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INTERRELATIONSHIPS BETWEEN GILL MORPHOLOGY AND
ACID-BASE REGULATION IN FRESHWATER FISH

By

Gregory Gerard Goss, B.Sc., M.Sc.

A thesis

Submitted to the School of Graduate Studies
in Partial Fulfilment for the Requirements for the Degree

Doctor of Philosophy

University of Ottawa/ Université d'Ottawa

Ottawa-Carleton Institute of Biology



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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

**INTERRELATIONSHIPS BETWEEN GILL MORPHOLOGY AND
ACID-BASE REGULATION IN FRESHWATER FISH**

Doctor of Philosophy (1993)
(Biology)

University of Ottawa
Université d'Ottawa

Title: Interrelationships Between Gill Morphology and Acid-Base Regulation in
Freshwater Fish

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Abstract

This thesis examines the branchial mechanisms utilized by freshwater fish to regulate internal acid-base status and presents a model to explain the underlying basis of the compensatory processes. Rainbow trout, *Oncorhynchus mykiss*, brown bullhead, *Ictalurus nebulosus*, and American eels, *Anguilla rostrata*, were examined under a variety of experimental treatments which induced both respiratory (hyperoxia, hypercapnia) and metabolic (post-hyperoxia, post-hypercapnia, HCl infusion, NaHCO₃ infusion) acid-base disturbances. Acid-base regulation was achieved by appropriate adjustments of Na⁺ and Cl⁻ net fluxes across the gills which, in turn, were accomplished by variable contributions of three different branchial mechanisms; i) morphological adjustments to the gill epithelium, ii) changes in internal (H⁺, HCO₃⁻) and external (Na⁺, HCO₃⁻) substrate availability, and iii) *differential* changes in Na⁺ versus Cl⁻ net fluxes through regulation of Cl⁻ efflux. This thesis determined the variable contribution of each of these mechanisms to overall compensation of acid-base disturbances.

In brown bullhead and trout, respiratory acidosis caused a reduction in chloride cell (CC) surface area whereas alkalosis was associated with increases in CC surface area. In addition, there were ultrastructural changes in the CCs during hypercapnia suggestive of reduced functioning at that time. These changes included an apparent reduction in the numbers of mitochondria and decreases in the density of the vesiculo-tubular network. These findings demonstrated that these two species vary the availability, and hence the activity, of the Cl⁻/HCO₃⁻ exchange mechanism by physical covering/uncovering of CCs by adjacent pavement cells (PVC).

Increases in the density of microvilli displayed on the external surface of the PVC coupled with ultrastructural modifications during hypercapnic acidosis were associated with

increases in Na^+ uptake ($J_{\text{in}}^{\text{Na}^+}$). These data, in addition to the lack of correlation between $J_{\text{in}}^{\text{Na}^+}$ and CC surface area during hypercapnia, suggest that the Na^+ uptake mechanism (Na^+/H^+ exchange - Na^+ channel/ H^+ ATPase) is PVC associated. Inter-specific and intra-specific differences in gill epithelial CC surface area may account for inter-specific and intra-specific differences in the ability of freshwater teleost fish to compensate acid-base disturbances. Increased rates of Cl^- uptake ($J_{\text{in}}^{\text{Cl}^-}$) (via increases in CC surface area) were associated with an increased capacity to regulate an alkalosis. A greater $J_{\text{in}}^{\text{Cl}^-}$ (induced by cortisol/ovine growth hormone treatment) increased the ability of trout to regulate a NaHCO_3 infusion-induced alkalosis while reduction in $J_{\text{in}}^{\text{Cl}^-}$ (through removal of the external Cl^-) impaired the ability of trout to regulate a NaHCO_3 infusion-induced alkalosis. These data confirm the dominant role of the $\text{Cl}^-/\text{HCO}_3^-$ exchange system in the regulation of acid-base status in freshwater fish.

Eels exhibited low CC surface areas and an inability to vary the CC surface area during acid-base disturbances. This was associated with low rates of $J_{\text{in}}^{\text{Cl}^-}$ and an inability to alter $J_{\text{in}}^{\text{Cl}^-}$ during acid-base disturbance. However, eels were as capable of base excretion during NaHCO_3 infusion-induced alkalosis as were trout; this was accomplished by manipulation of the rates of Cl^- efflux to allow for a net excretion of Na^+ with respect to Cl^- . The resultant *differential* loss of these two ions served as a mechanism to partially compensate the metabolic alkalosis. In trout with an impaired ability to manipulate $J_{\text{in}}^{\text{Cl}^-}$ (through removal of external Cl^-), manipulation of Cl^- efflux and the *differential* loss of Na^+ over Cl^- was invoked as a mechanism of acid-base regulation.

In addition to the effect that alterations in CC surface area have on the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange ($J_{\text{in}}^{\text{Cl}^-}$), it was demonstrated that changes in the concentration of the internal counter-ion (HCO_3^-) may alter the rates of acid-base compensation. When

[HCO₃⁻] is elevated, J_{max}^{Cl⁻} is elevated thereby increasing the capacity to excrete HCO₃⁻ *via* the Cl⁻/HCO₃⁻ exchanger over and above those determined by CC morphology. This is an important mechanism to increase the rate of acid-base compensation during metabolic alkalosis.

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List of Abbreviations

α - Greek letter alpha
Amm - total ammonia
ATP - Adenosine tri-phosphate
 β - Greek letter beta
CC - Chloride cell
 C_{CO_2} - total carbon dioxide content

CCFA - Chloride Cell Fractional Area
CFW - Chloride free water trout
 $^{\circ}C$ - degrees centigrade
h - hours
[HCO₃⁻] - bicarbonate concentration
IC - Intercalated Cell
J_{in} - unidirectional influx
J_{net} - unidirectional net flux
J_{out} - unidirectional efflux or outflux
J_{max} - maximal transport rate
K_m - inverse of affinity
MC - Mucous Cell
MR - Mitochondrial Rich Cell
OT - Ottawa tapwater trout
P_{CO₂} - Partial Pressure of Carbon Dioxide
P_{O₂} - Partial Pressure of Oxygen
pH_a - arterial pH
pH_e - extracellular pH
pH_i - intracellular pH
PVC - Pavement cell
SEM - Scanning Electron Microscope
TA - titratable alkalinity
TEM - Transmission Electron Microscope
[NaCl]_e - water NaCl concentration

CHAPTER 1
GENERAL INTRODUCTION

Homeostasis is the maintenance of a variable or set of variables at a particular level often in the face of frequent challenges. Freshwater fish maintain a high ionic concentration gradient between the extracellular fluids and the dilute external environment. Much attention has focused on the mechanisms for maintaining this gradient, particularly for Na^+ and Cl^- , while less attention has been paid to the regulation of internal acid-base balance. The gill is considered to be the predominant site of ionic and acid-base regulation in freshwater fish (Cameron, 1980; McDonald and Wood, 1981; Wood *et al*, 1984; Perry *et al*, 1987a; McDonald *et al*, 1989a) with other mechanisms such as renal excretion (Cameron, 1978; Wood and Caldwell, 1978; Wheatly *et al*, 1984; Perry *et al*, 1987b; Goss and Wood, 1990b) and intracellular buffering (Cameron, 1980; Heisler and Neumann, 1980) playing lesser but still significant roles. This thesis focuses primarily on the role of the gill in acid-base regulation.

i) Theoretical Considerations

Classically, changes in internal pH are thought to reflect alterations in either or both of the variable components of the Henderson-Hasselbalch equation, partial pressure of carbon dioxide (P_{CO_2}) or bicarbonate ion concentration ($[\text{HCO}_3^-]$). Alterations in P_{CO_2} predominantly reflect respiratory changes while changes in $[\text{HCO}_3^-]$ are caused largely by metabolic adjustments. An alternate view (Stewart 1978, 1983) attributes changes in pH to alterations in one or more of three independent variables- P_{CO_2} , total weak acids (A_{tot}), or strong ion difference (SID). SID is defined as the summated activity of all strongly dissociated cations (Na^+ , K^+ , Mg^{2+} , Ca^{2+}) minus that of all strongly dissociated anions (Cl^- , lactate⁻, HPO_4^{2-} , SO_4^{2-}) in solution. Therefore, in any system in which P_{CO_2} and A_{tot} are constant (*i.e.* no respiratory component to the acid-base disturbance), changes in the acid-base status in one solution must result from differential movements of strong ions between solutions to cause a change in the SID. These two

approaches should be viewed as complimentary rather than contradictory. The classical view provides a practical approach to the measurement and diagnosis of acid-base status. P_{CO_2} , pH, and total CO_2 can all be measured directly with reasonable accuracy and $[\text{HCO}_3^-]$ can be derived easily. The SID approach is more difficult to apply experimentally because of the cumulative error involved in multiple ion measurements, as well as the uncertainties in the quantification of A_{tot} (Cameron, 1989; Cameron and Iwama, 1989). However, it is extremely valuable in providing a theoretical basis for the linkage between ionic and acid-base regulation.

ii) Historical Development of Ionic and Acid-Base Regulation in Freshwater Fish.

Krogh (1938, 1939) suggested that the uptake of Na^+ and Cl^- by the gills of many freshwater animals were both independent of each other and were powered by active transport (primary or secondary). In addition, he suggested that Na^+ may be taken from the water in exchange for endogenous NH_4^+ and that Cl^- may be exchanged for endogenous HCO_3^- . Such so-called electroneutral exchanges are necessary to maintain electroneutrality in the internal compartments.

A generalized model of the fish gill epithelium showing the proposed mechanisms of ion and acid-base regulation in freshwater fish at the onset of the present thesis is shown in Fig 1.1. The gill is the principal site of respiratory gas and metabolic waste transfer (O_2 , CO_2 , NH_3) between the extracellular space and the external environment. In addition, the gill serves as the principle site of ion and acid-base regulation (Wood, 1988). In freshwater fish, there is a constant diffusive loss of Na^+ and Cl^- to the water *via* the gill epithelial paracellular pathways (indicated by the dashed lines in Fig 1.1: Eddy, 1985; McDonald, 1983; McDonald and Rogano, 1986; McDonald *et al*, 1991b). These losses have been shown to be exacerbated by lowered external NaCl concentrations (Laurent *et al*, 1985), lowered external Ca^{2+} concentrations (McDonald and Rogano,

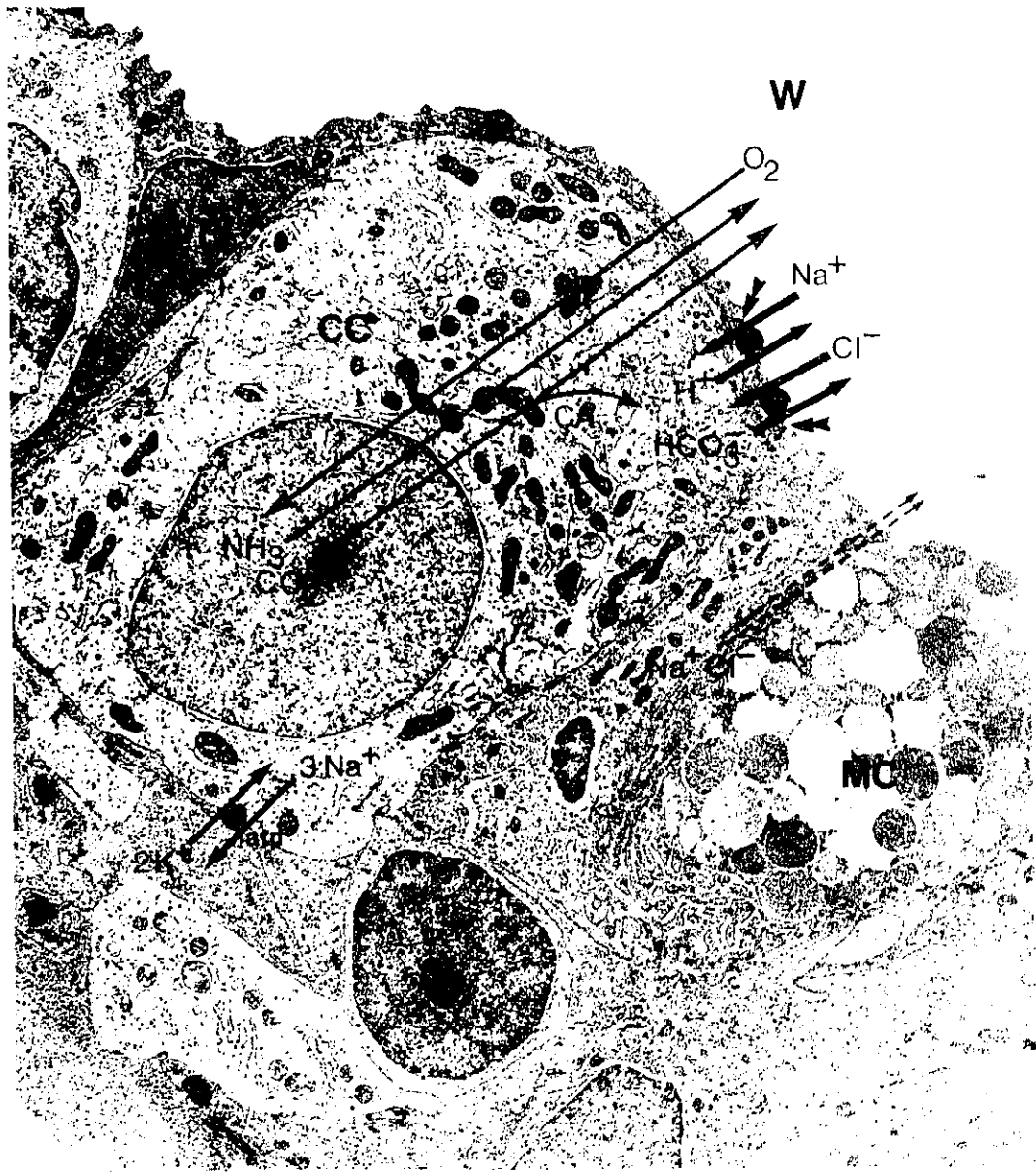
1986; Playle *et al*, 1989) or exposure to acidic water (McDonald and Wood, 1981). To maintain ion balance, these losses must be compensated by active uptake of Na^+ and Cl^- from the external water.

Maetz (1956) was the first to demonstrate uptake of Na^+ and Cl^- across the gills of goldfish by the use of radiotracer techniques. Maetz and Garcia-Romeu (1964) found that when the external concentration of ammonia in the water was raised, Na^+ influx was inhibited and when internal ammonia concentration was raised, Na^+ influx was stimulated. These observations supported Krogh's early suggestion for a $\text{Na}^+/\text{NH}_4^+$ exchange mechanism on the apical (water-facing) surface of the gill. Maetz (1971) later concluded that either H^+ or NH_4^+ may be exchanged for Na^+ and that ammonia may cross the gills as either NH_3 or NH_4^+ . Kirschner *et al* (1973) examined a variety of freshwater animals and concluded that H^+ was the predominant exchange counter-ion, although they suggested that NH_4^+ could also be exchanged to a significant extent.

Garcia-Romeu and Maetz (1964) observed that increasing the concentration of HCO_3^- in the external media reduced influx of Cl^- in goldfish while injection of HCO_3^- resulted in a stimulation of Cl^- influx. Ehrenfeld (1974) exposed crayfish to impermeant cations (choline chloride) or anions (sodium sulphate) and reported a 1:1 stoichiometry between base excretion and chloride uptake and acid excretion and sodium uptake. Thus, it was concluded that Na^+ is exchanged for H^+ predominantly while Cl^- is exchanged for HCO_3^- . These studies, however, did not focus on the problems of acid-base regulation but rather on ionoregulation and therefore, blood or haemolymph acid-base variables were not measured. From examination of Fig. 1.1, one can immediately see a linkage between

FIGURE 1.1

A transmission electron micrograph of a freshwater trout gill epithelium showing diagrammatically the roles of the fish gill in gas transfer, ionic regulation and acid-base balance as thought to occur at the **initiation** of this thesis. PVC = pavement cell, MC = mucous cell, CC = chloride cell, CA = carbonic anhydrase. See text for additional details.



ion and acid-base regulation because Na^+ is exchanged for an acidic equivalent ion (H^+ , NH_4^+) while Cl^- is exchanged for a basic equivalent (OH^- , HCO_3^-). Therefore, independent manipulation of these exchangers provides a direct mechanism for the regulation of internal acid-base status.

