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# Morphological responses of the rainbow trout (*Oncorhynchus mykiss*) gill to hyperoxia, base ($NaHCO_3$) and acid (HCl) infusions

Greg G. Goss[1], Chris M. Wood[2], Pierre Laurent[3] and Steve F. Perry[1]
[1]*Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, Canada, K1N 6N5*; [2]*Department of Biology, McMaster University, 1280 Main St. West, Hamilton, Ontario, Canada K1N 6N5*; [3]*Laboratoire de Morphologie Fonctionelle et Ultrastructurale des Adaptations, CNRS, 23 Rue de Loess, F-67037 Cedex, Strasbourg, France*

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## Abstract

Marked morphological responses occur in the gills of freshwater rainbow trout in response to experimental acid-base disturbance and these responses play an important role in acid-base correction. Compensated respiratory acidosis induced by 70h exposure to environmental hyperoxia (elevated water $PO_2$) caused a 33% decrease in branchial chloride cell fractional surface area (CCFA). Metabolic alkalosis induced by normoxic recovery (6h) from hyperoxia (72h) caused a 50% increase in CCFA, whereas metabolic alkalosis induced by infusion (19h) of $NaHCO_3$ caused a 70% rise. However, the largest increase (135%) in CCFA was seen in response to infusion (19h) of HCl. NaCl infusion had no effect. A particular goal was to assess the relative importance of changes in CCFA *vs.* changes in internal substrate ($HCO_3^-$) availability in regulating the activity of the branchial $Cl^-/HCO_3^-$ exchange system. For each of the experimental treatments, the accompanying blood acid-base status and branchial transport kinetics ($K_m$, $J_{max}$) for $Cl^-$ uptake had been determined in earlier studies. In the present study, a positive linear relationship was established between CCFA and $J_{max}^{Cl^-}$ in individual control fish in the absence of an acid-base disturbance. By reference to this relationship, observed changes in $J_{max}^{Cl^-}$ during metabolic acid-base disturbances were clearly due to changes in both CCFA and internal substrate levels (plasma $[HCO_3^-]$) with the two factors having approximately equal influence.

## Introduction

Freshwater teleosts manipulate their branchial ion exchange mechanisms in response to acid-base disturbances (Cameron 1976; Claiborne and Heisler 1984, 1986; Perry *et al.* 1987) with adjustment of $Cl^-/HCO_3^-$ exchange being the dominant mechanism under most conditions (Wood *et al.* 1984; Perry *et al.* 1987). During acidosis, $Cl^-/HCO_3^-$ exchange is reduced as a means of retaining base while during metabolic alkalosis, when $[HCO_3^-]$ is elevated, $Cl^-/HCO_3^-$ exhange is stimulated (Heisler 1984; Wood *et al.* 1984; Cameron and Iwama 1987; Perry *et al.* 1987; Goss and Wood 1990a, b). The mechanisms underlying the apparent manipulation of these transporters during acid-base disturbances remain unresolved.

In rainbow trout (*Oncorhynchus mykiss*), Goss and Wood (1991) developed a two-substrate model to describe branchial transport kinetics which sug-

*Correspondence to*: Greg G. Goss, Hospital for Sick Children, Division of Cell Biology, 555 University Avenue, Toronto, Ontario, M5G 1X8; Tel. (416) 813-5727; Fax: (416) 813-5028

gested that the availability of the internal substrate ($HCO_3^-$) played a large role in setting the rate of $Cl^-/HCO_3^-$ exchange during metabolic acid-base disturbances (*i.e.*, disturbances in which only plasma $[HCO_3^-]_a$, and not $PCO_2$, changes). On the other hand, in brown bullhead (*Ictalurus nebulosus*), Goss *et al.* (1992a) demonstrated that during respiratory acidosis induced by hypercapnia (high environmental $PCO_2$), $Cl^-$ uptake ($J_{in}^{Cl^-}$) was reduced *despite a progressive rise in plasma* $[HCO_3^-]_a$; this was associated with a reduction of the surface area of exposed branchial chloride cells (CC). During the subsequent pure metabolic alkalosis (*i.e.*, high plasma $[HCO_3^-]_a$ only) that accompanied removal of hypercapnia (Cameron 1976; Claiborne and Heisler 1984, 1986; Cameron and Iwama 1987; Perry *et al.* 1987), $J_{in}^{Cl^-}$ and CC surface area increased concomitantly. On the basis of these results, it was proposed (see review by Goss *et al.* 1992b) that i) the CC is the site of branchial $Cl^-/HCO_3^-$ exhange, ii) the rate of $Cl^-/HCO_3^-$ exchange during acid-base disorders is controlled, in part, by the surface area of CCs exposed to the water, and iii) the rate of $Cl^-/HCO_3^-$ exhange is also controlled, in part, by the availability of the internal substrate (*i.e.*, $[HCO_3^-]_a$).

Environmental hyperoxia [elevated water $PO_2(P_wO_2)$] results in an endogenously induced respiratory acidosis (Wood and Jackson 1980; Wood *et al.* 1984; Goss and Wood 1990a; Wood 1991; Wood and LeMoigne 1991) owing to hypoventilation and hypo-perfusion of the gill. Indeed, environmental hyperoxia induces internal acid-base disturbances similar to those observed for hypercapnia without the associated confounding effects of increasing water $[H^+]$ (decreasing pH) and elevated circulating catecholamine levels (Perry *et al.* 1987, 1989) which can independently alter net acid transfer (McDonald and Wood 1981; McDonald 1983; McDonald *et al.* 1983; Boutilier *et al.* 1986; Tang *et al.* 1988; McDonald and Milligan 1988; McDonald *et al.* 1989a, b).

Infusion of "fixed" acids (*e.g.*, HCl: Boutilier *et al.* 1986; Goss and Wood 1991; $NH_4SO_4$; McDonald and Prior 1988; McDonald *et al.* 1991; Milligan *et al.* 1991) and bases (*e.g.*, $NaHCO_3$; Claiborne and Heisler 1986; McDonald and Prior 1988; Goss and Wood 1990b) induce purely metabolic alterations in the acid-base status of the fish (*i.e.*, $PCO_2$ is virtually constant) and are useful in avoiding the possible effects of changes in the perfusive and convective properties of the gill surface that may occur as a result of hypercapnia or hyperoxia (Dejours 1973; Wood and Jackson 1980).

