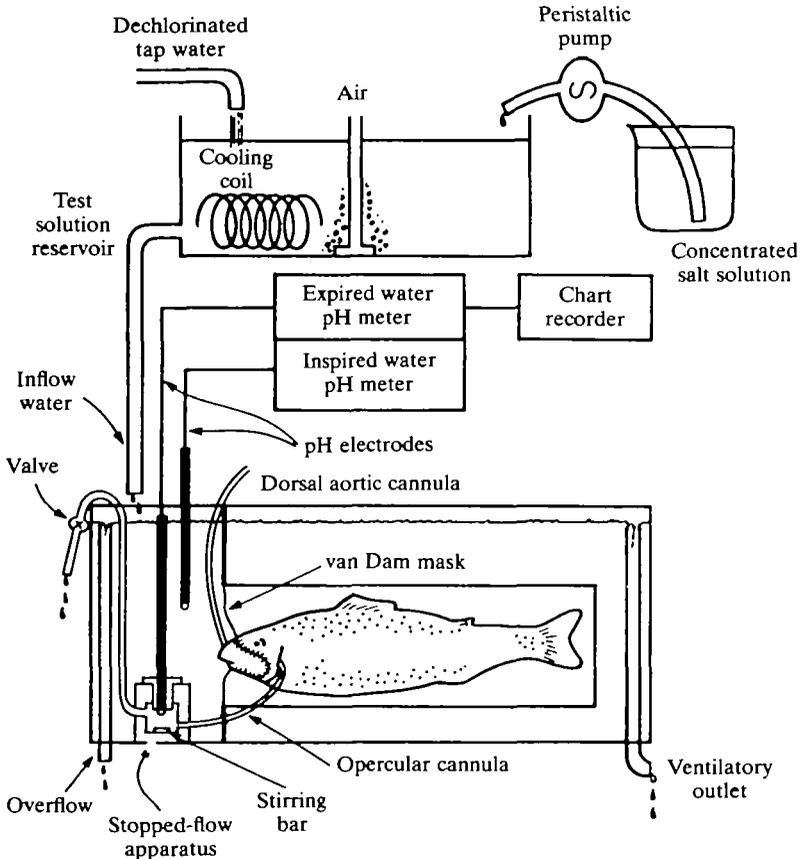


Fig. 1. The apparatus for measuring inspired, expired and stopped-flow water pH. Fish were prepared with a van Dam mask, a dorsal aortic cannula and an opercular cannula, and were placed in a two-chambered acrylic plastic box.

strength similar to that of the buffer solution used to calibrate the pH electrodes. By using this test solution, we reduced the response time of the pH electrode, increased its stability and thus obtained more precise water pH measurements.

### Experimental protocols and measurements

Fish were subjected to one of two treatments during the experimental periods, as follow. (1) NaOH was added to the reservoir to increase the pH of the test solution to $9.91\pm0.02$ for 90 min. (2) HCl was added to the reservoir to reduce the pH of the test solution to $3.88\pm0.02$ for 90 min.

Each series consisted of a 30-min control period (C1), two experimental periods (E1, E2), 45 min each, and a 30-min recovery period (C2).

The following measurements were performed in each of the control, experimental and recovery periods. (1) Inspired water pH (pHin), expired water pH (pHex) and stopped-flow water pH (pHst) were measured as by Wright *et al.* (1986). (2) Ventilation ($\dot{V}_G$) was measured by collecting the outflow water over 1-min periods from the standpipe in the back chamber of the van Dam apparatus. (3) Total carbon dioxide contents of the inspired water ($CO_{2,in}$) and expired water ($CO_{2,ex}$) were measured with a Carle gas chromatograph (model III) containing a $CO_2$ discriminating column (porapak Q) (Boutilier *et al.* 1985; Lenfant and Aucutt, 1966). (4) A blood sample (0.7 ml) was withdrawn from the dorsal aortic cannula and replaced with the same amount of heparinized saline. The whole-blood pH (pHB) was measured by using a Radiometer G297/G2 capillary electrode with a Radiometer PHM-71 acid–base analyzer. (5) The remaining blood sample was centrifuged and the plasma was removed. Total carbon dioxide content of the plasma ($CO_{2,pl}$) was measured by gas chromatography as described above for water. Total ammonia content ($T_{Amm}$) of the plasma was measured by micro-modification of a commercial diagnostic kit with an ultraviolet-visible recording spectrophotometer (L-glutamic dehydrogenase/NAD method; Sigma, 1982). (6) Inspired and expired water buffering capacities were determined separately in acid, alkaline and neutral water by titrating both the inspired and the expired water under each experimental condition using either $0.1\,mol\,l^{-1}$ HCl or $0.1\,mol\,l^{-1}$ NaOH as titrants. The water was open to the air and stirred during this procedure, resulting in small and uncontrolled changes in $P_{CO_2}$. However, there was no measurable difference between inspired and expired buffer curves for the same experimental condition. The buffer curves obtained from the titration were used to calculate the increase in proton concentration in expired water.