There are many methods available for experimental induction of acid-base disturbances in teleost fish. Exposure to hyperoxia (elevated P_{O_2} ; Wood and Jackson, 1980; Thomas, 1983; Höbe *et al*, 1984c; Wheatly *et al*, 1984; Wood *et al*, 1984; Goss and Wood, 1990a; Wood and LeMoigne, 1991; Wood, 1991b), hypercapnia (elevated P_{CO_2} ; Cameron, 1976; Perry, 1982; Claiborne and Heisler, 1984; Perry *et al*, 1987a, b), acid infusion (Cameron and Kormanik, 1982; McDonald *et al*, 1989a; Milligan *et al*, 1991; Goss and Wood, 1991) and base infusion (Cameron and Kormanik, 1982, Claiborne and Heisler, 1986; Goss and Wood, 1990b), have all been employed at various times. The possibility that the Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers might be dynamically manipulated during these acid-base disturbances was examined initially by a number of researchers with conflicting results. Kerstetter and Mize (1976) reported that lowering blood pH by 0.4 pH units had no effect on Na^+ or Cl^- influxes in artificially ventilated trout preparations and concluded that these exchangers did not respond rapidly to changes in blood acid-base status. Perry *et al* (1981) also found no changes in Na^+ and Cl^- influx during hypercapnic acidosis in the rainbow trout (*Oncorhynchus mykiss*). Cameron (1976), however, showed that in Arctic grayling (*Thymallus arcticus*), hypercapnic acidosis enhanced Na^+ influx while Cl^- influx was depressed. This would increase the rate of acidic equivalent loss and depress the rate of basic equivalent loss which would aid in correction of the acidosis. McDonald *et al* (1983) were the first to simultaneously measure unidirectional flux rates of Na^+ and Cl^- and the net acidic equivalent fluxes during acid-base disturbances. They found that during recovery of trout from exposure to low environmental pH, Na^+ influx

was stimulated relative to Cl^- influx and that this was associated with a net acidic equivalent excretion. Subsequently, it has been shown numerous times that branchial $\text{Na}^+/\text{H}^+(\text{NH}_4^+)$ and $\text{Cl}^-/\text{HCO}_3^-(\text{OH}^-)$ exchangers are dynamically manipulated to correct acid-base disturbances in fish (Wood *et al.*, 1984; Perry *et al.*, 1987a; Hyde and Perry, 1989; Avella and Bornancin, 1990; Goss and Wood, 1990a, b). In general, the effect on the gill ion fluxes during each of these disturbances is similar.

Respiratory acidosis [decreased extracellular pH (pH_e) resulting from increased P_{CO_2}] is usually associated with a negative net chloride flux ($J_{\text{net}}^{\text{Cl}^-}$) and a positive net sodium flux ($J_{\text{net}}^{\text{Na}^+}$) through a reduction in chloride uptake ($J_{\text{in}}^{\text{Cl}^-}$) and sometimes, but not always, a stimulation of sodium uptake ($J_{\text{in}}^{\text{Na}^+}$). This results in an increase in the SID and correction of the acidosis by increasing the $[\text{HCO}_3^-]$ in the extracellular fluid. During a metabolic acidosis [decreased pH_e resulting from "fixed" acids e.g. exercise, ammonium salt infusion, HCl infusion] the contribution of Na^+ uptake becomes more prominent. During metabolic alkalosis (increased pH_e due to elevated "fixed" base (HCO_3^-) e.g. during base (NaHCO_3) infusion or post-hypercapnia/post-hyperoxia in a fully compensated animal) compensation is normally associated with a positive $J_{\text{net}}^{\text{Cl}^-}$ and a negative $J_{\text{net}}^{\text{Na}^+}$ through a large stimulation of $J_{\text{in}}^{\text{Cl}^-}$ and reductions of $J_{\text{in}}^{\text{Na}^+}$. This would act to decrease the SID and, correspondingly, correct the alkalosis by reducing extracellular $[\text{HCO}_3^-]$. These alterations in the influx rate for both Na^+ and Cl^- were recently shown to occur mostly from increases/decreases in the maximal rate of transport (J_{max}) with few alterations in the affinity ($1/K_m$) of the transporter for the ions (Goss and Wood, 1990a, b; see below).

iii) Gill Epithelial Morphology of Freshwater Fish

The gill epithelium is comprised of at least five cell types (Conte, 1969; Dunel-Erb *et al.*, 1982; Laurent, 1984). Pavement or respiratory cells make up the largest single

fraction, often comprising more than 95% of the gill epithelium. These cells have been extensively studied with the scanning electron microscope (SEM)(Hughes, 1979; Hossler *et al*, 1979a, b; Olson and Fromm, 1973; Dunel and Laurent, 1980). Lamellar pavement cells (PVCs) generally do not have large numbers of mitochondria which are considered necessary for actively transporting cells, although they do have other ultrastructural features suggestive of high metabolic activity including a well developed Golgi apparatus, an abundance of rough endoplasmic reticulum and numerous vesicles (Laurent and Dunel, 1980). Their external morphology is characterized by microridges/microvilli whose function is unknown, although they may play a role in mucous adhesion (Hughes, 1979; Laurent, 1985).

Mucous cells (MCs) are probably not directly involved in either ion or acid-base regulation although they may have a role in modulating gill ion transport by providing a micro-environment rich in ions (Handy, 1989). Other cell types found in fish epithelia include serotonergic neuro-epithelial cells (Dunel-Erb *et al*, 1982; Bailly *et al*, 1989, 1992) and undifferentiated or stem cells for which no definitive roles have been described (Laurent, 1984).

The principle cell type thought to be responsible for active transport of ions in freshwater fish is the chloride cell, also termed the ionocyte (Laurent, 1984, 1989; Perry and Laurent, 1989). The chloride cell (CC) has a number of distinct ultrastructural features which are characteristic of ion transporting cells. These include large numbers of mitochondria and a dense network of tubules which are thought to be continuous with the basolateral membrane (Doyle and Gorecki, 1961; Philpott, 1966). These tubules have been postulated to be the site of Na^+/K^+ -ATPase activity in the CC (Ritch and Philpott, 1969). Na^+/K^+ -ATPase activity is necessary to establish and maintain the electrochemical gradients required for the functioning of the exchangers (Fig. 1.1). It has been shown *in*

in vitro that CCs have higher rates of oxidative metabolism compared with other cell types (Perry and Walsh, 1989) which is likely linked to increased levels of Na^+/K^+ -ATPase activity (Sargent *et al.*, 1975; Naon and Mayer-Gostan, 1983; Perry and Walsh, 1989) although the contribution of other ATPases (Ca^{2+} -ATPase: Mayer-Gostan *et al.*, 1983; $\text{Cl}^-/\text{HCO}_3^-$ -ATPase: De Renzis and Bornancin, 1984; Na^+/H^+ -ATPase: Balm *et al.*, 1988) should not be discounted. A distinguishing feature of the CC is the presence of a vesiculotubular system in the apical portion of the CC where mitochondria are generally scarce (Laurent, 1984).

Keys and Wilmer (1932) were the first to describe the "chloride secreting cells" as those cells involved in salt secretion in seawater fish but were unable to determine if they played any role in freshwater ion regulation. A role for the CC in freshwater osmoregulation was not elucidated until much later (Laurent and Dunel, 1980; Laurent *et al.*, 1985; Avella *et al.*, 1987; Laurent and Hebibi, 1989; Laurent and Perry, 1990). Recently, Perry and Laurent (1989) showed that rainbow trout exposed to water which contained lower levels of NaCl (~0.015 mmol/l) compared to normal levels of NaCl (~0.14 mmol/l) for a period of 30 days showed an increase in the rates of uptake of both Na^+ and Cl^- . This was associated with an increase in the exposed CC area on the filamental epithelium through an increase in individual CC area and a small increase in density of CCs. From their data, it was suggested that the CC is the site of uptake for both Na^+ and Cl^- .

iv) Analogous Epithelia - Structure and Function- What can we learn?

The freshwater fish gill shares a number of common features with some well characterized epithelial surfaces such as frog skin, toad and turtle urinary bladder, and cortical collecting duct of the mammalian kidney. These epithelia have an apical (mucosal) surface bathed in a very hypotonic solution (water or urine) while the basal

(serosal) surfaces are exposed to the extracellular fluid. These epithelia function effectively in the dynamic uptake of ions and the excretion of acidic and basic equivalents (Brodsky and Schilb, 1966; Gluck *et al*, 1982; Madsen and Tisher, 1984, 1986; Kniaz and Arruda, 1990). Thorough analysis of the similarities and differences in these animals may help to demonstrate the mechanisms of acid-base regulation in freshwater fish.

The high concentration gradients across these epithelia necessitate a high transmembrane resistance to passive ion flow in order to minimize passive ion movements. This has been shown for frog skin (Smith, 1971), turtle bladder (Rich *et al*, 1990) and cortical collecting duct (Turnheim, 1991). These structures are now grouped together as belonging to a family of "tight" epithelia. The gill serves the same function in freshwater fish as the above mentioned epithelia do. The apical surface is bathed by the water while the basolateral surface is exposed to the extracellular fluid creating a high concentration gradient for passive flow of ions. However, for a number of reasons, the fish gill has not been well characterized. There are no known measurements of the transmembrane resistance in the freshwater fish gill. Part of the problem is the lack of a suitable system to test the physiological functioning of the fish gill. Other transporting epithelia can be mounted as sheets either directly or grown in culture which allows for direct physiological and pharmacological characterizations. Comparable systems do not exist for the fish gill and are not feasible with present technology.

The frog skin and turtle bladder have been shown to perform electrically as a functional syncytium owing to the presence of gap junctions between cells (Ehrenfeld *et al*, 1990). This is very important in allowing for the separation of various components of the ion/acid-base transport systems into different cell types while allowing for electroneutrality to be maintained. It has not been demonstrated that gap junctions exist between the cells on the gills of freshwater fish.

Morphologically, the systems mentioned above, share some common features from which we can gain insight into the functioning of the freshwater fish gill. The majority of cells are similar in structure to the pavement cell of the fish gill. Pavements cells have a slightly different nomenclature depending on the system under study. They have variously been termed the principal cell (cortical collecting duct) or the granular cell (frog skin and toad/turtle bladder). The morphological/physiological features of the principal/granular cells in other species are similar to those listed in section iii for the pavement cell with one significant difference. In the fish gill, the pavement cell is rich in carbonic anhydrase (CA) activity especially in the apical regions of the cell (Rahim *et al*, 1988). The authors hypothesized that the presence of CA in PVCs was somehow linked to CO₂ excretion but overlooked the potential involvement of this enzyme in acid-base regulation. This enzyme is essential in providing the counter-ions (H⁺, HCO₃⁻) for the ion/acid-base transporting mechanisms. In the frog skin (Rosen and Freidley, 1973), the toad and turtle bladders (Fritsche *et al*, 1991), and the cortical collecting duct (Rosen, 1970, 1972), the analogous cell types do not possess significant CA activity and this is one factor used in the characterization of the cells. The principal/granular cells have been shown to be the site of Na⁺ uptake through apical Na⁺ conductive channels (frog skin: Ehrenfeld and Garcia-Romeu, 1977; Harvey *et al*, 1988; Ehrenfeld *et al*, 1990; turtle bladder: Steinmetz *et al*, 1967; collecting duct: Benos, 1982; Stokes, 1982; Kristensen and Ussing, 1985) but not the site of either apical Cl⁻/HCO₃⁻ exchange or apical H⁺-ATPase activity (Steinmetz, 1987; Weiner and Hamm, 1991).

In addition to the pavement type cells, these epithelia possess a mitochondria rich cell type similar to the chloride cell in the fish gill. These cells have been termed intercalated cells (IC) in the kidney or mitochondria rich (MR) cells in the toad/turtle bladder and frog skin and have been shown to be rich in CA activity (Rosen, 1970, 1972;

Rosen and Freidley, 1973; Dimberg *et al*, 1981; Rahim *et al*, 1988). In the MR cells of the turtle bladder and the IC cells of the collecting duct, there is a further sub-division into two distinct cell types (α - and β - sub-types) based on apical membrane characteristics (Stetson and Steinmetz, 1985). Currently, the frog skin is thought to consist of a homogeneous group of α -type MR cells (Ehrenfeld *et al*, 1990) although sub-division on the basis of intracellular ion concentrations has been suggested (Rick, 1992).

The α -type (acid excreting) cell is characterized by having an apical H^+ -ATPase, basolateral Cl^-/HCO_3^- exchanges (and Cl^- conductance) and an ability to rapidly endocytose the apical (luminal) membrane in acidic cytoplasmic vesicles (Gluck *et al*, 1982; Schwartz and Al-Aqwati, 1985; Schwartz *et al*, 1985; Al-Aqwati, 1986; Brown *et al*, 1987a, c). Morphologically they can be seen in the SEM as having numerous microplacae on their apical surface and in the transmission electron microscope (TEM) as having numerous rod-shaped intramembrane particles on the apical membrane (Stetson and Steinmetz, 1985). During hypercapnia (elevated P_{CO_2}), these cytoplasmic vesicles can rapidly re-insert themselves into the luminal membrane (Cannon *et al*, 1985) and they display more extensive microplacae on their apical surface (Fritsche *et al*, 1991). It has been shown immunocytochemically that these vesicles contain the H^+ -ATPase (Brown *et al*, 1987b, 1988a, b) and therefore, this system allows for rapid modulation of proton secretion.

A second type of MR cell is the β -type (base-secreting) which is characterized as having apical Cl^-/HCO_3^- exchanges and basolateral H^+ -ATPase (Stetson and Steinmetz, 1985), *i.e.* a reversed polarity to that of the α -type cell. These cells do not endocytose vesicles (Schwartz *et al*, 1985, 1988) and are characterized by their ability to bind peanut agglutinin to surface receptors (Lehir *et al*, 1982; Shuster *et al*, 1986). In the SEM, these cells can be distinguished as not having apical microplacae but sparse microvilli (Fritsche *et*

al., 1991), while in the TEM, they do not exhibit apical membrane associated rod-shaped particles (Stetson and Steinmetz, 1985)

Attempts have been made to subdivide the CC population in freshwater fish into different distinct sub-types based solely on their morphological appearance but definitive evidence for these distinctions has not been performed. Pisam *et al* (1987) distinguished two types of cells in the gills of freshwater guppies (*Lebistes reticulatus*). In their study, α -CC's (note: although they named their two sub-types α - and β -CC's, there was no attempt to correlate their structures with function or to relate these sub-types to the α - and β -sub-types of MR/IC cells found in turtle bladder and the collecting duct) were lighter staining, elongated cells located at the base of the lamellae and lay in close contact with the capillaries. The α - cells had more extensive basolateral infoldings forming an extended network. The vesiculotubular network was less prominent. The β -CC, was darker staining, more ovoid in shape and was located primarily in the inter-lamellar regions of the gill. The vesiculotubular network was quite prominent in these cells which were in contact only with the central venous sinus. In addition, Pisam *et al* (1987) stated that during seawater adaptation, α -CCs metamorphosed into seawater CCs while β -CC's degenerated and disappeared.

Franklin and Davison (1989), Perry and Laurent (1989) and Perry *et al* (1992b) have all noted that in salmonids at least, there appear to be varying surface morphologies displayed by the CC. In some CCs, the surface displays a complex series of microvilli, while in others, the surface is relatively smooth. These differences may represent different sub-types of CCs in this genus. Interestingly, many other genera (catfish, eel, tilapia) do not display the same degree of variability in their surface morphology (P. Laurent, pers comm). An alternate explanation for the varying surface morphology of the CC was put forward by Wendelaar-Bonga and van der Meij (1989) who suggested that the varying

degrees of staining noted by Pisam *et al* (1987) and the variability of the surface structures may be accounted for by differences in the life cycle of the CC. They contend that the variations in staining are due to apoptosis (controlled cell death) of the CC and that the darker staining cells represent cells which are undergoing degeneration.