The primary goal of the present study was to examine the relationship(s) between branchial $Cl^-$ fluxes and gill epithelial morphology during and after prolonged (70h) hyperoxia, and after 19h of continuous infusion of NaCl (sham control), base (as $NaHCO_3$), or acid (as HCl). The experiments were designed to determine whether morphological regulation of blood acid-base status as suggested by Goss *et al.* (1992a, b) is a generalized and co-ordinated response to acid-base disturbances, and not simply a peculiarity of hypercapnic acidosis. For each of the experimental treatments, we had earlier determined the accompanying blood acid-base status and the branchial transport kinetics for $Cl^-$ uptake, *i.e.*, the relationship between $J_{in}^{Cl^-}$ and the concentration of *external* substrate (water $[Cl^-]$) (Hobe *et al.* 1984; Goss and Wood 1990a, b, 1991). When calculated by classical one-substrate Michaelis-Menten analysis (which considers only variation in external substrate), the $J_{max}^{Cl^-}$ is traditionally thought to be an index of the capacity of the system when all transporters are recruited. One might therefore expect a direct relationship between the CC fractional area (CCFA) and $J_{max}^{Cl^-}$. However, if the *internal substrate* availability ($HCO_3^-$) is also important, then one might expect that significant deviations from such a relationship would occur *in a direction dictated by internal substrate availability*. To test these ideas, we constructed a relationship between CCFA and $J_{max}^{Cl^-}$ in control fish not subjected to acid-base disturbances and examined whether data from the experimental treatments conformed to this relationship. Therefore, a second goal of the present study was to examine the relative contributions of morphological modification of the gill epithelium and alterations of internal substrate availability to acid-base balance in fish experiencing diverse acid-base disturbances.

## Materials and methods

### *Experimental animals*

Rainbow trout (*Oncorhynchus mykiss*, 217–380 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario, Canada and acclimated for at least two weeks to flowing dechlorinated Hamilton tapwater ($Na^+$ = 0.6 mmol/l, $Cl^-$ = 0.8 mmol/l, $Ca^{2+}$ = 0.9 mmol/l, pH = 7.8). In series I (hyperoxia) the fish were placed directly in separate, darkened, well-aerated plexiglass boxes that were continuously supplied (0.5 l/min) with tapwater and allowed to recover for 72h prior to experimentation. Vigorous aeration thoroughly mixed the water. In series II (infusion series), the trout were first anaesthetized (MS222 1:10,000; Sigma), fitted with indwelling dorsal aortic cannula (Soivio *et al.* 1975) and placed in the boxes where they were allowed to recover for 72h prior to experimentation. Temperature was maintained 14 ± 1°C.

### *Exposure regime*

Series 1 – Exposure to hyperoxia: Series 1 was designed to examine the effects of exposure to hyperoxia on gill epithelial morphology and to relate these changes to alterations in branchial ion fluxes that are known to occur during such a treatment. After 72h of recovery from surgery, the fish box and the water flowing to the boxes was equilibrated with $O_2$, this elevated the water $PO_2$ within 0.33h from 140 torr to > 500 torr. After 72h of exposure to continuous hyperoxia, the $O_2$ was replaced with air, quickly returning $PO_2$ to normoxic levels. Fish were killed by a cephalic blow after exposure to control conditions (n = 11), 70h of continuous hyperoxia (n = 11), or 6h of recovery from hyperoxia (n = 11). The second right gill arch was removed and prepared for electron microscopic analysis as described below.

Series 2 – Acid/base infusions: Series 2 was designed to examine the effects of infusion (19h) of either A) 140 mmol/l NaCl (sham control, n = 10; 2.81 ± 0.10 ml/kg/h); B) 140 mmol/l $NaHCO_3$ (n = 7; 2.92 ± 0.11 ml/kg/h); or C) 70 mmol/l HCl / 70 mmol/l NaCl (n = 7; 2.99 ± 0.13 ml/kg/h) on the gill morphology. The goal was to induce an alkalosis or an acidosis of similar magnitude to those reported during exposure to, and recovery from, hyperoxia (Höbe *et al.* 1984; Goss and Wood 1990a; Wood and LeMoigne 1991), while minimizing the volume load. These rates of infusion and solution concentrations were selected on the basis of initial trial experiments (not shown). The concentration of $NaHCO_3$ (140 mmol/l) was chosen so as to minimize any changes in the osmolarity of the plasma. The concentration of HCl (70 mmol/l HCl / 70 mmol/l NaCl) was used instead of 140 mmol/l HCl as the fish did not tolerate infusion of the latter. The fish were infused *via* the dorsal aortic cannulae with a Gilson Minipuls peristaltic pump for a period of 19h.

Series 3 – Relationship between CCFA and $J^{Cl^-}_{max}$ in the absence of an acid-base disturbance: In order to describe a possible relationship between the maximal transport rates for $Cl^-$ ($J^{Cl^-}_{max}$) and the fractional area of CCs (CCFA) on the filamental epithelia under control conditions (non-infused) where no acid-base disturbance is present, the radioisotopic influx of $^{36}Cl^-$($J^{Cl^-}_{in}$) was examined over increasing external NaCl concentrations ($[NaCl]_e$) as described in Goss and Wood (1990a, b). The idea was to use the natural variation in CCFA to establish such a relationship. Six flux periods of increasing $[NaCl]_e$ (nominally 50, 150, 300, 600, 1200, 2400 µmol/l) were measured. Each lasted 0.5h except for the final flux period lasting 0.67h to increase accuracy. Samples (40 ml) were analyzed for $[Cl^-]_e$, and total radioactivity (cpms). The effect of increasing the $[NaCl]_e$ on the influx of $Cl^-$ showed distinctive saturation kinetic curves that obeyed Michaelis-Menten kinetics. The curves (not shown) were used to yield estimates of the uptake kinetic parameters ($K_m$, $J_{max}$) during the experimental regime. $K_m$ and $J_{max}$ were determined for each fish *via* transformation of the data by Eadie-Hofstee regression analysis (Michal 1985). Eadie-Hofstee analysis was used because it magnifies departures from linearity which might not be apparent from a

Lineweaver-Burk plot. At the end of the flux period, a portion of the second right gill arch was then excised and prepared for electron microscope analysis as described below.