### Calculations and statistics

The rate of carbon dioxide excretion ($\dot{M}_{CO_2}$) was calculated from the total $CO_2$ contents of inspired and expired water and the ventilation volume per hour by application of the Fick principle, and expressed per gram of fish mass.

Free ammonia concentration in plasma $[NH_3]$ was calculated from total ammonia concentration in plasma and whole-blood pH by use of the Henderson–

Hasselbalch equation, incorporating the $pK_{Amm}$ value of Cameron and Heisler (1983).

Carbon dioxide partial pressures of both plasma and water were calculated from the total $CO_2$ content and pH, using the Henderson–Hasselbalch equation and the $pK'_{CO_2}$ and $\alpha_{CO_2}$ values of Boutilier *et al.* (1985).

The increase in proton concentration in expired water was calculated from the differences in pHex and pHin and the appropriate buffer curve, and was expressed as the proton concentration increase per hour per gram of fish mass.

Data are presented as means±standard error. To compare the relationships in the data, Student's two-tailed *t*-tests, one-way and two-way ANOVAs (analysis of variance) and regression analyses were used. 5 % level of rejection was taken as the statistical limit of significance.

## Results

### Downstream water pH

At high pH, the inspired water was acidified as it passed through the gills (Fig. 2). During periods C1 and E1, there was no significant difference between pHex and pHst. However, in E2 and C2 there were small but significant differences in pH. The calculated proton concentration increase in expired water was significantly larger for fish exposed to alkaline water (Fig. 3). There was, however, no significant difference in the proton concentration increase in expired water between periods E1 and E2, or between periods C1 and C2.

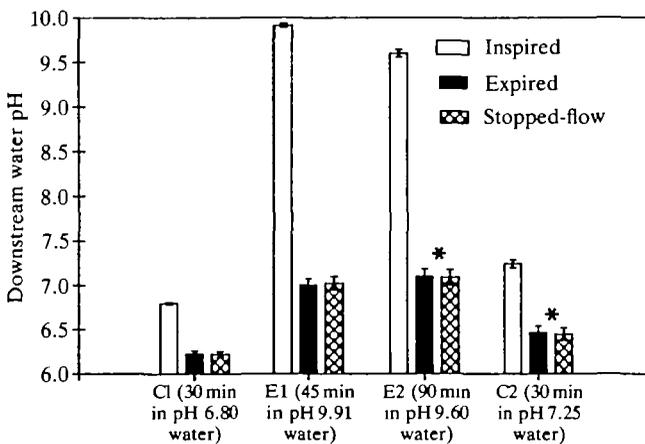During the low-pH treatment, the inspired water was alkalized rather than