From these various epithelia, an organized model for the maintenance of the acid-base status in other "tight" epithelia has emerged with significant differences to the fish gill model shown in Fig 1.1. In known "tight" epithelia, Cl^- uptake and base excretion are accomplished by a $\text{Cl}^-/\text{HCO}_3^-$ exchanger located on the apical surface of the β -type intercalated (MR) cell. Na^+ uptake and H^+ (proton) excretion, however, are accomplished on two different cell types by two different mechanisms. Na^+ uptake is accomplished through amiloride-sensitive Na^+ conductive channels located on the apical surface of the principal/granular cell while H^+ excretion is accomplished by a H^+ -ATPase on the apical surface of the α -type intercalated (MR) cell. Electroneutrality in the cell is maintained owing to the fact that the epithelium acts as a functional syncytium.

v) Morphology and Acid-Base Regulation

Although the responses of the gill epithelial $\text{Cl}^-/\text{HCO}_3^-$ exchange and Na^+/H^+ exchange (H^+ pump/ Na^+ channel) to changes in internal acid-base status are well characterized (see above), the mechanisms involved in the initiation of these responses are unresolved. Previous studies have examined the relationships between ionoregulation and morphology of the gill. However, no studies have been performed to investigate the possibility that morphological alteration of the gill epithelium is utilized as a mechanism for acid-base regulation.

Modification of the morphological characteristics of other "tight" epithelia occur during acid-base disturbances. In turtle bladder, regulation of acid excretion is accomplished by rapid insertion of H^+ pumps into the luminal membrane, thereby altering

the apical surface area of the α -mitochondria rich (MR) cells during acidosis (Gluck *et al.*, 1982). Similar changes occur in α -type intercalated cells in the mammalian kidney (Madsen and Tisher, 1986; Satlin and Schwartz, 1989) and in the frog skin (Ehrenfeld *et al.*, 1990).

Cameron and Iwama (1987) first suggested a relationship between gill morphology and acid-base regulation in fish based on results of a preliminary study using hypercapnic catfish (*Ictalurus punctatus*). Specifically, they suggested that during the acidosis induced by hypercapnia, CCs proliferated on the filamental epithelium as a mechanism for acid-base compensation. Unfortunately, this study used only two animals for the experimental group so conclusions could not be drawn.

The theory behind morphological alteration of the gill epithelium as a mechanism of acid-base regulation is straightforward. If the transporters responsible for acid-base regulation are present on a particular cell type, then by alteration of the apical exposure of those exchangers (cell types), the availability and, hence, the activity of the transporters can be modulated. An important point to note, however, is that increases in one type of transport correspond to simultaneous decreases in the other type (see section ii). This presents certain problems given the generally accepted model (Fig 1.1) showing $\text{Na}^+/\text{H}^+(\text{NH}_4^+)$ and $\text{Cl}^-/\text{HCO}_3^-(\text{OH}^-)$ exchanges operating on the same cell type, the chloride cell. There are a number of ways this could be solved. One solution would be to have the acidic and basic equivalent excreting transporters located on different cells thereby allowing independent regulation as is seen in other "tight" epithelia. A second solution is that CCs may actually modulate the relative number of each of the different exchanges exposed to the water through selective endocytosis and exocytosis, thereby altering the relative rate of acidic or basic equivalent excretion. A third possibility is that there exist sub-populations of CCs on the gill surface in a similar manner to those of the

α - and β - subtypes of intercalated cells in the mammalian cortical collecting duct (see above). Another possibility is that the number of transporters on a cell surface is relatively static but that the accessibility or functioning of these transporters is regulated.

vi) Role of the Pavement Cell in Acid-Base Regulation

It is possible that the pavement cell plays a significant role in acid-base regulation in freshwater fish and the intense localization of the CA in these cells functions to provide acid-base equivalents to an ion-transporting mechanism. In one of the few studies that has investigated the potential involvement of the pavement cell in acid-base regulation (Girard and Payan, 1980), it was demonstrated that Na^+ uptake was performed almost entirely by the cells located on the lamellae, while cells on the filament functioned in uptake of Cl^- . It was concluded that lamellar pavement cells, and not chloride cells, were the cells responsible for Na^+ uptake in freshwater fish. However, these observations were based on the assumption that CCs were found only on the filamental epithelia, an observation shown later to be untrue (Laurent *et al*, 1985). Based on observations using a perfused trout gill, Kerstetter and Keeler (1976) also concluded that the $\text{Cl}^-/\text{HCO}_3^-$ and Na^+/H^+ exchange mechanisms were located on the pavement cells rather than the chloride cells. These views were then reinforced in an influential paper by Payan *et al* (1984). However, Gardaire *et al* (1985) demonstrated that there were methodological problems associated with these experiments and interpretation of the earlier results was left in doubt. These authors concluded that ion uptake occurred on both the filamental and lamellar surfaces of the freshwater trout gill. Bartels (1989) found features in lamprey pavement cells which were analogous to those found in the granular cells in the frog skin epithelium, a well documented acid-base excreting epithelium. This author suggested that the pavement cell in freshwater lampreys is responsible for Na^+ uptake in a manner similar to that seen in the frog skin. One of the weaknesses of this study, however, was

the lack of physiological data to correlate with the morphological features. Therefore, the only conclusive statement one can make from these conflicting pieces of evidence is that the role of the pavement cell in freshwater ion regulation has not been determined.

vii) Na^+/H^+ Exchange or a Proton Pump?

A significant difference between the models outlined for the other "tight" epithelia and the fish gill (Fig 1.1) is the mechanism responsible for the uptake of Na^+ and the excretion of protons (H^+). Avella and Bornancin (1989) proposed that the mechanism for Na^+ uptake and H^+ excretion may not occur according to the classical Na^+/H^+ exchange mechanism as proposed by Krogh (1938, 1939: Fig 1.1). The Na^+/H^+ type of mechanism was first described for "leaky" epithelia such as rat intestine (Murer *et al*, 1976) and renal proximal tubule (Kinsella and Aronson, 1981) where the external $[\text{Na}^+]$ is very high (>20 mmol/l): the theory being that Na^+ entry along its electrochemical gradient will power the Na^+/H^+ exchange mechanism. Instead, Avella and Bornancin (1989) have proposed the indirect coupling of an active electrogenic proton pump and passive entry of Na^+ via a Na^+ conductive channel to maintain electroneutrality. Theoretically, this type of mechanism can be uncoupled at low external Na^+ levels thereby allowing H^+ loss to the environment without gain of Na^+ . It is proposed that the Na^+/H^+ exchange mechanism cannot function at low external $[\text{Na}^+]$ such as in freshwater because the gradient for Na^+ entry is too great to trigger the countertransport. A proton pump/ Na^+ channel type of mechanism as has been found for other "tight" epithelia has been suggested for the fish gill. However, direct evidence for either of these mechanisms in freshwater fish has not yet been attained although it is an area of research currently under investigation (Lin and Randall, 1991). Therefore, throughout this thesis, the Na^+ uptake mechanism will be referred to as the Na^+/H^+ (Na^+ channel/ H^+ pump) with no intent to infer either of these mechanisms as the predominant one.

viii) Inter-specific Variations in the Rates of Acid-Base Regulation

The rate of compensation from an acid-base disturbance is highly variable depending on the experimental conditions and the species. Using compensation of hypercapnic acidosis as an example, the marine elasmobranch, *Scyliorhinus stellaris* compensated fully within 8-10 h (Heisler *et al*, 1978), seawater adapted salmon, *Oncorhynchus kisutch*, required 24 h to fully compensate (Perry, 1982) while the freshwater catfish *Ictalurus punctatus* also required 24 hours (Cameron, 1980) or more (Cameron and Iwama, 1987). The freshwater rainbow trout, which has been studied to a much greater extent under a greater number of conditions, has been shown to require 24-48 h to compensate hypercapnic acidosis (Janssen and Randall, 1975; Eddy *et al*, 1977; Perry *et al*, 1988; Perry, 1982; Perry *et al*, 1987a). Each of these species were acclimated to varying environmental salinities and in addition, each species has a characteristic gill epithelial morphology relating to the environment to which they were acclimated. These factors may variably affect the mechanisms utilized and the rates at which correction of an acid-base disturbance may occur. The challenge, therefore, is to separate the relative contributions of differences in morphology from differences in environment with respect to rates of acid-base compensation.

In most species of freshwater fish, the predominant mechanism for the regulation of acid-base disturbances is modulation of the gill $\text{Cl}^-/\text{HCO}_3^-$ exchange system (Maetz and Garcia-Romeu, 1964; De Renzis and Maetz, 1973; Claiborne and Heisler, 1984, 1986; Wood *et al*, 1984; Perry *et al*, 1987a; Goss and Wood, 1990a, b). However, freshwater American eels (*Anguilla rostrata*) are an interesting species to examine because they lack appreciable Cl^- uptake (Kirsch, 1972; Bornancin *et al*, 1977; Hyde and Perry, 1987, 1989) and hence $\text{Cl}^-/\text{HCO}_3^-$ exchange activity. In addition, their relative rates of Na^+ uptake are low as are the rates of passive ion loss from the extracellular space to the external

environment. Therefore, comparison of the relative abilities of rainbow trout and American eels to regulate an acid-base disturbance provides a valuable opportunity to examine the relative roles of the Na^+ uptake/ H^+ extrusion and $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanisms in the maintenance of acid-base homeostasis and may give clues as to the mechanisms involved and their locations. These inter-specific differences in ionic uptake and acid-base compensation may be related to inter-specific differences in gill morphology. Indeed, while trout display varying abundance of CCs in direct response to variations in the external milieu (Mattheij and Stroband, 1971; Olivereau, 1971; Laurent *et al.*, 1985; Avella *et al.*, 1987; Spry and Wood, 1988; Perry and Laurent, 1989), eels possess only few CCs exposed to the water (Perry *et al.*, 1992a, b, P. Laurent, pers comm). It is believed that the paucity of CCs displayed in eels may account for the low rates of Cl^- influx.

ix) Intra-specific Variations in the Rates of Acid-Base Regulation

If variations in the amount of CCs displayed on the gill surface can result in **inter-specific** differences in the ability to compensate an acid-base disturbance, then to what degree do **intra-specific** variations in gill epithelial morphology account for alterations in the ability of fish to compensate? Adaptation to ion-poor freshwater (Höbe *et al.*, 1984b; Perry and Laurent, 1989; Avella and Bornancin, 1990), cortisol treatment (Perry and Wood, 1985; Laurent and Perry, 1990; Madsen, 1990a), combined cortisol/growth hormone treatment (Madsen, 1990b) and cortisol/thyroxine treatment (Madsen, 1990c) have all been shown to affect the number of CCs on the gill epithelial surface. In a recent study, Perry and Laurent (1989) showed that transfer of trout from normal freshwater ($\text{NaCl} \sim 0.14 \text{ mmol/l}$) to dilute water ($\text{NaCl} \sim 0.015 \text{ mmol/l}$) resulted in increases in plasma cortisol concentrations and elevations in the fractional area of CCs exposed on the gill. A subsequent study by these researchers (Laurent and Perry, 1990) showed that elevation of plasma cortisol titre through daily intramuscular injection resulted in increased rates of

uptake for both Na^+ and Cl^- . The increases in $J_{\text{in}}^{\text{Na}^+}$ and $J_{\text{in}}^{\text{Cl}^-}$ were correlated with increases in filamental CC fractional area (Laurent and Perry, 1990). This suggests that the CC is the major site of both Na^+ and Cl^- uptake in freshwater rainbow trout. It also suggested that a mechanism for alteration of ion flux rates by altering the surface area of exposed CCs and hence, availability of transport sites. This would act to alter the relative number and availability of transport mechanisms and thereby may be a means of altering the rates of ion transfer during acid-base disturbances.

x) Substrate Availability and Acid-Base Regulation

In addition to inter- and intra-specific variations in ion and acidic-basic equivalent transfer which may result from alterations in epithelia morphology, alterations in the salinity of the water are known to directly affect the rates of ion transfer and hence acid-base compensation. Separation of these two components is sometimes difficult owing to the effects of long-term acclimation to differing salinities on gill epithelial morphology (Perry and Laurent, 1989).

The relationship between ion influx and external ion concentration in freshwater animals has been studied numerous times for conditions involving long-term acclimation to varied salinities (Shaw, 1959; Kerstetter *et al*, 1970; Kerstetter and Kirschner, 1972; Maetz, 1972; De Renzis and Maetz, 1973; Wood and Randall, 1973; De Renzis, 1975; Perry and Wood, 1985; Frain, 1987). In general, increasing the external ion concentration in a stepwise fashion over short periods of time results in increased ionic fluxes. The relationships generated in this fashion can be approximated by typical first order kinetic curves as described by Michaelis-Menten kinetics (Potts and Parry, 1963). These results yield estimates for the affinity of the transporter for the external ion (K_m) and the maximal transport capacity when the carrier is saturated (J_{max}). These parameters have been shown to be appropriately altered in response to long-term acclimation to different

salinities. For example, at lowered external ion concentrations, J_{\max} is elevated and K_m lowered (increased affinity) compared to fish acclimated to higher salinities (Wood and Randall, 1973; cf. Lauren and McDonald, 1987). These alterations would act to increase transport capacity and aid in ionoregulation.

The mechanisms resulting in changes in the affinity ($1/K_m$) of the branchial Na^+ and Cl^- transporters are not central to this thesis, although they remain an intriguing problem. Possibilities include competitive inhibition with Ca^{2+} at the external surface as Ca^{2+} levels are changed (Maetz, 1972; De Renzis and Maetz, 1973; Avella *et al*, 1987), or differential recruitment of sub-populations of transporters on the epithelial surface (Frain, 1987) or direct modulation of the transporters by the animal (Goss and Wood, 1990a, b).

The dynamic regulation of J_{\max} of the branchial ion and acid-base transporters, however, is central to the thesis for a number of reasons. Classically, alterations in J_{\max} have been interpreted as resulting from alterations in the number of transport sites available (Shaw, 1959). Theoretically, there are a number of different mechanisms available for increasing the number of transport sites available. One possibility is that J_{\max} is altered by production of new transporters or by activation/deactivation of nascent transporters (Frain, 1987). If there is an increase in the numbers of transporters present, then one might expect alterations in the gill epithelial morphology in those fish showing an increase in ion uptake. Perry and Laurent (1989) noted that there was an increase in the capacity for both Na^+ and Cl^- uptake (J_{\max} was not measured but an increase was inferred) and that this was related to increases in the fractional area of CCs exposed on the gill filamental surface. Therefore, it was concluded that alteration of ion transport capacity was related to the morphological modification of the gill. An alternative mechanism for the regulation of J_{\max} was put forward recently by Wood and Goss (1991) and Goss and Wood (1991). They showed that by increasing the concentration of internal

counter-ion (H^+ , HCO_3^-), J_{max} was automatically increased by mass action. This indicates that the internal counter-ion is not at saturating concentrations (K_m is greater than physiological levels) and therefore, in any treatment where internal counter-ion concentrations are altered (acid-base disturbance), J_{max} is altered for that population.

Therefore, in any situation where the transport capacity of an animal is altered, there are interactive effects of alterations of morphology, external ion concentrations and internal counter-ion concentrations which directly affect the rates of acid-base compensation.

xi) Goals of the Thesis

In light of the above discussion, the goal of the present thesis was to determine the mechanism(s) involved in the regulation of blood acid-base status in freshwater teleost fish. Although it is well documented that branchial influxes of Na^+ and Cl^- are regulated in opposite directions during acid-base disturbances, the mechanisms involved in the regulation of these transporters remain unresolved. Based on knowledge that morphological modification is an important feature of acid-base regulation in analogous "tight" epithelia, the possibility that this mechanism was actively utilized by teleost fish was investigated. The general approach was to use multiple treatments to induce acid-base disturbances (hypercapnia, hyperoxia, acid infusion, base infusion) to determine whether modification of the gill epithelial morphology occurs and to employ morphological (scanning and transmission electron microscopy) and physiological tools to discern the relative contributions of these mechanisms to overall acid-base regulation.

CHAPTER 2
PHYSIOLOGICAL AND MORPHOLOGICAL REGULATION OF ACID-BASE STATUS
DURING HYPERCAPNIC ACIDOSIS IN BROWN BULLHEAD CATFISH (*ICTALURUS*
***NEBULOSUS*)**

INTRODUCTION

Environmental hypercapnia is often used as a tool to induce acid-base disturbances in aquatic animals (Janssen and Randall, 1975; Cameron 1976; Cameron and Iwama 1987; Perry *et al*, 1987a, b). This treatment induces a respiratory acidosis that is gradually compensated over several days owing to an elevation of plasma $[\text{HCO}_3^-]$, with the fish ultimately attaining a new steady-state pH. Subsequent removal of the hypercapnic stimulus causes a rapid relative metabolic alkalosis since the respiratory acidosis dissipates rapidly, whereas the metabolic base excess (accumulated HCO_3^-) is only cleared gradually. These changes in $[\text{HCO}_3^-]$ occur mainly by dynamic manipulation of branchial $\text{Cl}^-/\text{HCO}_3^-$ exchange. During acidosis, $J_{\text{in}}^{\text{Cl}^-}$ is reduced as a means of retaining base while during metabolic alkalosis, when $[\text{HCO}_3^-]$ is elevated, $J_{\text{in}}^{\text{Cl}^-}$ is stimulated (Heisler, 1984, 1986; Wood *et al*, 1984; Cameron and Iwama, 1987; Perry *et al*, 1987a; Goss and Wood, 1990a, b; McDonald *et al*, 1991b). However, the mechanisms underlying the apparent manipulation of these transporters during acid-base disturbances are unknown.