*Analytical techniques and calculations*

Water $[Cl^-]_e$ was measured by the mercuric thiocyanate method (Zall *et al.* 1956). Duplicate 5 ml $^{36}Cl^-$ water samples were measured directly by scintillation counting (LKB Rackbeta). Unidirectional influxes ($J_{in}$) were calculated as described by Wood (1988). Correction for radioisotope backflux (Maetz 1956) was not necessary because internal specific activity never exceeded 5% of the external specific activity. At the end of the kinetic study in control (non-infused) fish, after 70h hyperoxia or 6h after return to normocapnia, and after 19h of infusion with either solution, A, B or C, the fish were killed by a blow to the head and the second right gill arch was removed and prepared for electron microscope analysis as described below.

*Morphological methods*

Examination of gill CC morphology: A portion of the second gill arch was excised from the fish and the arch tissue was cut off using a razor blade leaving the anterior and posterior filaments attached by the septum of the arch. The tissue was then cut into individual filaments (for transmission electron microscopy: TEM) or pairs of filaments still attached at the septum (for scanning electron microscopy: SEM) and fixed in 5% glutaraldehyde buffered with 0.15 mol/l sodium cacodylate (pH = 7.4; osmotic pressure of fixative = 292 mOsm) for 1h at 4°C. For TEM, the individual filaments were then postfixed in 1% osmium tetroxide in water and embedded in Araldite to allow cross-sectioning of the lamellae or the filament. Ultrathin sections were prepared with an automatic ultramicrotome (Ultracut, Reichert) and poststained with uranyl acetate followed by lead citrate for examination in the TEM (Elmiskop 101, Siemens). For SEM, pairs of filaments still attached at the septum were dehydrated completely in a series of ethanol baths and subsequently placed in two successive baths (2 min) of 1,1,1,3,3,3 hexamethyldisilazan (Aldrich) and then air-dried. The pairs of filaments were then glued to a specimen stub suitable for a Stereoscan 100 scanning electron microscope (Cambridge Ltd) in a manner that maintained the lateral side of the filaments parallel with the stub plate. The portion of the filament epithelium on the trailing edge of the filaments near the junction of the two filaments was then focused on the screen and photographed at a 1000 × magnification. At least 4 non-contiguous fields were randomly photographed from each fish for morphometric analysis. Mean CC surface area (defined as the two-dimensional opening of the apical crypt of one cell ($\mu m^2$) and surface CC density (amount of cells/$mm^2$) were determined by tracing the CC perimeters using a digitizing tablet (Numonics 2210) and a morphometric software program (Sigmascan, Jandel). In addition, the fractional surface area of CCs per unit epithelium ($\mu m^2/mm^2$) was calculated from these measured parameters for each photo. The mean values for each fish were then calculated and this value was then used in the determination of the mean for that period. The trailing edge of the filament epithelium was chosen for the morphometrical analysis for practical reasons because this epithelium is generally flat and can be mounted parallel to the stub allowing more precision in the measurement of CC morphometry.

*Statistical analysis*

All values are presented as means ± 1 SEM. Comparisons between means were performed by a two-way analysis of variance (ANOVA), followed by Student Neuman-Keuls tests for multiple comparison. Linear regression relationships were generated by the method of least squares. 5% was accepted as the fiducial limit of significance.

## Results

*Morphological responses to hyperoxia (Series 1)*

The effects of hyperoxia on gill CC morphology are shown in Fig. 1. After 70h, CCFA was decreased by