Fig. 2. pH of inspired, expired and stopped-flow expired water of rainbow trout during high-pH treatment. Measurements were taken when the fish had been in the neutral control water for 30 min, after the fish had been exposed to pH 9.91 experimental water for 45 and 90 min, and 30 min after the fish had been returned to the neutral control water. pHin was significantly different from pHex in all cases, whereas pHst was significantly different from pHex only in periods E2 and C2, marked with an asterisk ($\bar{x}\pm$s.E.).
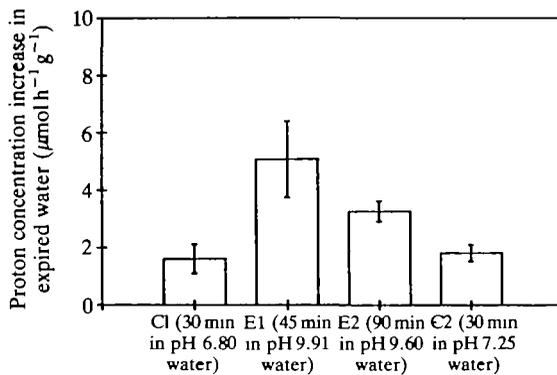
Fig. 3. Proton concentration increase in the expired water during high-pH treatment of rainbow trout expressed in $\mu$mol per hour per gram of fish mass. Data were calculated using the pH values in Fig. 2 and buffer curves for water over the appropriate pH range. Significant differences were exhibited between periods E1 and C1 and between E2 and C2 ($\bar{x} \pm$ s.e.).
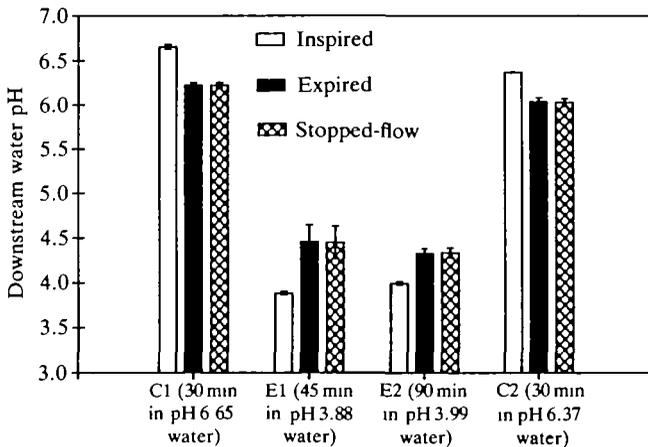


Fig. 4. pH of inspired, expired and stopped-flow expired water of rainbow trout during low-pH treatment. Measurements were taken after the fish had been in the neutral control water for 30 min, after the fish had been exposed to pH 3.88 experimental water for 45 and 90 min, and 30 min after the fish had been returned to the neutral control water. pHin was significantly different from pHex in all cases but pHst was significantly different from pHex only in period E2 ($\bar{x} \pm$ s.e.).

acidified, as it passed over the gills (Fig. 4). There was no significant difference between pHex and pHst except during period E2. The decrease in proton concentration in expired water was larger than the increase observed in neutral water (Fig. 5). Once again, there were no significant differences in the proton concentration changes in expired water between either E1 and E2 or C1 and C2.
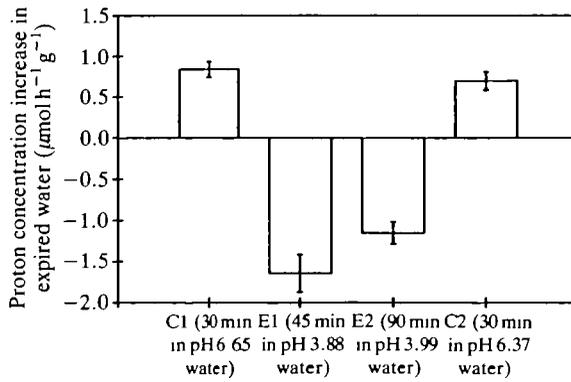
Fig. 5. Proton concentration increase in the expired water during low-pH treatment of rainbow trout expressed in $\mu$mol per hour per gram of fish mass. Data were calculated using the pH values in Fig. 4 and buffer curves for water over the appropriate pH range. Significant differences were exhibited between periods E1 and C1 and between E2 and C2 ($\bar{x}\pm$s.e.).
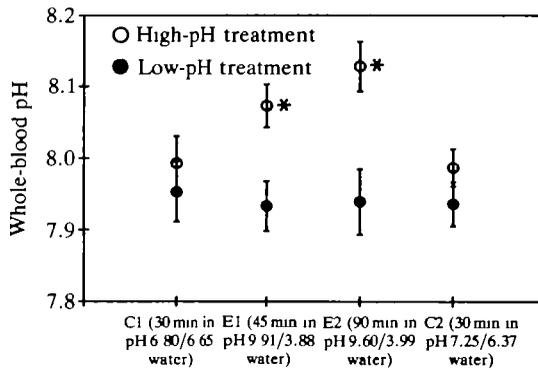