Cameron and Iwama (1987), in a study using channel catfish (*Ictalurus punctatus*) suggested that morphological modification of the gill could be involved in regulation of hypercapnic acidosis. This was based on their observations of increases in the numbers and sizes of "apical pits" of CCs at times of increased net acid excretion. More recently, Cameron (1989) suggested that future directions in the study of the mechanisms of acid-base regulation in fish should involve determination of the relationships between morphology and physiology. Therefore, the goal of the present study was to examine simultaneously the relationships between gill epithelial morphology (chloride cells and pavement cells) and the physiological changes ($J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$) occurring during environmental hypercapnia.

METHODS

Experimental Animals

Brown bullhead catfish (*Ictalurus nebulosus*) of either sex [mean weight = 198 ± 8.8 g (standard error (SE), $n = 48$)] were obtained from ponds in the Haute-Marne region of France and kept indoors in large fibreglass tanks supplied with flowing aerated dechlorinated Strasbourg tap water (Table 2.1). Temperature was maintained at 18 ± 1 °C (SE) during the acclimation period. Fish were fed twice a week with silurid pellet. Photoperiod was kept constant at 12 h light: 12 h dark.

Experimental Protocol

Ionic fluxes and gill morphology during environmental hypercapnia

Bullheads were placed into individual opaque acrylic boxes and allowed to recover for 36 h prior to experimentation. At the start of the experiment, the flux boxes (volume ~ 2 l) were flushed with water prepared by the method of Goss and Wood (1990a) which had reduced levels of titratable alkalinity compared to normal Strasbourg tap water (Table 2.1). This protocol was utilized in an attempt to increase the precision of the measurement of titratable alkalinity for the estimation of whole body net acidic equivalent flux. Unfortunately, reliable measurements could not be obtained and are not presented here. The reduction in water $[Ca^{2+}]$ from 2.24 mmol/l to 1.11 mmol/l would have negligible effects on gill ionic permeability (Potts 1984). Following this flushing, flow to the boxes were stopped and $^{36}Cl^-$ (1.0 μCi) was added to each box from a 1 mol/l NaCl stock solution to yield final Na^+ and Cl^- levels equivalent to those in Strasbourg tap water (Table 2.1). After an initial mixing time of 0.5 h, water samples (40 ml) were withdrawn from the box at the beginning of the flux period and 3 h later. The boxes were again thoroughly flushed with prepared water and the procedure was repeated using $^{22}Na^+$.

TABLE 2.1

Comparison of the measured chemical composition of Strasbourg tap water with the prepared water used in determination of the flux measurements. All measurements are mmol/l.

	<u>Strasbourg Tap Water</u>	<u>Prepared Water</u>
[Na ⁺]	0.59	0.63
[Cl ⁻]	0.68	0.70
[Ca ²⁺]	2.24	1.11
[Mg ²⁺]	0.60	0.63
[Titratable Alkalinity]	4.24	2.11
pH	8.46	8.28

The water samples were analyzed for $[\text{Na}^+]$ or $[\text{Cl}^-]$ and $^{22}\text{Na}^+$ or $^{36}\text{Cl}^-$ activity, whichever was appropriate. $J_{\text{in}}^{\text{Na}^+}$ and $J_{\text{in}}^{\text{Cl}^-}$ were monitored by the disappearance of either $^{22}\text{Na}^+$ or $^{36}\text{Cl}^-$ from the water over the 3 h period. Eight periods were examined in the present study: Control (pre), 3, 6, 12, 27, and 48 h of hypercapnia (2% CO_2 in air; $P_{\text{wCO}_2} = 15$ torr), and 3 and 6 h recovery after the return to normocapnia. The 3- and 6-h hypercapnic and post-hypercapnic (recovery) periods were repeated using an inverse order of addition of isotope to permit estimates of $J_{\text{in}}^{\text{Na}^+}$ and $J_{\text{in}}^{\text{Cl}^-}$ at the 0-3 h hypercapnia and 0-3 h recovery time periods and $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ at the 3-6 h hypercapnia and 3-6 h recovery time periods, respectively. Hypercapnic acidosis was achieved by equilibrating the inflowing water to a level of 2% CO_2 . This was accomplished by bubbling 13% CO_2 supplied from a custom-built gas mixer through a large surface area gas exchange column as described by Perry *et al* (1987a) to achieve a final P_{wCO_2} of 2%. P_{wCO_2} was continuously monitored by passing inflowing water by a P_{CO_2} electrode (E5037; Radiometer) connected to a digital meter (PHM 72; Radiometer). During the flux periods, when the inflow to the boxes had stopped, boxes were individually aerated with 2% CO_2 to maintain the hypercapnia.

After each flux period, fish were removed from the boxes and killed by spinal section. The gills were then excised and fixed for microscopic analysis (see below).

Analytical procedures

Water $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ activities were determined on 5 ml samples using liquid scintillation counting (Intertechnique SL38). Total $[\text{Na}^+]$ was measured by atomic absorption spectrophotometry (Perkin-Elmer 2380). Water $[\text{Cl}^-]$ was determined by the mercuric thiocyanate method of Zall *et al* (1956). Unidirectional influxes (J_{in}) and net fluxes (J_{net}) were determined as described by Wood (1988).

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 1 h 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 magnification of 1000X. 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 (μm^2) and surface CC density (amount of cells per mm^2) were determined by tracing the CC perimeters using a digitizing tablet (Summagraphics) and a morphometric software program (Sigmascan, Jandel). In addition, the fractional surface area of CCs per unit epithelium ($\mu\text{m}^2/\text{mm}^2$) 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 morphometric analysis for practical reasons because this epithelium is generally flat and can be mounted parallel to the stub allowing more precision in CC morphometry.

Comments on the methodology for determination of CC fractional area

The apical surface of the CC is obviously a three dimensional structure yet quantification of the apical membrane was accomplished by two-dimensional analysis. This type of analysis probably underestimates the true area of the CC apical membrane and is further confounded by varying degrees of convolution of the apical membrane. Clearly it would be advantageous to analyze the structures in a three dimensional analysis and examine the entire gill surface but this would be entirely impractical given the time and expense needed. Nevertheless, a two-dimensional analysis is probably a reliable method for estimation of relative changes in the fractional areas of CC's exposed to the water. Occasionally, in this and subsequent chapters, identification and differentiation between CC's and mucous cells (MC's) was difficult. If a cell could not be positively identified as a CC, then it was assumed to be a MC and was not used in the calculation of fractional area.

It would have been optimal as well to estimate the total gill epithelial CC fractional area (filamental and lamellar) as both these components are potential sites of ionic uptake and acidic/basic equivalent transfer. However, the fractional area of the filament and the lamellae change in parallel (Laurent and Perry, 1990) and therefore, only the filamental surface was used for CC morphometry.

Examination of gill pavement cell morphology

The same preparations used to access CC morphometry were also used to quantify

several PVC morphometric parameters (see below). Frontal scanning photos were taken from both the filament and the lamellar epithelium at high magnification (10,000X). Three to five photos were examined from each fish and non-adjacent areas of $9000 \mu\text{m}^2$ were used in the quantification. The built-in tilt control of the microscope was used to compensate the tilt angle of the epithelium with the stub surface. The angle of the stub with the incident beam was set at 45° . Compensation was deemed satisfactory when circular structures (microvilli, for instance) did not display any obvious visible deformation. Observations of the lamellar epithelium required the selection of favourable areas where lamella (trailing edge) were positioned in such a way as to permit access of the beam to the surface. Photos were always taken from the first third of the lamella (trailing edge) at approximately equal distance from the base of the top of the lamella. To be consistent and reduce sampling error, photos were taken from the PVC surface displaying the highest density of microvilli visible in the sampling area.

The microvilli surface density was used to characterize the functional state or activity of the PVCs; the larger the surface area developed by microvilli, the higher the functional state of the PVC. This characteristic has been used to define qualitatively the activities of other cell types (e.g., intercalated cells; Madsen and Tisher, 1985). Microvilli surface density was measured according to the methods outlined by Weibel *et al* (1966) and Laurent and Hebibi (1989) by counting intercepts of boundary profiles of microvilli within segments of a test grid. The grid was superimposed on the scanning electron microscope photos and the intercepts of the microvilli with the grid segments were counted, this was repeated three times with different blind orientation of the grid. The surface density of microvilli is directly proportional to the number of intercepts between the test grid and the photograph.

Statistical analyses

All values are presented as means \pm 1 standard error of the mean. Results for Figs 2.1-2.3 and 7 were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons where the ANOVA indicated significance. 5% was accepted as the fiducial limit of significance in all cases.

RESULTS

A) Physiology

Exposure to environmental hypercapnia resulted in a significantly negative $J_{\text{net}}^{\text{Cl}^-}$ and positive $J_{\text{net}}^{\text{Na}^+}$ (Fig 2.1). The change in $J_{\text{net}}^{\text{Cl}^-}$ persisted throughout the 48 h hypercapnic period (except for 27 h) whereas the changes in $J_{\text{net}}^{\text{Na}^+}$ were significant only for the first 12 h. During the post-hypercapnic recovery period, there was no change in $J_{\text{net}}^{\text{Na}^+}$ while $J_{\text{net}}^{\text{Cl}^-}$ increased (*i.e.* became more positive, Fig 2.1). The changes in net fluxes during and after environmental hypercapnia were almost entirely accounted for by changes in $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ (Fig 2.2). Exposure to hypercapnia caused a rapid and marked reduction of $J_{\text{in}}^{\text{Cl}^-}$ and a stimulation of $J_{\text{in}}^{\text{Na}^+}$. $J_{\text{in}}^{\text{Cl}^-}$ remained depressed throughout the duration of hypercapnia, whereas $J_{\text{in}}^{\text{Na}^+}$ returned to pre-hypercapnic levels after 27 h. During recovery, $J_{\text{in}}^{\text{Na}^+}$ remained unchanged, while $J_{\text{in}}^{\text{Cl}^-}$ was greatly stimulated compared to the 48 h hypercapnic period.

B) Chloride cell morphology

Exposure to hypercapnia resulted in a 60% reduction in the area of individual CCs by 6 h and reached a maximum of 95% reduction after 48 h (Fig 2.3a). Surface CC density initially was unaffected by the hypercapnia. However, after 27 h, there was a significant decrease in the number of CCs exposed on the filament epithelium (Fig 2.3b). The changes in these two parameters caused a pronounced reduction of CC fractional area of 50% by 6 h due solely to a change in individual CC surface area. By 48 h, CC fractional area was reduced to 5% of its original value owing to both the reduction in the area of individual CCs area and the reduction in the number of exposed CCs (Fig 2.3c). During post-hypercapnic recovery, there was a rapid increase in CC surface area and a rapid reappearance of CCs on surface of the filament epithelium.

FIGURE 2.1

The temporal effects of environmental hypercapnia (2% CO₂) and the subsequent return to normocapnia (recovery) on the whole body net fluxes of (A) chloride ($J_{\text{net}}^{\text{Cl}^-}$) and (B) sodium ($J_{\text{net}}^{\text{Na}^+}$). * indicates significant difference from control (Pre) value (P<0.05, n=6).

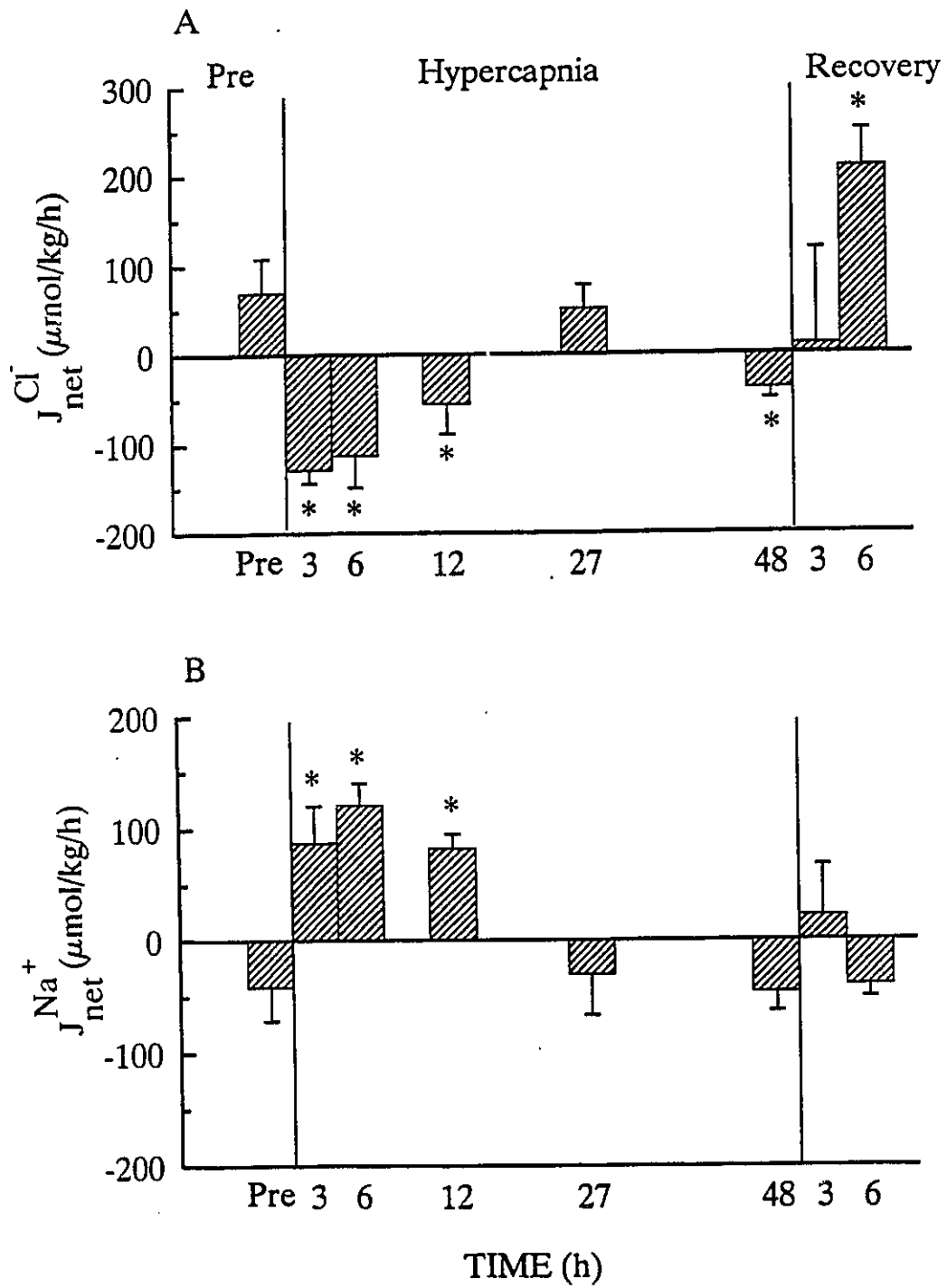


FIGURE 2.2

The temporal effects of environmental hypercapnia (2% CO₂) and the subsequent return to normocapnia (recovery) on the whole body influxes of (A) chloride ($J_{in}^{Cl^-}$) and (B) sodium ($J_{in}^{Na^+}$). * indicates significant difference from control (Pre) value ($P < 0.05$, $n=6$).