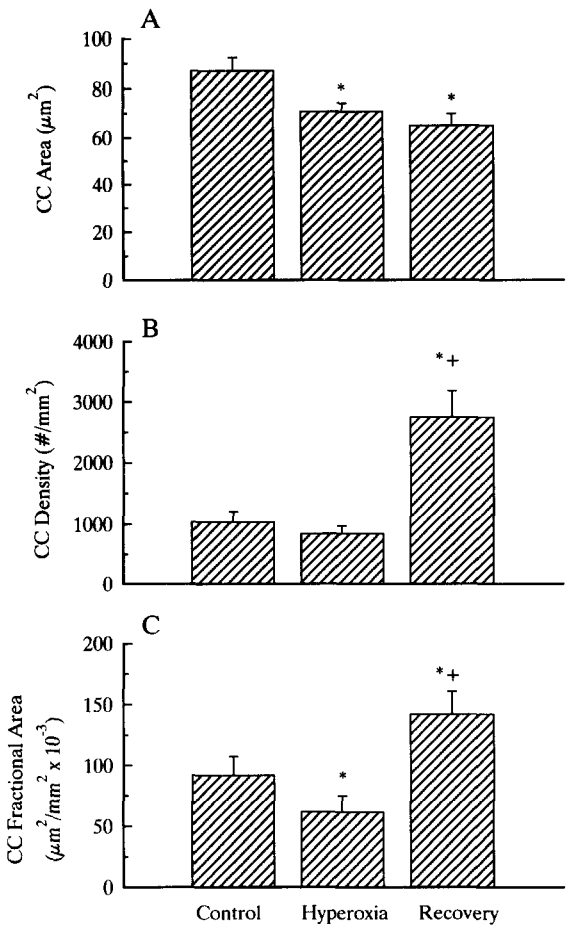


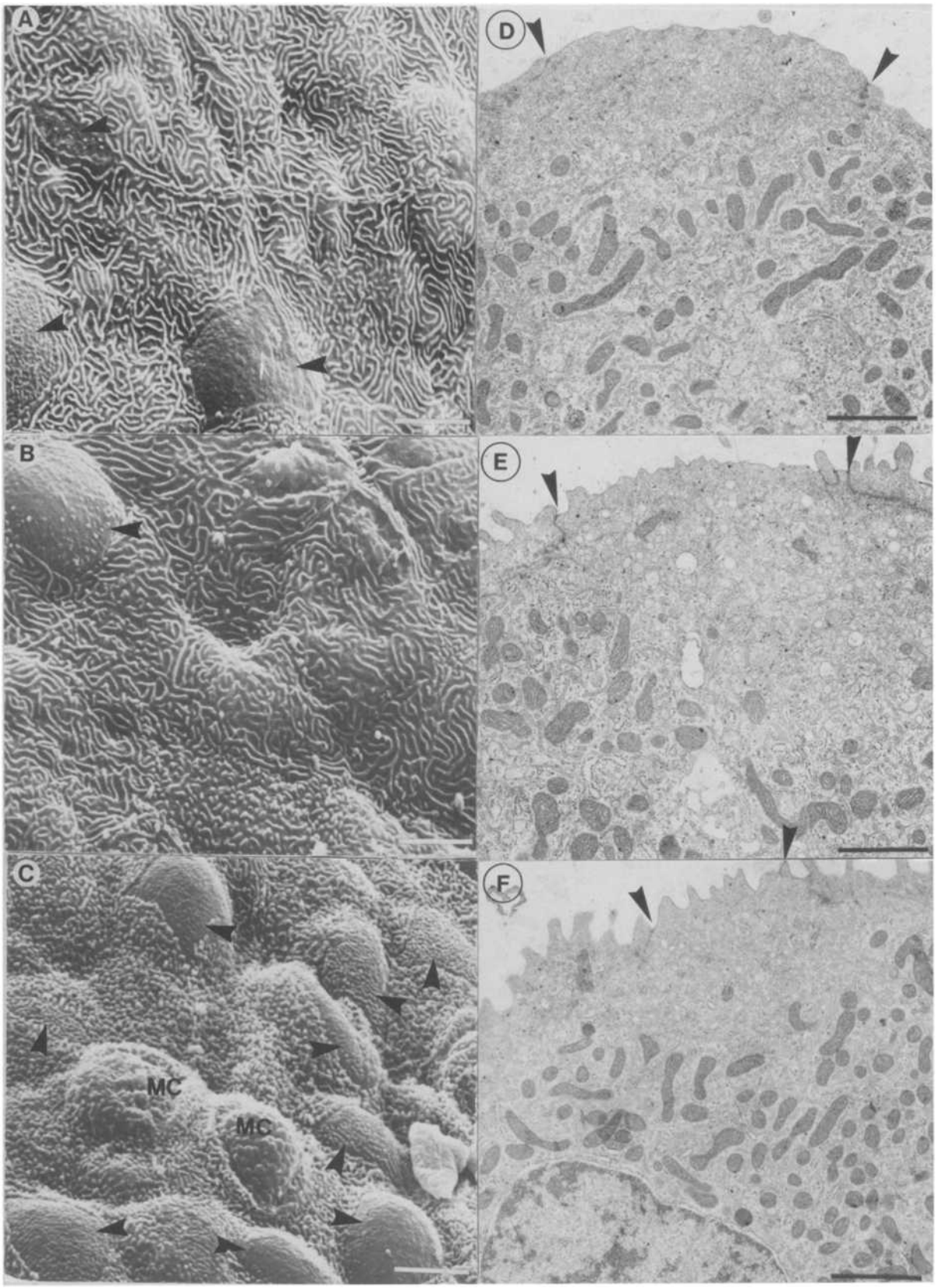

*Fig. 1.* The effects of exposure to 70h of environmental hyperoxia ($PO_2$ > 500 torr in water) and the subsequent 6h post-hypercapnic recovery period on gill filament chloride cell (CC) morphometry including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area. * indicates significant difference from control (Pre) value. + indicates significant difference from 70h value. Means ± SE.

33% (from 91771 ± 15415 to 61415 ± 13113 μm²/mm²; Fig. 1C). The decrease in CCFA was caused largely by a decrease in the average area (Fig. 1A) of exposed CCs on the filamental epithelia; CC density was not significantly altered (Fig. 1B). In the post-hyperoxic period, there was a large increase in the density of filamental CCs displayed on the filamental epithelium from 841 ± 133/mm² at the end of hyperoxia to 2746 ± 432/mm² only 6h after the removal of the hyperoxia stimulus (Fig. 1B). This resulted in a large increase in the CCFA to 142146 ± 18551 μm²/mm² at this time (Fig. 1C); the increase was significantly greater than both the control and the 70h hyperoxia periods. Representative SEM and TEM photographs showing the effects of exposure to environmental hyperoxia are shown in Fig. 2. The CCs in control fish displayed few apical membrane microvilli and were frequently raised above adjacent pavement cells (PVCs). The ultrastructural characteristics of the CC include numerous mitochondria and a well developed apical tubular network (Fig. 2D). The changes which occurred during hyperoxia included a decrease in CCFA owing to a small decrease in average cell area (Fig. 2B) and a general absence of microvilli (Fig. 2E). The return of normoxia was marked by a large increase in the density of filamental CCs (Fig. 2C) in accordance with the morphometrical analysis (Fig. 1B), prominent microvilli and numerous mitochondria, and a tendency for the CCs to bulge above the surface of adjacent pavement cells (Fig. 2F).