Fig. 6. Dorsal aortic blood pH of rainbow trout. Measurements were taken after the fish had been in the neutral control water for 30 min, after the fish had been exposed to pH 3.88 or 9.91 experimental water for 45 and 90 min, and 30 min after the fish had been returned to the neutral control water. * indicates a significant difference from the control (C1) value ($\bar{x}\pm$s.e.).

## Whole-blood pH

The blood of the fish was alkalized when the inspired water pH was raised to 9.91, and returned to normal during recovery. There was no significant difference in blood pH between periods E1 and E2 (Fig. 6).

No significant change in blood pH was observed when the inspired water pH was dropped to 3.88 and then returned to 6.37 (Fig. 6).

## Carbon dioxide excretion

During exposure to pH 9.91 water, there was no change in plasma total CO

Table 1. *Carbon dioxide excretion during high-pH and low-pH treatments in rainbow trout*

| | N | Control 1 | Experiment 1 | Experiment 2 | Control 2 |
|---|---|---|---|---|---|
| **High-pH treatment** | | | | | |
| Total $CO_2$ $(mmol\,l^{-1})$ | | | | | |
| Plasma | 6 | 19.4±0.9 | 20.1±1.0 | 20.2±1.1 | 19.4±0.9 |
| Inspired | 7 | 0.38±0.02 | 0.45±0.02* | 0.50±0.02* | 0.40±0.02 |
| Expired | 7 | 0.53±0.02 | 0.60±0.02* | 0.64±0.03* | 0.54±0.02 |
| $T_{CO_2(ex-in)}$ $(mmol\,l^{-1})$ | 7 | 0.15±0.01 | 0.15±0.02 | 0.14±0.02 | 0.14±0.01 |
| $P_{CO_2}$ (kPa) | | | | | |
| Plasma | 6 | 0.64±0.03 | 0.54±0.02* | 0.48±0.03* | 0.65±0.04 |
| Inspired | 7 | 0.19±0.01 | 0.00±0.00* | 0.00±0.00* | 0.11±0.01* |
| Expired | 7 | 0.59±0.04 | 0.21±0.03* | 0.21±0.04* | 0.47±0.05* |
| $\dot{V}_G$ $(ml\,min^{-1})$ | 7 | 146±38 | 145±31 | 117±17 | 126±15 |
| $\dot{M}_{CO_2}$ $(\mu mol\,g^{-1}\,h^{-1})$ | 7 | 3.18±0.89 | 2.91±0.74 | 2.27±0.45 | 2.46±0.28 |
| **Low-pH treatment** | | | | | |
| Total $CO_2$ $(mmol\,l^{-1})$ | | | | | |
| Plasma | 6 | 17.1±1.4 | 16.3±1.7 | 16.2±1.7 | 16.5±1.7 |
| Inspired | 6 | 0.40±0.02 | 0.32±0.02* | 0.32±0.02* | 0.41±0.03 |
| Expired | 6 | 0.54±0.03 | 0.46±0.04* | 0.44±0.04* | 0.52±0.04 |
| $T_{CO_2(ex-in)}$ $(mmol\,l^{-1})$ | 6 | 0.14±0.02 | 0.14±0.03 | 0.12±0.03 | 0.11±0.02 |
| $P_{CO_2}$ (kPa) | | | | | |
| Plasma | 6 | 0.61±0.04 | 0.60±0.06 | 0.59±0.06 | 0.61±0.05 |
| Inspired | 6 | 0.25±0.01 | 0.62±0.04* | 0.61±0.04* | 0.38±0.03* |
| Expired | 6 | 0.60±0.04 | 0.87±0.07* | 0.83±0.07* | 0.66±0.08 |
| $\dot{V}_G$ $(ml\,min^{-1})$ | 6 | 110±18 | 128±22 | 122±15 | 126±22 |
| $\dot{M}_{CO_2}$ $(\mu mol\,g^{-1}\,h^{-1})$ | 6 | 2.03±0.14 | 2.39±0.46 | 2.05±0.44 | 1.85±0.35 |