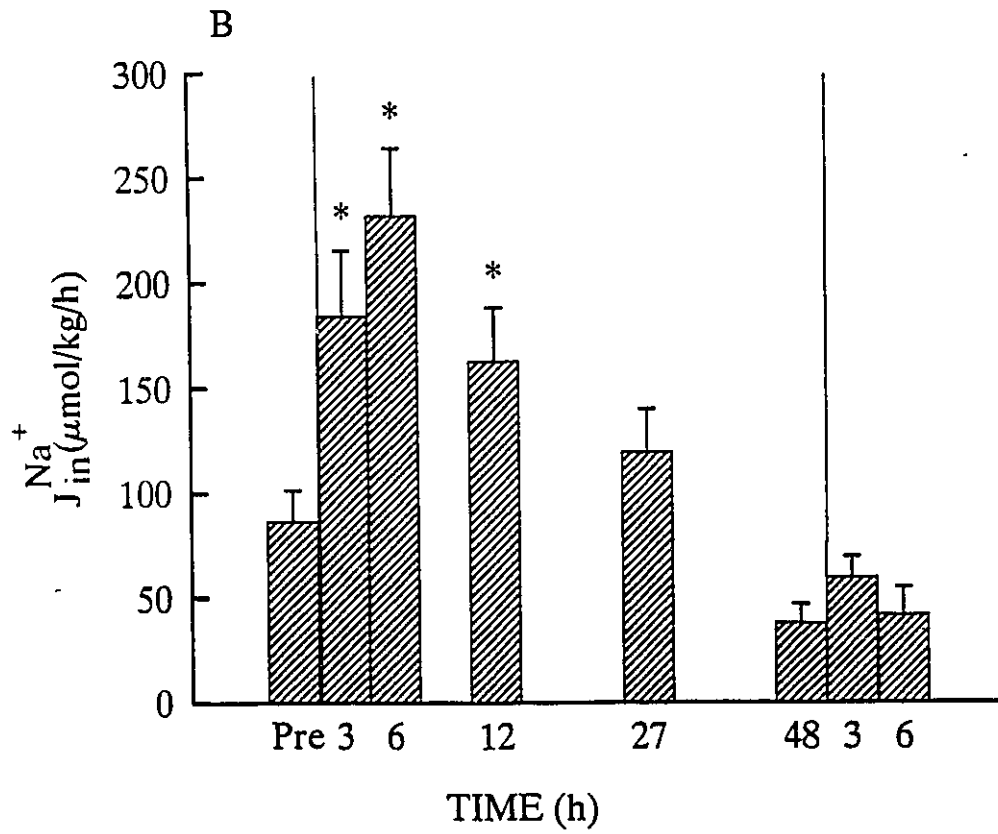
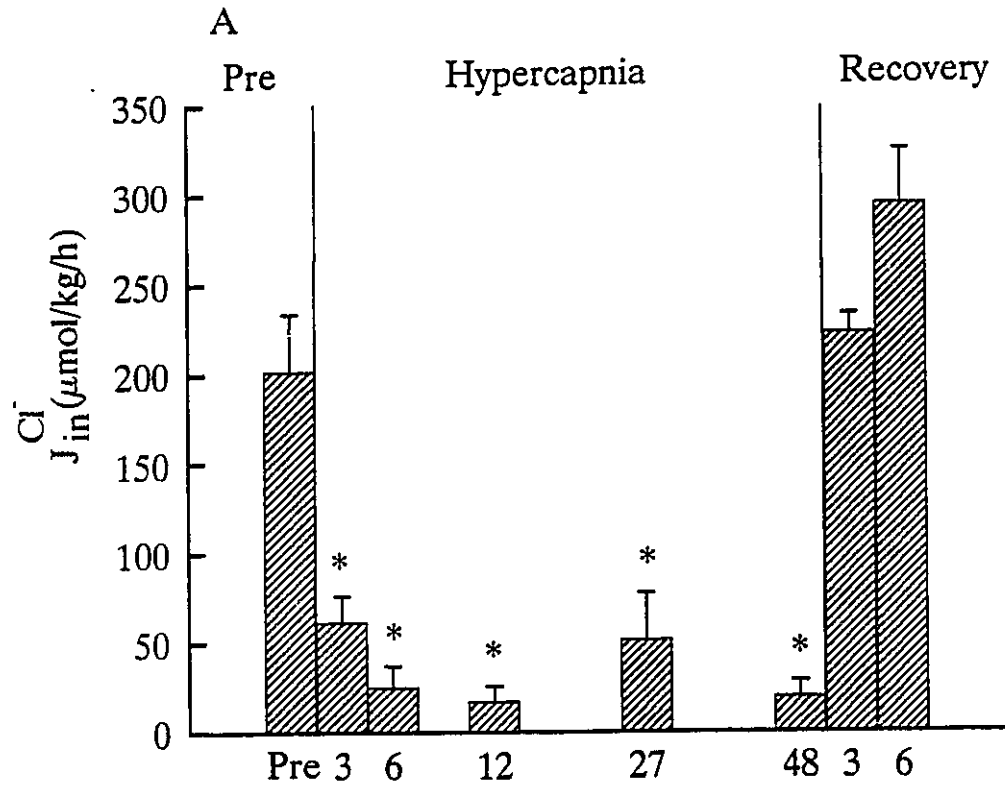
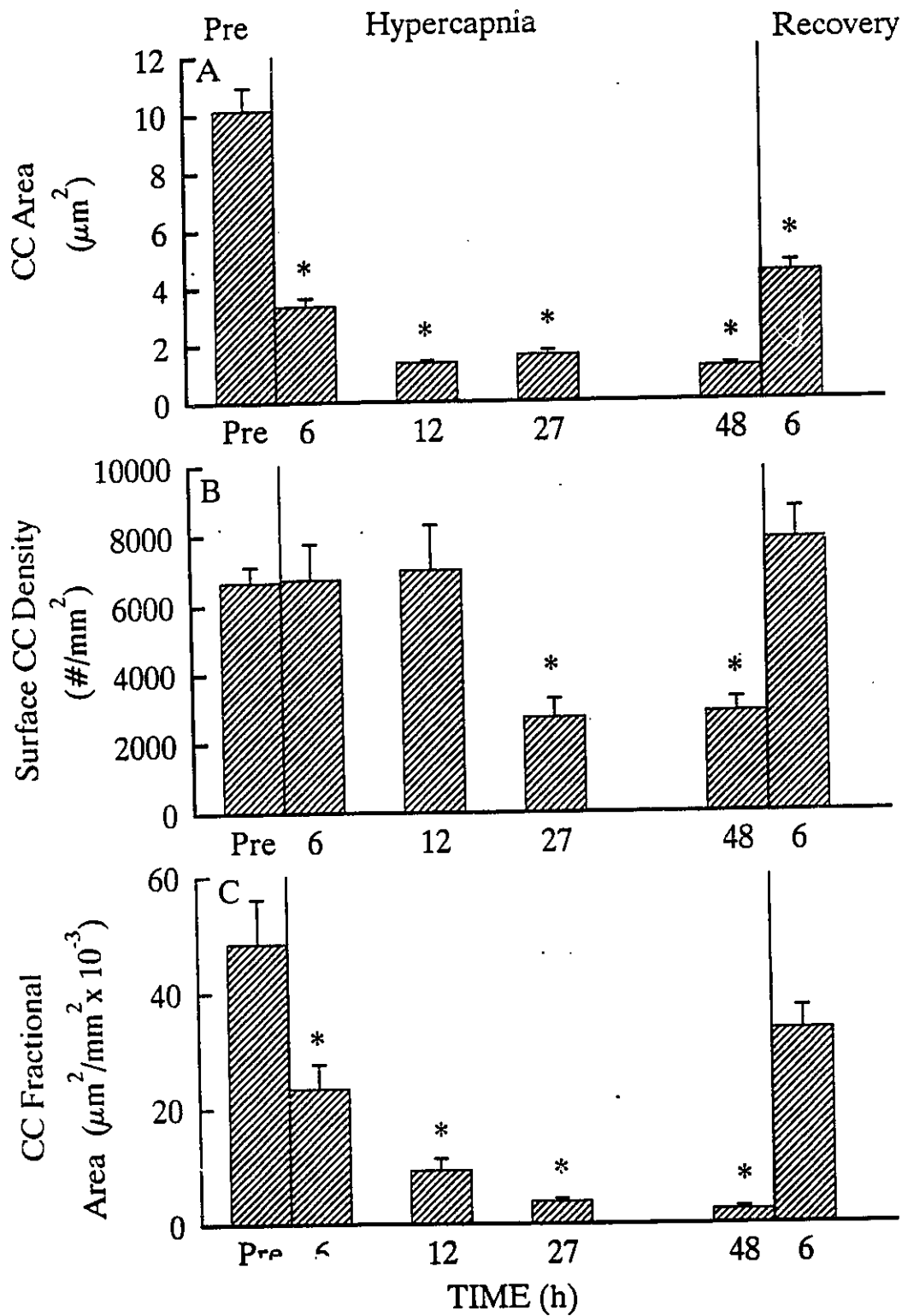


FIGURE 2.3

The temporal effects of environmental hypercapnia (2% CO₂) and the subsequent return to normocapnia (recovery) 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 (P<0.05, n=6). See text for further details.



Representative SEM photographs from various time periods and the measured $J_{in}^{Cl^-}$ for that particular fish are illustrated in Figs 2.4 and 2.5. Under control conditions, the filament epithelium exhibited a distinctive appearance (Figs 2.4a, b) with the CCs appearing spongy and generally relatively flush with the surrounding PVCs. Exposure to hypercapnia resulted in smaller individual CC surface areas initially (Fig 2.4c, d) and, as the hypercapnia continued, the CC surface density was reduced, in agreement with the results of Fig 2.3 (Figs 2.5a, b). In addition, the CCs appeared to become recessed into the filament epithelium (see high magnification: Figs 2.4d, 5b), being covered by the surrounding PVCs. The post-hypercapnic recovery period was associated with the reappearance of CCs on the filament epithelium (Figs 2.5c, d). These re-emergent CCs had a very atypical villous appearance as seen at higher magnification (Fig 2.5d).

TEM micrographs (see below) aided in the positive identification of these cells as CCs. Representative TEM micrographs from control, hypercapnic, and post-hypercapnic bullheads are shown in Figs 2.6. The CCs of control bullheads, like CCs of other freshwater teleosts, had a characteristic ultrastructure; in particular, the cells possessed numerous mitochondria and an extensive tubular network near the apical membrane (Fig 2.6a). In addition, many (but not all) of the filamental CCs were in direct contact with the water owing to a large, uncovered and highly digitated apical membrane. After 6 h of hypercapnia, the apical surface of contact with the external water decreased as the CC was now partially covered by adjacent PVCs although there was still a characteristic, organized tubular network in the apical portion of the CC (Fig 2.6b). This corresponds to the reduction in CC surface area seen at this time (Fig 2.3a). In addition, there appeared to be fewer mitochondria and a disorganization of the CC tubular system as the hypercapnia progressed (intermediate periods not shown) suggesting a reduced function of the CC. By

FIGURE 2.4

Representative low and high magnification scanning electron microscopy (SEM) photographs of the filament epithelium (interlamellar area) under control normocapnic conditions (Figs 2.4a, b; $J_{in}^{Cl^-} = 341 \mu\text{mol/kg/h}$) and after 6 h of exposure to environmental hypercapnia (Figs 2.4c, d; $J_{in}^{Cl^-} = 28.9 \mu\text{mol/kg/h}$). Note the reduction in the CC apical surface area and change in the appearance of the CCs (compare *white arrows*; Fig 2.4b with Fig 2.4c) after only 6 h of environmental hypercapnia. CC = chloride cell, PVC = pavement cell. Figs 2.4b, c scale bar = 20 μm , Figs 2.4a, d scale bar = 2 μm . See text for further details.

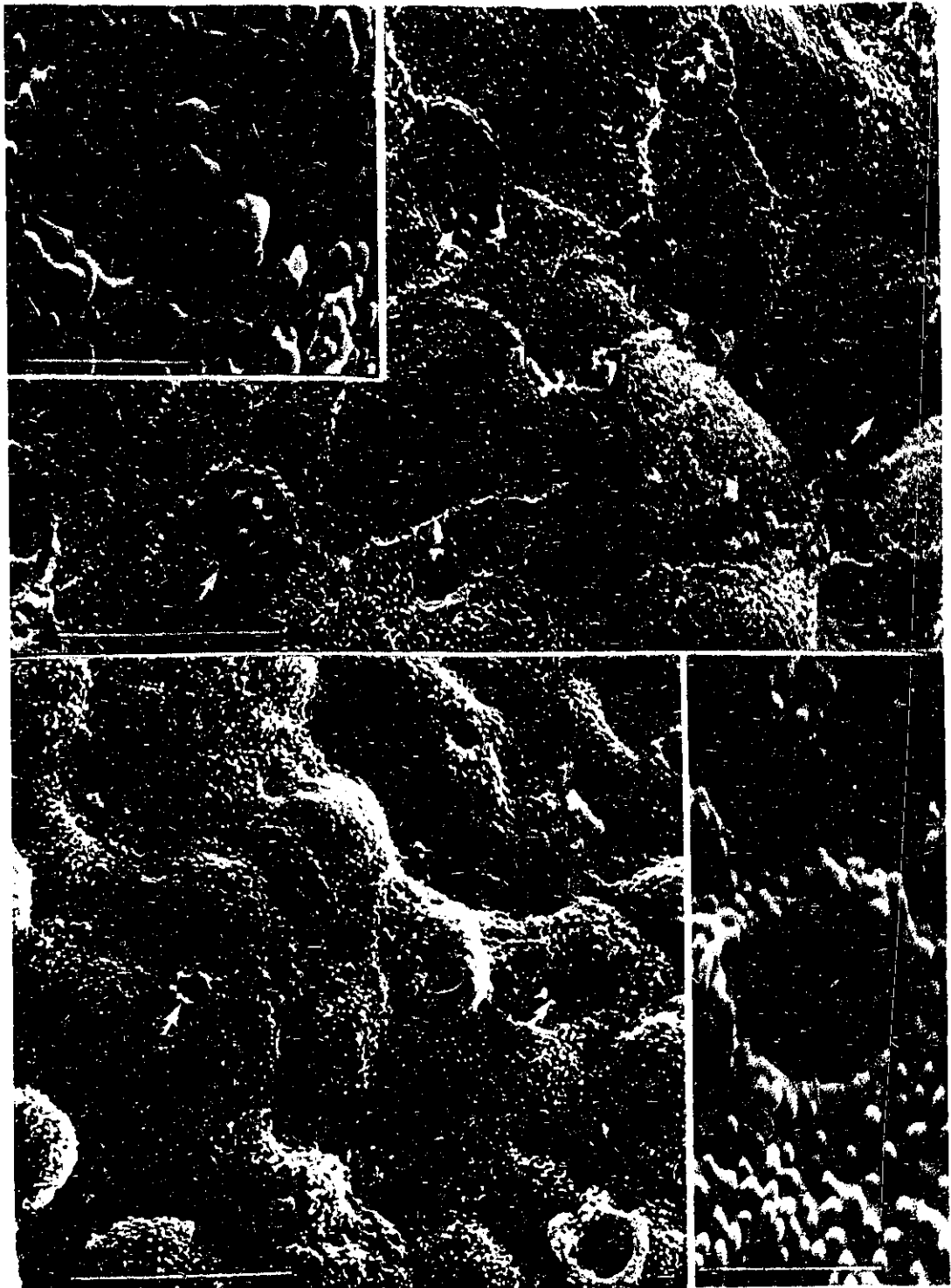


FIGURE 2.5

Representative low and high magnification SEM photographs of the filament epithelium after 48 h exposure to environmental hypercapnia (Figs 2.5a, b, $J_{in}^{Cl^-} = 28 \mu\text{mol/kg/h}$; compare with Figs 2.4a, b). Note the extreme reduction in the CC apical surface area and CC surface density by these time periods *black arrowhead*, Fig 2.5b). After 6 h of normocapnic recovery following 48 h exposure to environmental hypercapnia (Figs 2.5c, d, $J_{in}^{Cl^-} = 378 \mu\text{mol/kg/h}$) there are numerous atypical CCs (*black arrowhead*, Fig 2.5c) emerging from the epithelium. Note in addition, the villous surface of these functionally new CCs (Fig 2.5d). MC = mucus cell. Figs 2.5a, c scale bar = 20 μm , Figs 2.5b, d scale bar = 2 μm . See text for further details.