### *Morphological responses to base ($NaHCO_3$) or acid (HCl) infusion (Series 2)*

Infusion of $NaHCO_3$ caused a 45% increase in the density of filamental CCs from 1613 ± 262/mm² in NaCl-infused fish to 2364 ± 222/mm² in $NaHCO_3$-infused fish (Fig. 3B). In addition, there was a trend (not significant) toward an increase in the average CC area (Fig. 3A). The net result was a significant 70% increase in the CCFA from 83959 ± 14693 μm²/mm² in NaCl-infused fish to 143098 ± 20633 μm²/mm² in $NaHCO_3$-infused fish (Fig. 3C; *cf.* recovery from hyperoxia: Fig. 1). Infusion of acid (HCl) caused a significant increase in both the area (90.6 ± 7.2 μm²; Fig. 3A) and density (2304 ± 108/mm²; Fig. 3B) of filamental CCs and together these two alterations resulted in a large 135% increase in the CCFA in the HCl-infused fish. Of all groups examined, CCFA was greatest in the HCl-infused fish (197719 ± 18731 μm²/mm²).

Representative SEM (Fig. 4A–C) and TEM (Fig. 4D–F) photographs showing the effects of NaCl, base ($NaHCO_3$), or acid (HCl) infusion are presented in Fig. 4. In NaCl-infused fish, gill morphology was similar to that in the non-infused con-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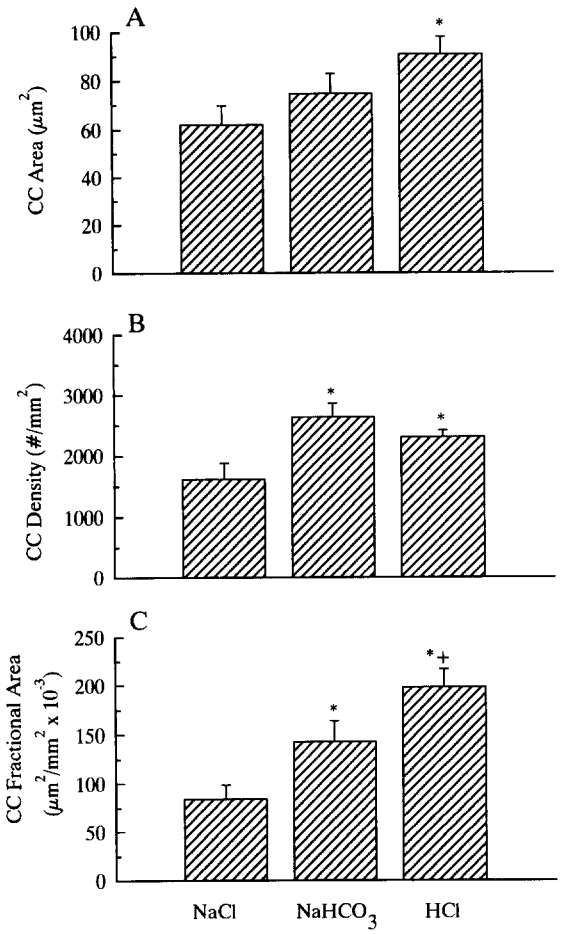


*Fig. 3.* The effects of infusion of 140 mmol NaCl, 140 mmol $NaHCO_3$, or 70 mmol HCl/70 mmol NaCl on gill filament chloride cell (CC) morphometry including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area. * indicates significant difference from control (Pre) value. Means ± SEM.

trols of the hyperoxia study (compare Fig. 4A, D with Fig. 2A, D). In the base-infused group, the CCs had a very characteristic smooth apical surface presenting few apical microvilli projections while in the acid-infused group, the CCs appeared to have increased numbers of apical microvilli (Fig. 4C). Occasionally in the acid-infused group, the CCs protruded from the filamental epithelium (Fig. 4F). In addition, these CCs appeared to be active as indicated by the large numbers of mitochondria and a very densely organized tubular network in the apical portion of the cell (Fig. 4F).

### *Relationship between $J^{Cl^-}_{max}$ and CCFA (Series 3)*

Table 1 summarizes the arterial blood acid-base status and the kinetic parameters, $K_m$ and $J_{max}$ accompanying each of the experimental treatments. $K_m$ and $J_{max}$ were calculated by classic one-substrate Michaelis-Menten analysis. These data are taken from previous publications (Hobe *et al.* 1984; Goss and Wood 1990a, b, 1991). In those studies, no attempt was made to assess the role of variations in gill CC morphology on the changes in $J^{Cl^-}_{max}$. The mechanisms underlying the changes in $J^{Cl^-}_{max}$ were attributed to variation in internal substrate ($HCO_3^-$). The present study attempts to resolve the relative contribution of each of these mechanisms to overall acid-base regulation by comparing gill morphology during acid-base disturbances with measured $J^{Cl^-}_{max}$ during that treatment.

By 70h, the respiratory acidosis due to greatly elevated $P_aCO_2$ induced by environmental hyperoxia was almost entirely compensated by a large increase in plasma $[HCO_3^-]_a$ (Table 1). Post-hyperoxic recovery (6h), resulted in a persistence of high plasma $[HCO_3^-]$ but a return of $P_aCO_2$ close to control values (still slightly elevated). The accompanying elevation in $pH_a$ was, therefore, an almost pure metabolic alkalosis. NaCl infusion had no effect on blood acid-base status, but $NaHCO_3$ infusion caused an almost pure metabolic alkalosis similar to but larger than that seen during post-hyperoxic recovery. Again, $P_aCO_2$ was slightly elevated. HCl infusion caused a pure metabolic acidosis; plasma $[HCO_3^-]_a$ and $pH_a$ were significantly depressed without change in $P_aCO_2$.

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*Fig. 2.* Representative low magnification scanning electron microscopy (SEM; A–C) and high magnification transmission electron microscope (TEM; D–F) photographs of the filament epithelium under control normoxic conditions (Fig. 2A, D), after 70h (Fig. 2B, E) and the subsequent post-hyperoxic recovery period (Fig. 2C, F). Note in particular the increase in CC fractional area during the post-hyperoxic recovery period. MC = mucous cell. Fig. 2A–C, arrowheads indicate CCs, Fig. 2D–F, arrowheads indicate cell-cell junctions. Fig. 2A–C scale bar = 10 μ. Fig. 2D–F scale bar = 1 μ.