* Indicates a significant difference from the control (C1) value ($\bar{x}\pm$s.e.).

content, but plasma $P_{CO_2}$ was reduced. Inspired and expired water total $CO_2$ were increased because carbon dioxide was trapped as $HCO_3^-$ in this alkaline water, even though inspired water $P_{CO_2}$ was lower than control values (Table 1). There was, however, no change in the difference between inspired and expired water $T_{CO_2}$ between or within the control and experimental periods; that is, carbon dioxide excretion was unaffected by the elevation in water pH and $T_{CO_2}$. The ventilation volume ($\dot{V}_G$) varied greatly among animals but there were no significant differences between the control and experimental groups (Table 1).

Exposure of the fish to acid conditions also produced no significant changes in plasma total $CO_2$ content, plasma $P_{CO_2}$, $\dot{V}_G$ and $\dot{M}_{CO_2}$ (Table 1). $P_{CO_2}$ of the inspired acid water was raised to twice that of control water, but total $CO_2$ content decreased. However, there was no significant difference in $T_{CO_2(ex-in)}$ between fish in control and acidified water (Table 1).

*Plasma ammonia*

Ammonia was accumulated within the body of the fish during both high-pH and

Table 2. *Plasma ammonia levels during high-pH and low-pH treatments in rainbow trout*

|  | N | Control 1 | Experiment 1 | Experiment 2 | Control 2 |
|---|---|---|---|---|---|
| **High-pH treatment** |  |  |  |  |  |
| Total ammonia in plasma ($\mu$mol l$^{-1}$) | 6 | 48.9±8.2 | 85.0±15.4* | 81.8±17.6* | 54.5±11.1 |
| [NH$_3$] in plasma ($\mu$mol l$^{-1}$) | 6 | 0.81±0.15 | 1.70±0.37* | 1.80±0.36* | 0.88±0.19 |
| NH$_3$/$T_{Amm}$ ratio |  | 0.017 | 0.020 | 0.022 | 0.016 |
| **Low-pH treatment** |  |  |  |  |  |
| Total ammonia in plasma ($\mu$mol l$^{-1}$) | 6 | 57.3±14.9 | 173±47.0* | 213±49.2* | 95.4±15.3* |
| [NH$_3$] in plasma ($\mu$mol l$^{-1}$) | 6 | 0.86±0.24 | 2.46±0.69* | 2.96±0.69* | 1.36±0.29* |
| NH$_3$/$T_{Amm}$ ratio |  | 0.015 | 0.014 | 0.014 | 0.014 |

*Indicates a significant difference from the control (C1) value ($\bar{x}$±S.E.).

low-pH treatments. Total ammonia content of plasma increased by 70% in fish in pH 9.91 water but returned to normal during recovery (Table 2). Non-ionic ammonia content [NH$_3$] increased along with the [NH$_3$]/$T_{Amm}$ ratio, reflecting the elevation in blood pH (Table 2). Total ammonia content in plasma rose continuously during the pH 3.88 treatment, increasing above the initial control value by 203.4% in E1 and 272.8% in E2. It was still 66.4% higher than the initial control value during recovery (Table 2). [NH$_3$] increased with total ammonia content. There was no change in the [NH$_3$]/$T_{Amm}$ ratio as blood pH remained constant (Table 2).

## Discussion

### Downstream water pH

In all our experiments, the differences between pHst and pHex values were quantitatively negligible, although in some cases they were statistically significant due to a small disequilibrium of the $CO_2$:$HCO_3^-$ reaction, as observed by Wright *et al.* (1986). The similarity of pHst and pHex values indicated, however, that the $CO_2$ hydration/dehydration reaction was catalyzed by carbonic anhydrase at the gill surface over a wide range of environmental water pH.

In inspired water of approximately neutral pH, expired water was acidified by excreted $CO_2$, which formed $HCO_3^-$ and protons, the reaction being catalyzed by carbonic anhydrase (Wright *et al.* 1986). At the same time, protons were consumed by excreted $NH_3$, which formed $NH_4^+$. As the $CO_2$ excretion rate is about 10 times greater than the ammonia excretion rate in fish (Wright and Wood, 1985), the overall result was acidification of the expired water.