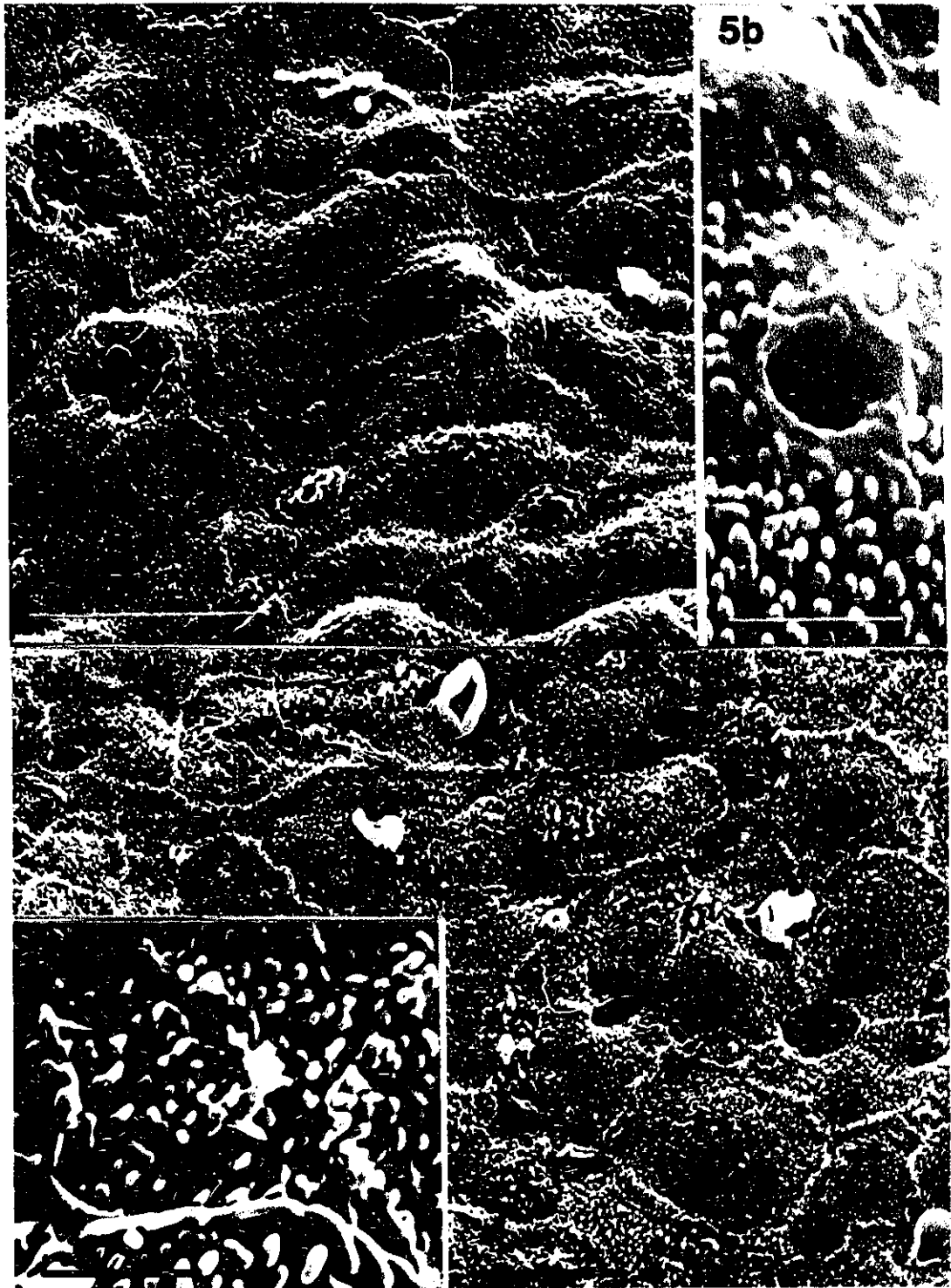


FIGURE 2.6.

High magnification TEM photographs of the filament epithelium demonstrating the ultrastructural changes in CC morphology during environmental hypercapnia. Under control conditions (Fig 2.6a; $J_{in}^{Cl^-} = 186 \mu\text{mol/kg/h}$) there is a characteristic organization of the apical membrane facing the external water. After 6 h exposure to environmental hypercapnia (Fig 2.6b; $J_{in}^{Cl^-} = 29 \mu\text{mol/kg/h}$) the apical membrane is now *apparently* separated from the external water in this plane of section (*). Organelles, including mitochondria, are still present. After 48 h exposure to environmental hypercapnia (Fig 2.6c; $J_{in}^{Cl^-} = 1 \mu\text{mol/kg/h}$), the apical contact of the cell with the external water is severely reduced. After 6 h of normocapnic recovery there was a rapid increase in CC function (Fig 2.6d; $J_{in}^{Cl^-} = 379 \mu\text{mol/kg/h}$). Typically, apical contact with the external water is again present with a somewhat different organization, more villar when compared to control fish (Fig 2.6a). The 6-hour recovery period was characterized by a rapid return of CCs to the surface of the filament epithelium (see Fig 2.3) and a return of CC function. Fig 2.6e demonstrates the reappearance of chloride cells after 6 hours of recovery by retraction of the overlying PVC sheet leaving access of the CC to the water. *Black arrowheads* indicate cell-cell junctions. CC = chloride cell, PVC = pavement cell, w = water surface. Bar = 1 μm .

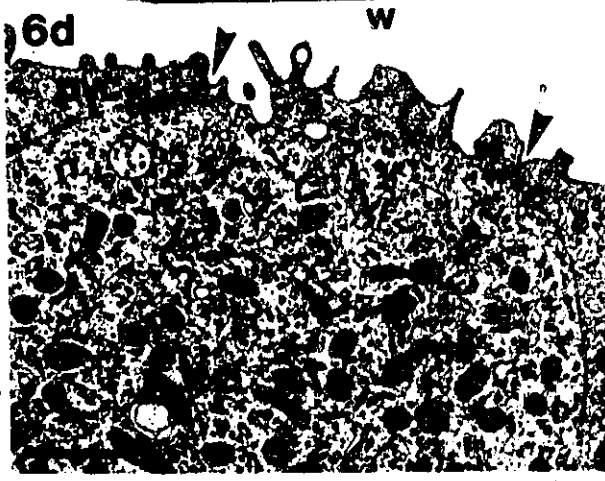
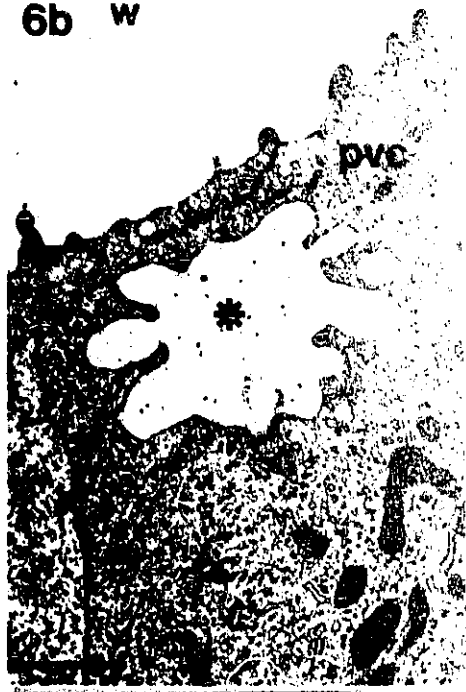
6a

w

6b

w

DVC



48 h, the apical contact of the CC with the external water had almost completely disappeared (Fig 2.6c), an observation consistent with both the physiological and morphometric data (Figs 2.2, 2.3). Return to normocapnia caused pronounced changes in the morphology of the CC. There was a rapid reappearance of CCs at this time and an increase in the individual CC surface area (Fig 2.6d). Fig 2.6d shows a CC with a large apical surface in contact with the external water. In addition, the apical membrane of the representative CC in Fig 2.6d was flush or even slightly elevated above the remainder of the filament epithelium and displayed numerous microvillar-like projections, observations that are consistent with the SEM micrographs (Fig 2.5c, d) for this period. The CCs at this time also showed a distinct, organised tubular network and increased numbers of mitochondria consistent with a more active cell type. The 6 h recovery period was noted for an increase in CC fractional area due to both an increase in CC surface area and a rapid increase in the CC density (Fig 2.3). This micrograph, apparently showing CCs re-establishing direct contact with the external water at distinct locations, suggests an epithelium in transition and is consistent with the re-emergence of CCs on the filament epithelium at this time.

C) Pavement cell morphology

Exposure to hypercapnia resulted in distinct morphological changes to both the lamellar and filament PVCs. Only the results for the filament PVCs are presented here, as the results for both areas (lamellar and filamental) were essentially identical. Fig 2.7 shows the results of the quantitative morphometric study by grid analysis for the filament PVCs. There was a 30% increase in the density of PVC apical microvilli after only 6 h of hypercapnia. As the hypercapnia continued, there was a progressive increase in the density of microvilli ultimately attaining a level two-fold higher than under control conditions. Qualitatively, the microvilli on some cells at the 48 h period were present in

very dense concentrations with the microvilli appearing to form more elongate, microplicae-like structures at this time. Return to normocapnia caused a reduction in PVC microvilli density similar to that seen under control conditions. Fig 2.8a-d are representative SEM photomicrographs showing changes in lamellar PVC microvilli density during exposure to hypercapnia and the subsequent recovery.

There were significant ultrastructural changes in the PVCs during and after hypercapnia (Fig 2.9). Under control conditions, the mitochondria were sparse and distributed uniformly throughout the cell. In addition, apical microvilli were poorly developed (Fig 2.9a). After 6 h of hypercapnia, the mitochondria were more abundant, the apical microvilli were more highly developed, and vesicles were more numerous within the cytoplasm (Fig 2.9b). By 48 h these trends had progressed further, the PVCs showing an abundance of organelles demonstrates a polarization toward the apical region of the cell (Fig 2.9c). After only 6 h of recovery in normocapnic water, the PVC morphology was similar to that seen in control bullheads (Fig 2.9d).

D) Relationship between Ion Uptake and Gill Morphology

To examine the relationships between Cl^- and Na^+ influxes and CC morphometry during acid-base disturbances, correlations were calculated between $J_{\text{in}}^{\text{Cl}^-}$ and the CC fractional area (Fig 2.10a) and $J_{\text{in}}^{\text{Na}^+}$ and CC fractional area (Fig 2.10b) during control (normocapnic) situations when no acid-base disturbance is present. In order to generate a large range of values for $J_{\text{in}}^{\text{Cl}^-}$, $J_{\text{in}}^{\text{Na}^+}$ and CC fractional area, some bullheads were first pre-treated with cortisol (4 mg/kg/day for 10 days) to manipulate these physiological parameters experimentally. This treatment has been shown to increase the levels of all three parameters in rainbow trout (*Oncorhynchus mykiss*) under control normocapnic

FIGURE 2.7

The temporal effects of environmental hypercapnia (2% CO₂) and the subsequent return to normocapnia on microvilli density of filament pavement cells (PVC). * indicates significant difference from control (Pre) value (P<0.05, n=6). See text for further details.

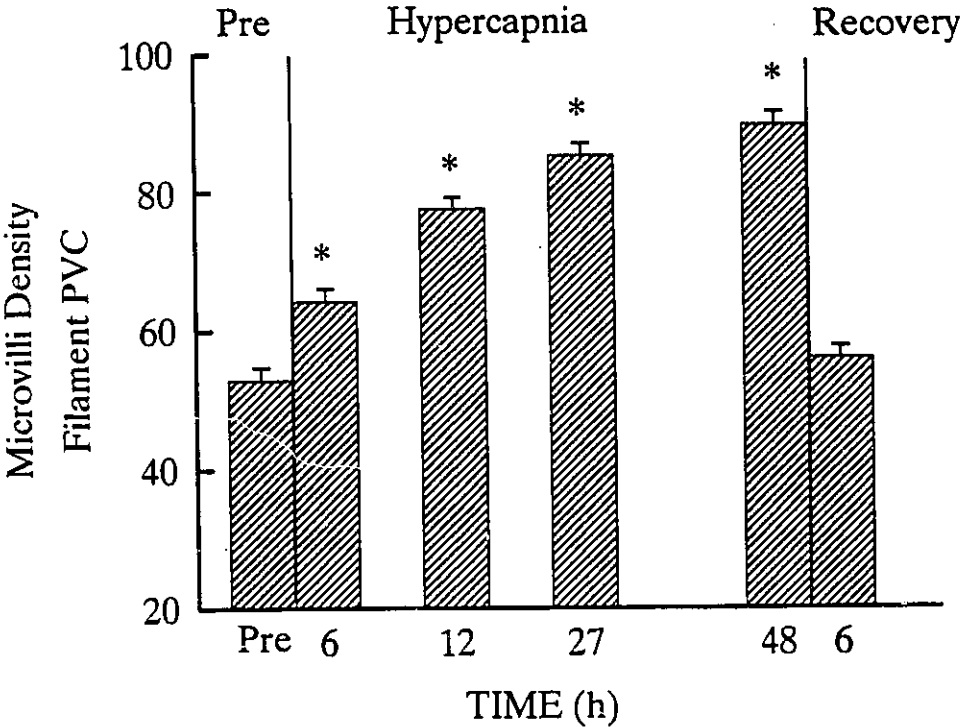


FIGURE 2.8

Representative SEM photographs of the surface epithelium of the bullhead gill filament during exposure to environmental hypercapnia. Note the significant and progressive increase in pavement cell (PVC) microvilli density during hypercapnia (Figs 2.8b, c) compared to control (Fig 2.8a). During the normocapnic recovery period following 48 h exposure to environmental hypercapnia, there is a reduction of PVC microvilli (Fig 2.8d) to a level seen under control conditions (Fig 2.8a). Bar = 2 μ m.