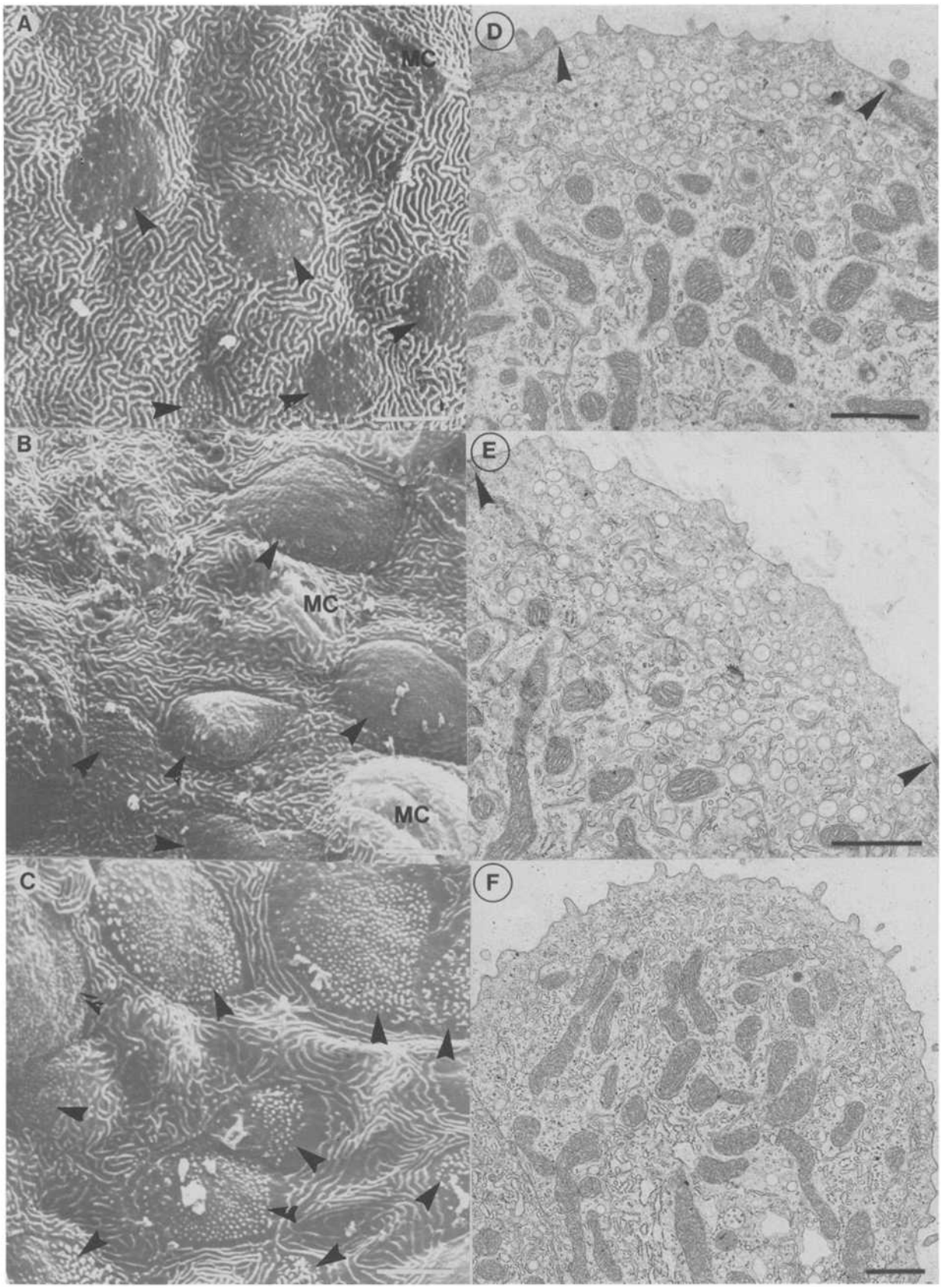
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*Table 1.* The effects of various acid-base disturbances on the kinetic parameters ($K_m$ and $J_{max}$) for the $Cl^-$ uptake mechanism ($Cl^-/HCO_3^-$ exchange) on the gills of rainbow trout acclimated to Hamilton tapwater (taken from Goss and Wood 1990a, b; 1991). * = significantly different from appropriate control.

| Treatment | $pH_a$ | $P_aCO_2$(torr) | $[HCO_3^-]_a$ (mmol/l) | $K_m^{Cl^-}$ (μmol/l) | $J_{max}^{Cl^-}$ (μmol/kg/h) | Reference |
|---|---|---|---|---|---|---|
| Control | 7.907 ± 0.018 | 2.17 ± 0.10 | 7.10 ± 0.42 | 127 ± 20 | 286 ± 27 | Goss and Wood (1990a) |
| Hyperoxia (70h) | 7.820 ± 0.021* | 8.65 ± 0.61* | 22.10 ± 2.15* | 248 ± 42* | 272 ± 49 | Goss and Wood (1990a); Hobe *et al.* (1984) |
| Post-hyperoxia (6h) | 8.084 ± 0.038* | 2.84 ± 0.13* | 14.21 ± 1.57* | 137 ± 13 | 445 ± 54* | Hobe *et al.* (1984) |
| NaCl infusion | 7.884 ± 0.033 | 2.35 ± 0.04 | 8.20 ± 1.47* | 121 ± 13 | 303 ± 68 | Goss and Wood (1990b) |
| $NaHCO_3$ infusion | 8.161 ± 0.025* | 2.75 ± 0.36* | 18.21 ± 2.26* | 135 ± 12 | 674 ± 89* | Goss and Wood (1990b) |
| HCl infusion | 7.740 ± 0.029* | 1.94 ± 0.15 | 4.54 ± 0.35* | 178 ± 53 | 336 ± 39 | Goss and Wood (1991) |