When fish were exposed to pH 9.91 water, the alkalization of expired water du

to $NH_4^+$ formation was reduced because, at this pH, less than half of the excreted $NH_3$ will form $NH_4^+$, whereas essentially all the molecular carbon dioxide excreted will form bicarbonate, and the remainder will form carbonate. In pH 3.88 water, in contrast, acidification caused by $HCO_3^-$ formation was negligible, for no more than 1 % of the total excreted $CO_2$ will be converted to $HCO_3^-$ at this pH. Alkalization by $NH_4^+$ formation, however, will occur because almost all the excreted ammonia is converted to $NH_4^+$. As a result, expired water pH showed a small but significant increase.

We translated the differences of expired and inspired water pH into proton concentration increases in expired water by using the appropriate buffer curve.

The total acid equivalents added to the neutral water as it passed over the gills was in the same range as carbon dioxide excretion, indicating that acidification was largely the result of $CO_2$ hydration. Exposure to alkaline water resulted in a marked increase in the acid equivalents added to the water (Fig. 3) without any change in carbon dioxide excretion (Table 1). This is to be expected because of a marked increase in proton production due to carbonate formation ($pK_2=10$). As a result, proton production at this pH will be approximately 1.5 times $CO_2$ excretion. Other explanations for this observed increase are an expected decreased formation of $NH_4^+$ at high pH, and/or a reduced $HCO_3^-$ excretion and/or increased proton efflux. A net acid efflux across the gills is indicated by the rise in blood pH in fish exposed to alkaline conditions. If $HCO_3^-$ efflux across the gills is reduced, $CO_2$ excretion will be maintained by an elevated excretion of molecular $CO_2$ augmented by the increased $P_{CO_2}$ difference between water and blood (Table 1).

The number of alkaline equivalents added to the water passing over the gills when fish were exposed to acid water (Fig. 5) was greater than the expected ammonia excretion, which is known to be reduced in acidic environments (Wright and Wood, 1985). Bicarbonate (excreted *via* a $Cl^-/HCO_3^-$ exchange process) dehydration will also contribute to this process and may account for the unexpectedly high levels of alkaline equivalents added to the water under acid conditions.

When inhalant water pH and exhalant water pH are plotted against inspired water pH (Fig. 7), the pHin/pHex regression intersects the line of identity at pH 5.3. This shows that water was acidified in the neutral and alkaline environments and alkalized in the acidic environment as it passed over the gills. At environmental water pH around 5.3, about one-tenth of the excreted $CO_2$ will be converted to $HCO_3^-$, acidifying the expired water, but almost all the excreted $NH_3$ (about one-tenth of the amount of excreted $CO_2$) will be converted to $NH_4^+$ and raise expired water pH, the overall effect being no change in water pH as it flows over the gills.

If one assumes that $NH_3$ excretion is 10 % of $CO_2$ excretion, that only $NH_3$ and $CO_2$ (but not $NH_4^+$ and $HCO_3^-$) are excreted, and that $\dot{V}G$ and excretion rates are unaffected by inhalant water pH, it can be calculated that, although the relationship between inhalant and exhalant water pH is non-linear, the lines will
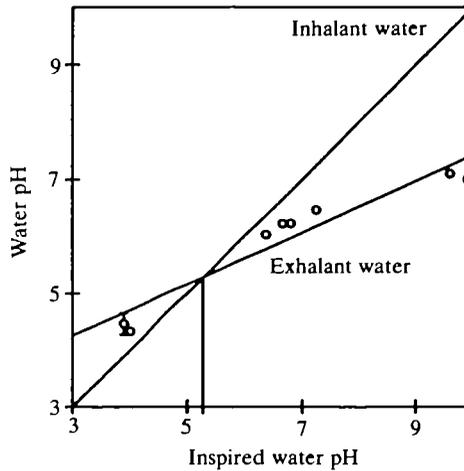
Fig. 7. The relationship between pH of exhalant water (O) and inhalant water of rainbow trout. The exhalant water line is the regression line of the mean pH values collected from both high-pH and low-pH treatments ($y=0.453x+2.894$, $r^2=0.96$). The inhalant and exhalant lines cross at pH 5.3, which may be equivalent to the point where no pH change occurs when water flows over the gills.