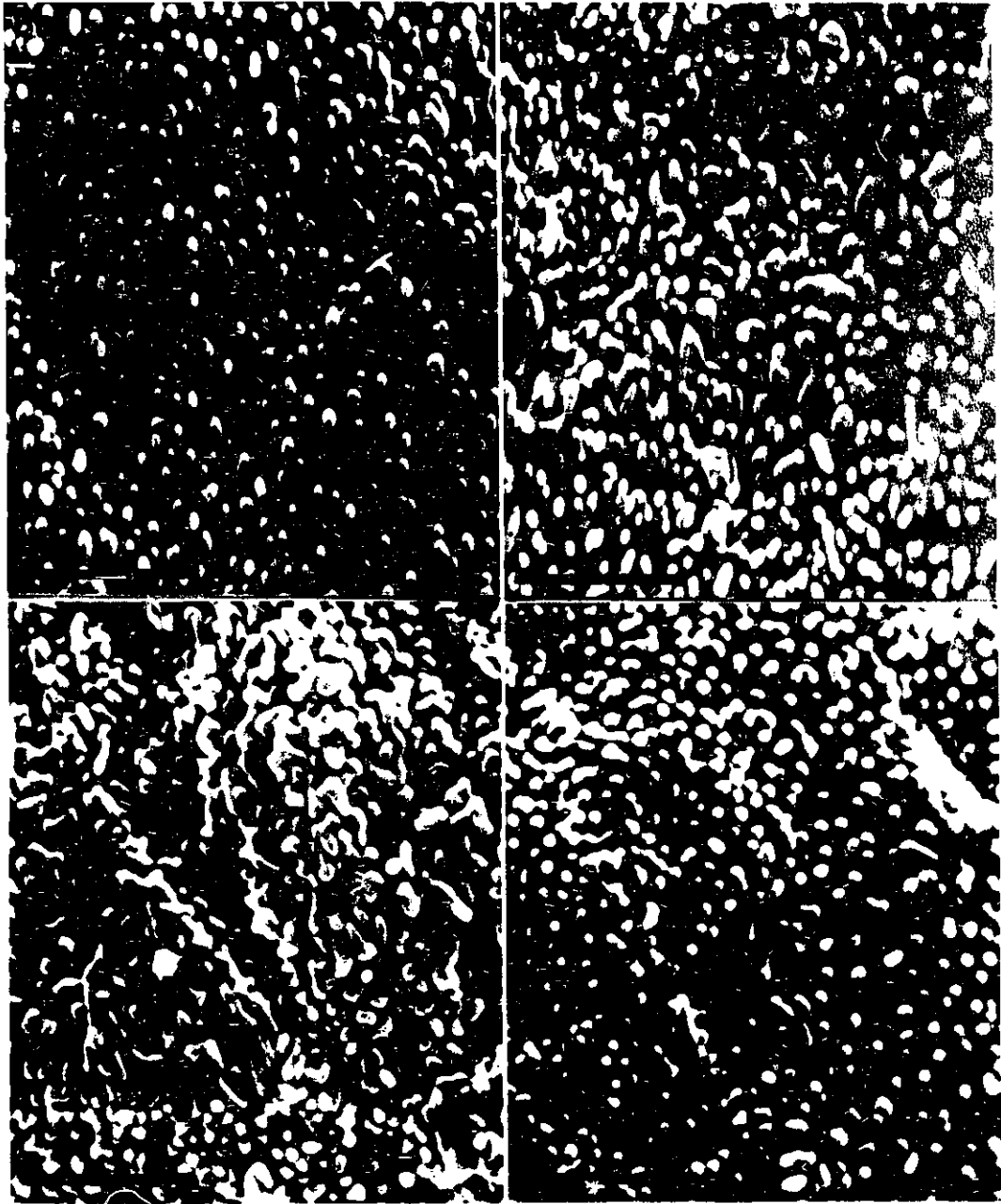
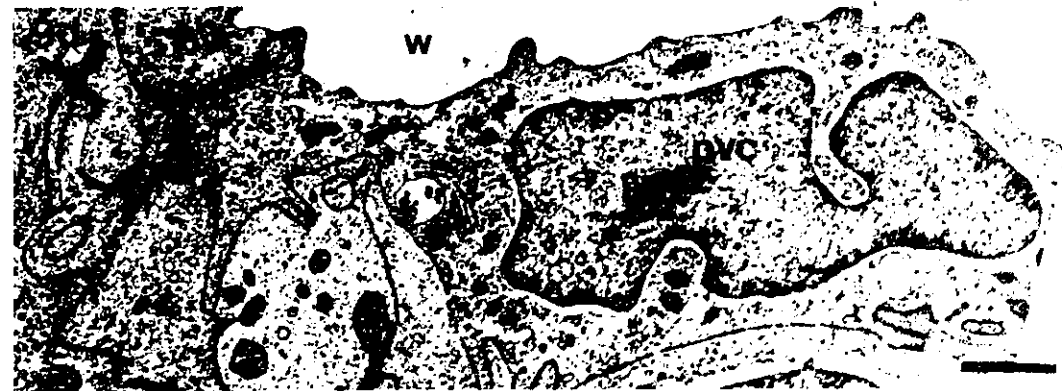
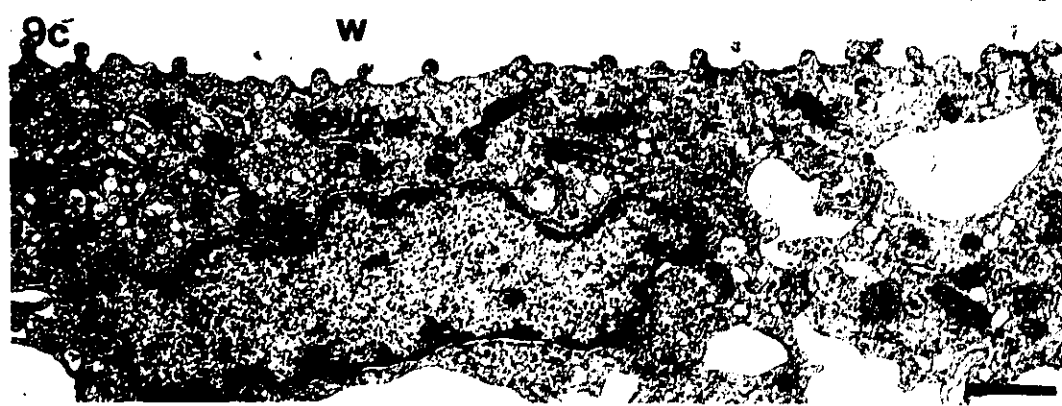
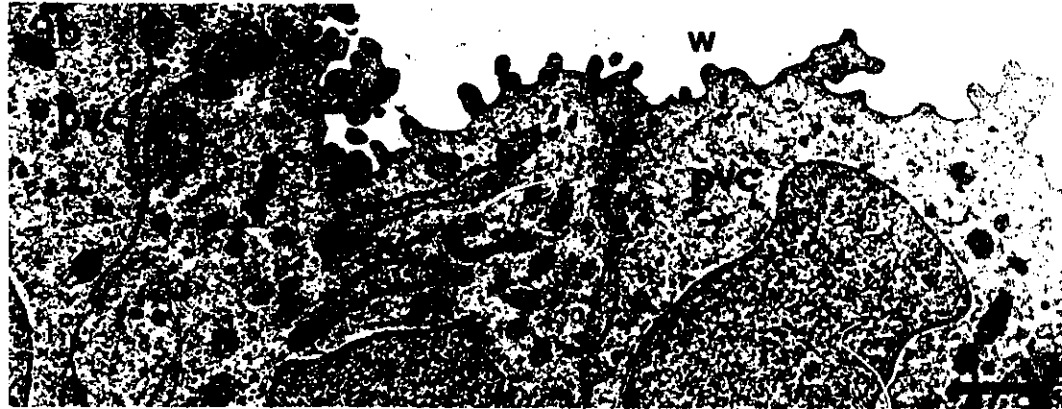
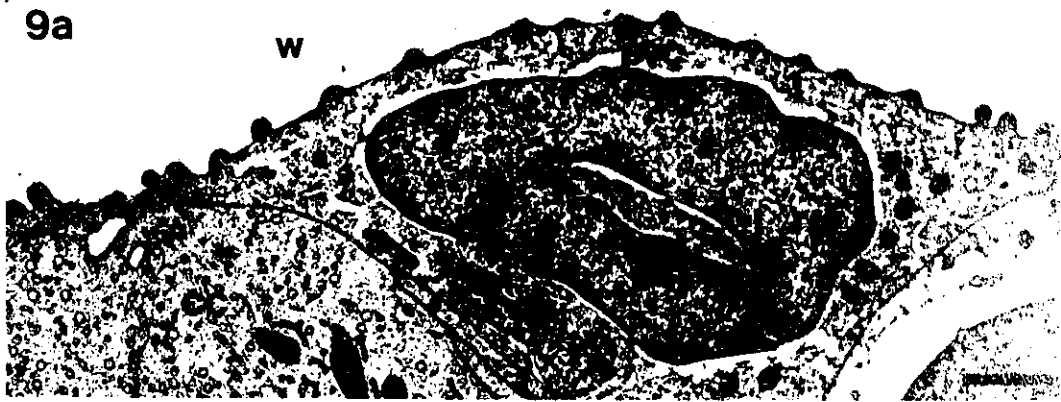


FIGURE 2.9

Representative TEM photographs of pavement cells during and after exposure to environmental hypercapnia. Control bullheads (Fig 2.9a) have PVCs characterized by sparse, evenly distributed mitochondria and poorly developed apical microvilli. After 6 h of environmental hypercapnia (Fig 2.9b), the mitochondria are more abundant and tend to be localized towards the apical area in one of the PVCs. In addition, note the developing apical villi, and the numerous vesicles within the cytoplasm. After 48 h of environmental hypercapnia, there was an increase in the density of PVC microvilli and numerous apical mitochondria were noticed (Fig 2.9c). After 6 h recovery, the PVC morphology was similar to that seen in control bullheads (Fig 2.9d). Bar = 2 μ m.

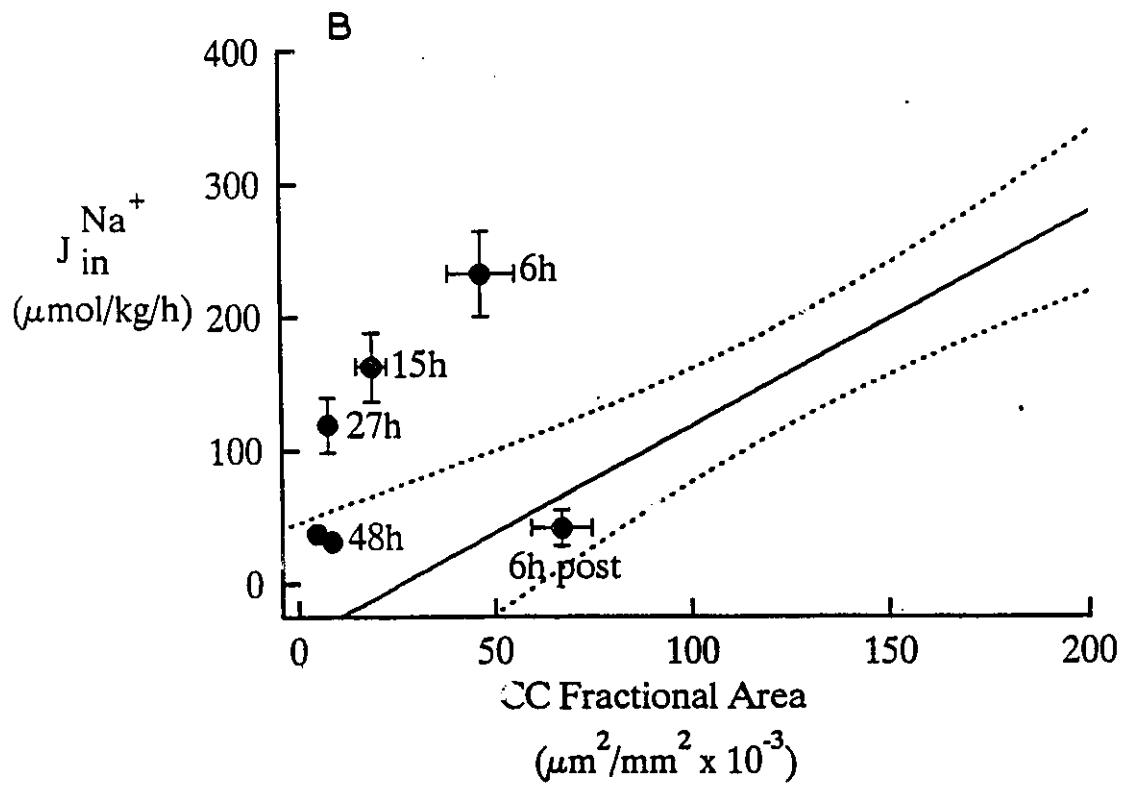
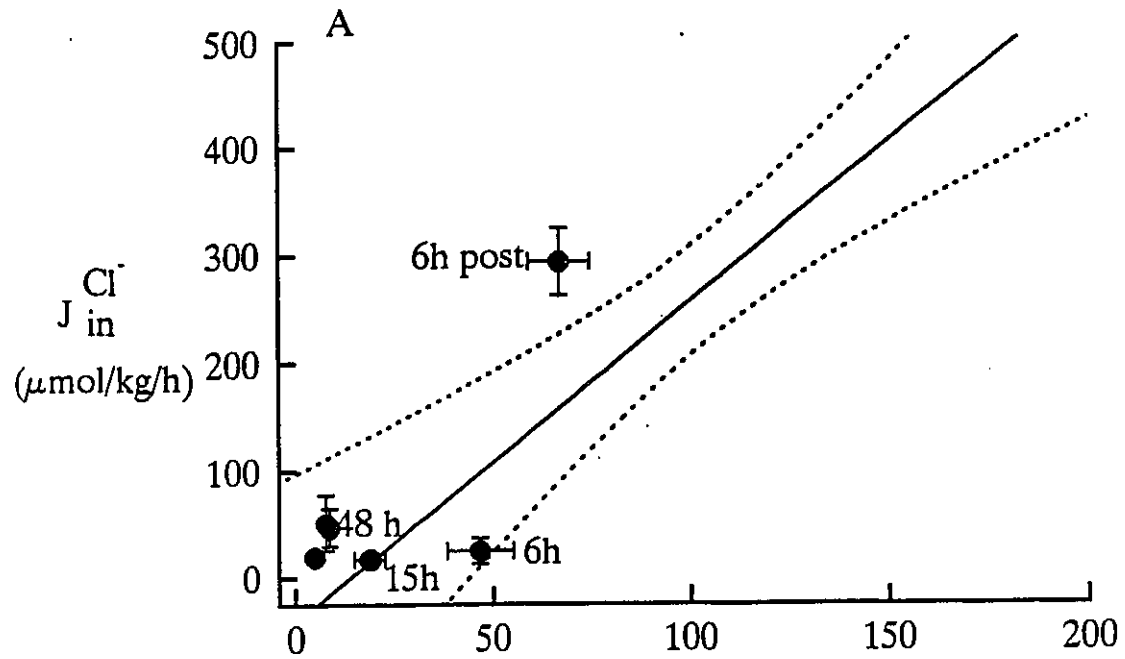


conditions (Laurent and Perry, 1990). The mean values from the hypercapnic and post-hypercapnic fish were then plotted on the appropriate graphs to enable comparisons with the influx rates predicted from the correlation analysis. Both $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$ correlated significantly with CC fractional area under control (normocapnic) conditions. During hypercapnia, $J_{in}^{Cl^-}$ at each period fell within the 95% confidence limits suggesting that changes in CC fractional area could account entirely for the changes in $J_{in}^{Cl^-}$ at this time. In the recovery period, $J_{in}^{Cl^-}$ was slightly higher than predicted from the CC fractional area, possibly owing to increased internal substrate (HCO_3^-) availability for the Cl^-/HCO_3^- exchanger (Wood and Goss 1990; Goss and Wood 1991). On the other hand, during the initial 27 h of hypercapnia, $J_{in}^{Na^+}$ was consistently higher than predicted by the regression between CC fractional area and $J_{in}^{Na^+}$ (Fig 2.10b).

FIGURE 2.10

Correlation (\pm 95% confidence limits) between whole body influx of (A) chloride ($J_{in}^{Cl^-}$) and (B) sodium ($J_{in}^{Na^+}$) with gill filament CC fractional area in control and cortisol-treated bullheads during normocapnia. Filled circles (●) represent the measured mean values (\pm 1 SE) during environmental hypercapnia and the subsequent 6 h post-hypercapnic recovery (n=6 at each point). Where not shown, the SE lies within the data point.

Fig 2.10a, $y=0.0059x - 37$, $r = 0.800$; Fig 2.10b, $y = 0.0032x - 42$, $r = 0.797$.



DISCUSSION

This is the first study to examine in detail the morphological responses of the fish gill epithelium to acid-base disturbances and relate them to the compensatory changes in branchial ionic fluxes. The results show that during hypercapnic acidosis, there is a net Cl^- loss and Na^+ gain while during the post-hypercapnic period, there is a net Cl^- gain. These are appropriate responses to compensate the acid-base disturbances. The changes in the net fluxes occurred mainly through alterations in the influx component to ion balance. These findings are in general agreement with the results of other studies on the physiological responses of freshwater fish to hypercapnia of external or internal origin (Cameron and Iwama 1987; Perry *et al.*, 1987a, b; Wood 1988; Goss and Wood 1990a, b).

Cameron and Iwama (1987), in a study on progressively hypercapnic channel catfish (*Ictalurus punctatus*), suggested that a relationship may exist between gill morphology and acid-base regulation. This proposal was based on the observation that during hypercapnic acidosis, CCs appeared to proliferate on the filament epithelium. In contrast, we observed a profound reduction in CC fractional area during hypercapnic acidosis and a reappearance in the post-hypercapnic recovery period. The reasons for the difference in results between the present study and those of Cameron and Iwama (1987) are unknown. However, the combination of quantitative morphometric SEM analysis and the support of TEM analysis strengthens the results of this particular study. The results are consistent with those of Goss and Wood (1990a, b) who found that increases/decreases in the uptake of Na^+ and Cl^- during hyperoxia, hypercapnia or intravascular base infusion were mainly the result of alterations in the maximal transport rate. Theoretically, increases in the maximal transport rate can occur either by an increase in the numbers of transport sites present (Shaw 1959; Maetz 1974) or through an increase in the substrate availability and the cycling rate of the transporter (Wood and Goss 1990; Goss and Wood

1991). Alteration of the gill epithelium provides a simple mechanism for the regulation of the number of transport sites available.

The results of this study suggest that an important mechanism utilized in the regulation of hypercapnic acidosis and post-hypercapnic alkalosis is the modulation of the $\text{Cl}^-/\text{HCO}_3^-$ exchange system. Morphological modification of the gill epithelium would allow for direct regulation of a CC-associated $\text{Cl}^-/\text{HCO}_3^-$ exchanger by controlling its availability to the external water and hence, its function. This regulation occurs by rapid covering of CCs by adjacent PVCs and a subsequent reduction in CC function during acidosis. During alkalosis, the CCs re-emerge and reassume the ultrastructural characteristics of active cells. The rapid covering/retractions of the CCs by adjacent PVCs have been shown to occur during acute freshwater-seawater transfer in the young adult lamprey, *Geotria australis*, and was suggested as a mechanism of ion regulation at such times (Bartels and Potter, 1991).