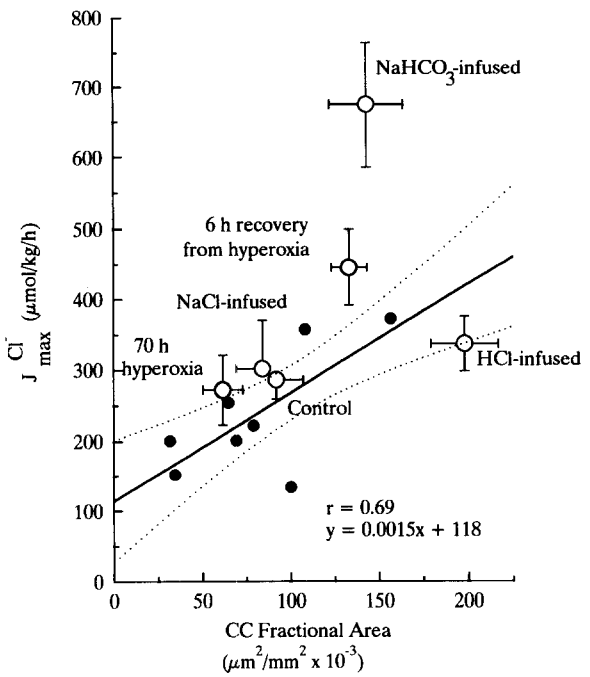


*Fig. 5.* Relationship between maximal transport rate for chloride ($J_{max}^{Cl^-}$) with gill filament CC fractional area under control conditions (internal counterion i.e. $[HCO_3^-]$, is unchanged (filled circles)). Solid line = linear regression (y = 0.0015x + 118, r = 0.690) + 95% confidence intervals. In addition, the measured $J_{max}^{Cl^-}$ values from Table 1 are plotted at the CC fractional area for each treatment group listed above (mean ± SEM).

Acidosis, arising from either respiratory (hyperoxia) or metabolic (HCl infusion) perturbations, was associated with increases in $K_m$ (decreases in affinity) of the proposed $Cl^-$ transporter; $J_{max}^{Cl^-}$ was not significantly altered by acidosis (Table 1). Metabolic alkalosis, arising from either post-hyperoxia or $NaHCO_3$ infusion, on the other hand, was associated with significant increases in $J_{max}^{Cl^-}$ while $K_m^{Cl^-}$ was not significantly altered.

Figure 5 illustrates that there was a significant positive relationship between CCFA and $J_{max}^{Cl^-}$ as determined on 8 individual fish under control conditions. The regression line (y = 0.0015x + 118; r = 0.69) represents the coupling between the two variables *in the absence of an acid-base disturbance* (*i.e.*, when $[HCO_3^-]_a$ is unchanged) and can be used to predict the effect of changes in CCFA alone on $J_{max}^{Cl^-}$ (the relation between CCFA and $J_{max}^{Cl^-}$). Superimposed on this graph are the measured values of $J_{max}^{Cl^-}$ for each treatment group from Table 1 and the measured values of CCFA from the present study. Those values that follow this relationship suggest that alterations in $J_{max}^{Cl^-}$ are the result of alterations in CCFA. If a value is above or below this relationship, then the difference represents the variation in $J_{max}$ which can be attributed to al-

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*Fig. 4.* Representative low magnification scanning electron microscopy (SEM; A–C) and high magnification transmission electron microscope (TEM; D–F) photographs of the filament epithelium during NaCl (sham) infusion (Fig. 4A, D), $NaHCO_3$ infusion (Fig. 4B, E) and HCl infusion (Fig. 4C, F). Note the increase in the CC fractional area during infusion of both $NaHCO_3$ and HCl. Figure 4A–C, arrowheads indicate CCs, MC = mucous cell. Figure 4D–F, arrowheads indicate cell-cell junctions. Figure 4A–C scale bar = 10 μ. Figure 2D–F scale bar = 1 μm.

teration in internal counterion concentration ($[HCO_3^-]_a$).

As anticipated, the mean values for the control treatment (different fish from those used to establish the regression line) are nearest to the line. The values for the NaCl infusion and 70h hyperoxia treatment did not significantly deviate from the line. Metabolic alkalosis as a result of removal of the hyperoxic stimulus resulted in an elevated $J^{Cl^-}_{max}$ compared to NaCl and hyperoxia compensated fish while $NaHCO_3$ infusion caused an even greater deviation. HCl infusion, on the other hand, did not result in a value significantly different from the control relationship.

## Discussion

### *Morphological modification of the gill CCFA in response to acid-base disturbances*

The present study confirms and extends the results of Goss *et al.* (1992a, b) showing that regulation of blood acid-base status is associated with morphological modification of branchial CCFA. CCFA was reduced significantly during hyperoxia-induced respiratory acidosis, a condition similar to the hypercapnic acidosis utilized in previous studies. The decrease in CCFA during hyperoxia were due to alterations in the average CC area with no significant alterations in the density of CCs. This is similar to the morphological response elicited by hypercapnic acidosis in brown bullhead catfish (Goss *et al.* 1992a). Metabolic alkalosis, caused either by return of hyperoxic fish to normoxic conditions, or by $NaHCO_3$ infusion was associated with an increase in CCFA similar to that observed in the post-hypercapnic catfish (Goss *et al.* 1992a). In the present study, however, the increases in CCFA were the result of significant increases in the density of exposed cells alone, rather than to a combined increase in average area and density of CCs. Owing to the short time period required for the increase of CCFA in the post-hyperoxic period, the increase in density was likely accomplished by uncovering of CCs already present within the filamental epithelium, rather than production of new CCs. This also appears to be the mechanism responsible for the increase in CC density during metabolic alkalosis resulting from post-hypercapnic alkalosis in brown bullhead catfish (Goss *et al.* 1992a). This is further supported by the study by Bartels and Potter (1990) in which rapid covering/uncovering of the CC by adjacent pavement cells was suggested as a mechanism for regulating ionic uptake during freshwater-seawater transfer in lamprey (*Geotria australis*).