intersect at pH 5.39 ($t=10\,°C$, ionic composition of water as in methods, pK values from Boutilier *et al.* 1985; Cameron and Heisler, 1983). This calculated value of 5.39 is similar to the graphically derived value of 5.3, indicating that the ratio of $NH_3$ to $CO_2$ excretion was 0.1 and only the non-ionic forms were excreted by the fish under these acidic conditions.

## $CO_2$ excretion

In high-pH water, a decrease in water and, therefore, plasma $P_{CO_2}$ was expected. When the water pH was increased from 6.80 to 9.91, the $CO_2/HCO_3^-$ ratio went down from about 0.2 to 0.002. As a result, there was a decrease of water $P_{CO_2}$ when pH was increased. This significant decrease in inspired water $P_{CO_2}$ was associated with a lowered plasma $P_{CO_2}$. In low pH water, however, $P_{CO_2}$ was expected to increase, for only 1 % of the total $CO_2$ will be as $HCO_3^-$. This increase occurred but had no impact on plasma $P_{CO_2}$. Arterial plasma $P_{CO_2}$ and inspired water $P_{CO_2}$ were not significantly different from each other, whereas expired water $P_{CO_2}$ was higher than arterial plasma $P_{CO_2}$ (but might not be higher than venous plasma $P_{CO_2}$). Changes in water pH, whether acid or alkaline, had no effect on carbon dioxide excretion by the fish, although high water pH might be expected to facilitate $CO_2$ excretion while low water pH inhibits it. It appears that $Cl^-/HCO_3^-$ exchange across the red blood cell membrane is the rate-limiting step in carbon dioxide excretion (Perry *et al.* 1982) and this will be unaffected by changes in water pH. This may also explain the absence of any effect of a rise in water $P_{CO_2}$ on plasma $P_{CO_2}$ in acid water.

The plasma $T_{CO_2}$ and $P_{CO_2}$ values obtained from these experiments were high i█

comparison with other published data. This might be due to the application of a van Dam mask which stressed the fish.

### Ammonia

At an environmental water pH of 9.91, more than half the ammonia will be in a non-ionic state ($NH_3$). [$NH_3$] will be 100 times greater than that of the control inspired water (pH 6.80). This will greatly inhibit the passive diffusion of $NH_3$ out of the fish. In addition, Wright and Wood (1985) concluded that the $Na^+/NH_4^+$ exchange mechanism was inhibited by high environmental pH. The observed accumulation of total ammonia in plasma, therefore, was expected in fish exposed to high water pH. Blood pH increased during the high-pH treatment, which raised the blood $NH_3/T_{Amm}$ ratio. This facilitated the passive diffusion of ammonia and ameliorated ammonia accumulation in fish plasma.

In water of pH 6.65 (control) the ratio $NH_3/NH_4^+$ is 0.02 but when the water pH is reduced to 3.88 the ratio is only 0.00005. The actual [$NH_3$] difference between water of pH 3.88 and pH 6.65 at constant total ammonia content, however, is very small. This small decrease in water [$NH_3$] might be expected to reduce blood [$NH_3$], but blood [$NH_3$] increased despite the reduction in water [$NH_3$] when the fish were exposed to acid conditions. Wright and Wood (1985), however, showed that $Na^+/NH_4^+$ exchange was abolished in fish exposed to water of pH 3.88, which led to a reduction in total ammonia excretion. Ammonia concentrations in blood increased throughout the period of acid exposure.

The overall result of $CO_2$ and $NH_3$ excretion is to ameliorate the magnitude of the change in water pH next to the gills in the face of changes in pH of the environmental water. Inspired water pH varied from 3.88 to 9.91 but expired pH varied only from 4.33 to 7.10. Thus, the high permeability of the gill epithelium to non-ionic but not ionic forms of carbon dioxide and ammonia maintains a relatively stable pH in the micro-environment of the fragile gill epithelium of fish.

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