Alternatively, the changes in CC area may be caused by exocytosis/endocytosis of the apical membrane as has been shown to occur in α-type mitochondrial rich (MR) cells of the turtle bladder (Gluck *et al.* 1982; Stetson and Steinmetz 1985; Kniaz and Arruda 1990, 1991). However, the β-type of MR cell, the cell type responsible for $HCO_3^-$ secretion (*via* $Cl^-/HCO_3^-$ exhange) in the turtle bladder and mammalian kidney cortical collecting duct (possibly analogous to the CC in freshwater fish) does not show apical endocytosis when viewed in the electron microscope (Schwartz *et al.* 1985, 1988). On the other hand, Satlin and Schwartz (1989), have presented evidence to indicate that cellular remodelling does occur in β-type MR cells of turtle bladder in response to an acidosis. Rich *et al.* (1990), have demonstrated increases in membrane conductance of β-MR cells (an indicator or surface area) associated with increases in $HCO_3^-$ secretion. Therefore, the exact mechanisms responsible for the changes in trout CC area during acid-base disturbances remain unresolved.

Metabolic acidosis induced by HCl infusion resulted in an unexpected rise in the CCFA in rainbow trout. This increase was even higher than that seen in $NaHCO_3$-infused fish. The physiological significance of this response is unclear given the role of the CC in base ($HCO_3^-$) excretion via $Cl^-/HCO_3^-$ exchange. Clearly, an increase in the CCFA would act to increase $Cl^-/HCO_3^-$ exchange capacity which would be detrimental to the regulation of the metabolic acidosis. This observation helps explain the observations of Goss and Wood (1991) that HCl infusion was not associated with reduced $Cl^-$ uptake but instead, the acidosis was compensated by large increases in $Na^+$ uptake and hence "acid" excretion. A possible explanation for

the increase in CCFA is that acid infusion increases plasma cortisol levels owing to the stressful nature of the protocol and the well-documented effect of increased stress on cortisol release (Pickering *et al.* 1991). Increases in cortisol titre are known to cause increases in CCFA (Perry and Wood 1985; Laurent and Perry 1990; Madsen 1990). An alternative explanation is that the CCs on the trout gill epithelium are not a homogeneous population of β-type CCs but instead are a combined population of both β- and α-type MR cells. The increase in CCFA noted during acid infusion may therefore represent a selective increase in α-type CCs. The presence of varying surface morphologies under control conditions (Franklin and Davison 1989; Perry and Laurent 1989; Perry *et al.* 1992) may suggest a mixed population of cells in rainbow trout similar to that found for turtle bladder (Lehir *et al.* 1982; Stetson and Steinmetz 1985; Schwartz *et al.* 1985, 1988; Satlin and Schwartz 1989; Fritsche *et al.* 1991). To date, no studies have separated the gill CC population into different sub-groups based on physiological function although Pisam *et al.* (1987) suggested a sub-division based on staining characteristics and ultrastructure. An alternative explanation for the varying morphologies in freshwater fish may be that the differences represent variations in the life cycle of the cell (Wendelaar-Bonga and van der Meij 1989).

*Effect of variations in CCFA and internal substrate availability on* $J_{max}^{Cl^-}$

Although there was a significant positive relationship between $J_{max}^{Cl^-}$ and CCFA under control conditions, alteration of the acid-base status of the fish produced deviations from this relationship (Fig. 5). These deviations, most apparent during metabolic alkalosis resulting from 6h post-hyperoxic recovery and $NaHCO_3$-infusion, were likely caused by alterations in internal substrate (*i.e.*, $HCO_3^-$) availability because plasma $[HCO_3^-]_a$ was significantly elevated in both treatments (Table 1). The larger deviation occurred in the $NaHCO_3$ treatment where the elevation in plasma $[HCO_3^-]_a$ was greatest. According to the two-substrate model (Wood and Goss 1990; Goss and Wood 1991), the internal counterion ($HCO_3^-$) for the $Cl^-/HCO_3^-$ exchanger limits the rate of $Cl^-$ transport. This occurs because $K_m^{HCO_3^-}$ is greater than physiological levels ($K_m^{HCO_3^-}$ = 33.3 mmol/l; Goss and Wood 1991). Therefore, elevations in internal $[HCO_3^-]$ result in increases in $J_{max}^{Cl^-}$ independent of, or additive to, alterations in CC morphology. In comparison to NaCl-infusion and control treatments, when no acid-base disturbance is present, $J_{max}^{Cl^-}$ was elevated both by an increase in CCFA (right shift along the determined relationship) and an increase in the substrate availability (upward shift from the determined relationship). These two mechanisms act in concert to clear to excess $[HCO_3^-]$ from the extracellular space.

HCl infusion resulted in an increase in CCFA in the present study, a response not appropriate for the compensation of an extracellular acidosis. However, at the same time, the small but significant decrease in plasma $[HCO_3^-]_a$ caused a downward deviation from the determined relationship, in accordance with the two-substrate model. The net effect was that $J_{max}^{Cl^-}$ did not change significantly (Table 1). Overall, these results suggest that variation in CCFA and variation in internal substrate availability are of approximately equal importance in setting the activity of the $Cl^-/HCO_3^-$ exchange system during acid-base disturbances which are primarily metabolic in nature.

The one significant exception to the conclusion was the 70h hyperoxia treatment. The mean point was not significantly different from the determined relationship despite the presence of the highest plasma $[HCO_3^-]_a$ in this group (Table 1). However, this treatment represented a state of almost completely compensated respiratory acidosis in which blood pH had returned to almost normal by the accumulation of plasma $[HCO_3^-]_a$. A high rate of $Cl^-/HCO_3^-$ exchange would clearly have been an inappropriate response in such a situation. This result suggests that extracellular pH may have an overriding effect on the system.

Finally, the results of this study on the rainbow trout, together with those of Goss *et al.* (1992a, b) on hypercapnic fish suggest that morphological modification of the gill epithelium is a generalized

response to acid-base disturbances and not just peculiar to hypercapnia or the brown bullhead. Specifically, manipulation of the $Cl^-/HCO_3^-$ exchange is accomplished by variable adjustments of the surface area of CCs exposed to the external water allowing for rapid regulation of the availability, and hence the functioning, of the branchial $Cl^-/HCO_3^-$ exchangers.

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