The results of the present study may explain an apparent paradox generated by Cameron and Iwama's (1987) suggestion of structural alteration as a means of acid-base regulation. If the uptake of both Na^+ and Cl^- occur on the CC, then how can morphological alteration of one cell type account for the bi-directional regulation of the two ion-transporting mechanisms during an acid-base disturbance? This would involve greatly increasing the activity of the Na^+ uptake/acid excretion at a time when CC fractional area was severely (95%) reduced. The present paper suggests the presence of a CC-associated $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism but does not support a relationship between the transitory increase in the function of the Na^+ /acid excretion mechanism and changes associated with the CCs during environmental hypercapnia. Of note, however, is the homogeneous nature of the response of the CC to hypercapnic acidosis. In these animals, the external morphology (SEM) did not display the heterogeneity noticed in the

gills of salmonids (Laurent *et al*, 1985; Franklin and Davison, 1989; Franklin, 1990; Laurent and Perry, 1990; Perry *et al*, 1992a, b). This homogeneity of surface ultrastructure and response to hypercapnia would suggest a single population of CCs in the brown bullhead catfish.

Keys and Wilmer (1932) first described the "chloride secreting cells" as the cells responsible for salt secretions in seawater teleost fish. However, the role for the CC in freshwater osmoregulation was not shown until much later (Laurent and Dunel 1980; Laurent *et al*, 1985; Avella *et al*, 1987; Laurent and Hebibi 1989; Laurent and Perry 1990). Recently, Perry and Laurent (1989) demonstrated that trout exposed to water containing lower levels of NaCl (~0.015 mmol/l) compared to normal levels of NaCl (~0.14 mmol/l) for a period of 30 days showed an increase in both the rates of Na⁺ and Cl⁻ uptake and gill filament CC area, simultaneously. Laurent and Perry (1990), using cortisol-treated fish showed direct correlations between CC fractional area and the uptake of both Na⁺ and Cl⁻. However, these observations were under steady-state conditions when no acid-base disturbance was present. According to McDonald *et al* (1989a), the net flux of Na⁺ must equal the net flux of Cl⁻ when no acid-base disturbance is present in order to obey the constraints of electroneutrality (Stewart 1983; Cameron 1989). Therefore, it is possible that the relationship of Na⁺ uptake with CC fractional area could be coincidental under steady-state conditions, the result of the necessity to maintain equivalent net fluxes of strong anions and strong cations (McDonald *et al*, 1989a).

In many studies on acid-base regulation during respiratory acidosis (hyperoxia: Wood *et al*, 1984; Goss and Wood 1990a; hypercapnia: Perry *et al*, 1987a) and in this study, $J_{in}^{Na^+}$ returned to lower levels after compensation from acidosis than those seen under control conditions. These reductions in $J_{in}^{Na^+}$ were tentatively ascribed to either a reduction in the perfusive and ventilatory characteristics (Wood *et al*, 1984; Goss and

Wood 1990a) or used as evidence for the lack of importance of the Na^+ uptake/acid excretion mechanism in compensation of the acidosis (Perry *et al*, 1987a). If the CCs are covered as a means of restricting the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism and hence, base excretion, and both the covering of the CCs and reduction of $J_{\text{in}}^{\text{Cl}^-}$ are maintained throughout the experimental regime, then $J_{\text{net}}^{\text{Na}^+}$ must be concomitantly reduced to match $J_{\text{net}}^{\text{Cl}^-}$ (McDonald *et al*, 1989a). This usually occurs through changes in both the influx and efflux components of ion balance (Goss and Wood 1990a, b) and explains the general reduction in the magnitude of the ion fluxes after compensation from a respiratory acidosis.

Although alteration of $J_{\text{in}}^{\text{Cl}^-}$ was a major mechanism in compensating the hypercapnic acidosis, there was still a substantial role for alteration of $J_{\text{in}}^{\text{Na}^+}$ and the subsequent acid efflux as a mechanism for pH compensation. The elevation of Na^+ uptake in the present study would result in the net excretion of acidic equivalents through either a coupled Na^+/H^+ exchange as originally proposed by Krogh (1938) or through a H^+ pump/ Na^+ channel type mechanism as proposed by Avella and Bornancin (1989). This thesis does not attempt to determine the mechanism of Na^+ uptake and H^+ excretion in freshwater fish but does provide clues as to the possible location of these transport systems. The lack of correspondence between $J_{\text{in}}^{\text{Na}^+}$ and CC fractional area during acidosis strongly suggests that the Na^+ uptake mechanism and the corresponding increased acid excretion is not CC related (at least not during hypercapnia).

This thesis presents evidence that the PVC undergoes morphological change during acidosis and alkalosis induced by exposure to environmental hypercapnia and its subsequent removal. The increase in PVC microvilli density noted during hypercapnia may correspond to an increase in the net acid excretion at this time. PVCs develop a structure that is more polarized toward the external water, and there are more

mitochondria and a more extensive Golgi present in PVCs of fish exposed to hypercapnia. These changes in the ultrastructural features suggest an increase in the activity of the PVCs possibly indicating a linkage between the Na^+ uptake and acid excretion mechanism and the PVC during hypercapnic acidosis.

There is little evidence in the literature to support the contention that the fish PVC is responsible for Na^+ uptake. However, if one accepts that the fish gill has similar physiological characteristics to other epithelia with analogous functions (*i.e.* those having the apical membrane exposed to a dilute medium such as frog skin or turtle urinary bladder), then there is evidence to support the proposition that Na^+ uptake occurs *via* the PVC (see Introduction). In frog skin, the granular cells (analogous to the PVC) have Na^+ channels on their apical (water-facing) membranes (Lindemann and Van Driessche 1978) and Na^+/K^+ -ATPase and K^+ channels on their basolateral membranes (Kristensen and Ussing, 1985). These characteristics are necessary for transepithelial Na^+ transport and indicate that the granular cell plays a major role in the uptake of Na^+ from the water and hence, acid-base regulation in frog skin (see review by Ehrenfeld *et al.*, 1990). From these studies, the similarities that exist in structure and function between the fish gill and these other epithelia, and the results of the present study, we suggest that the site responsible for increased Na^+ uptake during hypercapnic acidosis in fish is the PVC.

Although there were similarities between $J_{\text{in}}^{\text{Na}^+}$ and PVC microvilli density during the hypercapnia regimen, the correspondence was not ideal. After the initial hypercapnic acidosis, when $J_{\text{in}}^{\text{Na}^+}$ and PVC microvilli density were simultaneously elevated, $J_{\text{in}}^{\text{Na}^+}$ began to decrease while PVC microvilli density remained high. There are several possible explanations for this result, all of which are highly speculative. Since one mechanism for acid-base regulation, the covering of CCs and the subsequent near total inhibition of $\text{Cl}^-/\text{HCO}_3^-$ exchange, has already been fully engaged to combat the initial acid-base stimulus,

then the maintenance of secondary acid excreting mechanisms might be required in the event of a future, further acidosis. Therefore, the acid excreting mechanisms are retained even though the acidosis has been compensated. Another possibility is that although PVC microvilli density is a good general indicator of PVC activity, it is not the only characteristic that defines the activity of the PVC. There may also be additional mechanisms acting to regulate Na^+ uptake/acid excretion. These very complex problems should form the basis for future research into the relationships between Na^+ uptake and gill morphology.

The present study strongly supports the existence of a chloride cell-associated $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism in freshwater teleosts that can be regulated through morphological modification of the gill epithelium. Physical covering or uncovering of the CCs regulates the availability of the transporters and hence, the rate of base excretion. The data presented here do not support the existence of a chloride cell-associated Na^+ uptake mechanism during hypercapnia. Instead, we suggest that the pavement cell is responsible for the uptake of Na^+ and the excretion of H^+ during hypercapnic acidosis.

CHAPTER 3

**ROLE OF THE CHLORIDE CELL IN COMPENSATION FROM HYPERCAPNIC
ACIDOSIS IN BROWN BULLHEAD CATFISH (*ICTALURUS NEBULOSUS*).**

INTRODUCTION

The results of Chapter 2 indicate that morphological regulation of the gill epithelium is utilized by brown bullhead catfish to regulate the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism during acid-base disturbances. Specifically, acid-base compensation during hypercapnic acidosis was accomplished, at least in part, by a reduction in CC fractional area and a corresponding reduction in $J_{\text{in}}^{\text{Cl}^-}$. This would act to reduce the amount of base (HCO_3^-) excreted and correct the acidosis. These findings are in contrast to those of Cameron and Iwama (1987) who reported increased numbers of "apical pits", and therefore, CCs on the gill epithelium of channel catfish (*Ictalurus punctatus*) in response to hypercapnia. These authors suggested that increases in the CC might be related to an increased net H^+ transport. Therefore, the goal of the present Chapter is to resolve this discrepancy in the literature. The experimental approach was to assess the physiological and morphological responses of the gill epithelium to hypercapnia in groups of brown bullhead catfish displaying widely different gill chloride cell morphologies. This was achieved by using daily administration of exogenous cortisol as a tool to increase the numbers and surface area of CCs exposed on the gill epithelium (Doyle and Epstein, 1972; Perry and Wood, 1985; Laurent and Perry, 1990). If the CC is, indeed, involved in H^+ secretion, then an increased CC surface area would presumably impart an increased ability to compensate hypercapnic acidosis and the CCs present initially would be retained or even increased during the acidosis. Alternately, if the CCs are indeed uncoupled from the water during hypercapnia as a mechanism to reduce $\text{Cl}^-/\text{HCO}_3^-$ exchange and thus indirectly, raise H^+ excretion, then an increase in CC surface area would not aid in the compensation from hypercapnic acidosis.

The results of Chapter 2 showed that the fish gill pavement cell (PVC) undergoes morphological changes that appeared to be related to the acid-base status of the fish.

During hypercapnia, PVCs showed an increased surface microvilli density and ultrastructural modifications consistent with an increase in cellular activity. It was suggested that these alterations might be associated with an increased acid excretion and Na^+ uptake to help compensate the hypercapnic acidosis. The second goal, therefore, was to assess the effect of cortisol pre-treatment on PVC morphology under control conditions and during hypercapnia-induced acidosis and to relate these alterations to changes in Na^+ uptake.

METHODS

Experimental Animals

Brown bullhead catfish (*Ictalurus nebulosus*) of either sex [mean weight = 187 ± 10 g (standard error (SE), $n = 24$)] were obtained from ponds in the Haute-Marne region of France and kept as described in Chapter 2.

Experimental Protocol

Catfish were separated into two groups, one group ($n=12$) served as controls. A second group ($n=12$) were given daily intramuscular injections of cortisol [0.1 ml: 4 mg/kg/per day of hydrocortisone 21-hemisuccinate (Sigma) for 10 days] to elevate the gill epithelial chloride cell (CC) fractional area (CCFA) and the influx rates for both Na^+ and Cl^- (see Laurent and Perry, 1990). This treatment was utilized only as a tool to cause variations in the rates of ionic (Na^+ , Cl^-) uptake and CCFA. It was not meant to assess the role of cortisol in acid-base regulation in freshwater fish. Therefore, untreated fish rather than NaCl-injected fish were used as controls. However, it was shown previously in our lab (unpublished observations) that an identical protocol using sham (0.9% NaCl) injections did not result in an increase in CCFA in either trout, catfish or eels.

Catfish were placed into individual opaque acrylic boxes and allowed to recover for 36 h prior to experimentation. At the start of the experiment, the flux boxes (volume ~ 2 l) were flushed with water prepared by the method of Goss and Wood (1990a) which had reduced levels of titratable alkalinity compared to normal Strasbourg tap water (see Table 2.1). This protocol was utilized in an attempt to increase the precision of the measurement of titratable alkalinity for the estimation of whole body net acidic equivalent flux. Unfortunately, reliable measurements could not be obtained and thus, are not presented. The reduction in water $[\text{Ca}^{2+}]$ from 4.48 mmol/l to 2.22 mmol/l would have negligible effects on gill ionic permeability (Potts, 1984; McDonald and Rogano, 1986).

After the flushing, flow to the boxes was stopped and $^{36}\text{Cl}^-$ (1.0 μCi) was added from a 1 mol/l NaCl stock solution to yield final Na^+ and Cl^- levels equivalent to those in Strasbourg tap water (Table 2.1). After an initial mixing time of 0.5 h, water samples (40 ml) were withdrawn from the box at the beginning of the flux period and 3 h later. The boxes were again thoroughly flushed with prepared water and the procedure was repeated using $^{22}\text{Na}^+$. The water samples were analyzed for $[\text{Na}^+]$ or $[\text{Cl}^-]$ and $^{22}\text{Na}^+$ or $^{36}\text{Cl}^-$ activity, whichever was appropriate. $J_{\text{in}}^{\text{Na}^+}$ and $J_{\text{in}}^{\text{Cl}^-}$ were monitored by the disappearance of either $^{22}\text{Na}^+$ or $^{36}\text{Cl}^-$ from the water over the 3 h period. Catfish were then exposed to either 6 h of hypercapnia [$P_{\text{wCO}_2} = 2\%$ (15 torr)] or to normocapnic conditions in the control experiments. Physiological and morphological measurements were carried out as outlined in Chapter 1. At the end of the experimental period, fish were removed from the boxes and killed by spinal section. The gills were then excised and fixed for microscopic analysis as described in Chapter 2. The 3- and 6- h hypercapnic periods were repeated using an inverse addition order of isotope to estimate $J_{\text{in}}^{\text{Na}^+}$, $J_{\text{in}}^{\text{Cl}^-}$ at the 0-3 h hypercapnia time period and $J_{\text{in}}^{\text{Cl}^-}$, $J_{\text{in}}^{\text{Na}^+}$ at the 3-6 h hypercapnia periods, respectively.

Analytical procedures

Water $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ activities, $[\text{Na}^+]$, and $[\text{Cl}^-]$ were determined as described in Chapter 2. Unidirectional influxes (J_{in}) and net fluxes (J_{net}) were determined as described by Wood (1988). Morphological methods were performed as outlined in Chapter 2.

Statistical analyses

All values are presented as means \pm 1 SE. Results were analyzed by two-way ANOVA followed by Student Neuman-Keuls tests for multiple comparisons. 5% was accepted as the fiducial limit of significance in all cases.

RESULTS

A) Chloride cell morphology

The effects of cortisol treatment on CC morphological variables (CC area, CC density, CC fractional area) are illustrated in Fig 3.1. Treatment with cortisol caused a large increase (170%) in the CC fractional area on the filamental epithelium from $48431 \pm 7699 \mu\text{m}^2/\text{mm}^2$ to $130889 \pm 15214 \mu\text{m}^2/\text{mm}^2$. This increase was caused entirely by an increase in the area of individual CCs (from 10 to $27 \mu\text{m}^2$) in addition to a small, but significant, decrease in the density of CCs that displayed apical membrane exposed to the water (Fig 3.1). During hypercapnia, there were large decreases in CC fractional area in both the untreated and the cortisol-treated groups, reaching the same level by 6 h (23262 ± 4248 and $29241 \pm 4590 \mu\text{m}^2/\text{mm}^2$ in untreated and cortisol-treated groups, respectively). These changes in CC fractional area were caused solely by changes in CC area (Fig 3.1A) with no change in density noted in either group (Fig 3.1B).

Representative SEM micrographs from untreated pre-hypercapnic fish and cortisol-treated pre-hypercapnic fish are shown in Figs 3.2a, c, respectively. These micrographs show that the cortisol-treated fish displayed CCs with larger surface areas (Fig 3.2c) than in the untreated fish (Fig 3.2a). After 6 h of hypercapnia, the CCs in both groups showed a marked reduction in the average area exposed to the external environment. In addition, the CCs appeared to be recessed into the filamental epithelium suggesting some sort of functional alteration at this time (Figs 3.2b, d). The reductions in CC area and hence, CC fractional area, are clearly evident in these micrographs. Representative TEM micrographs from an untreated pre-hypercapnic fish and a cortisol-treated pre-hypercapnic fish are shown in Figs 3.3a, c, respectively. Ultrastructurally, CCs from the cortisol-treated fish (Fig 3.3C) possessed similar characteristics to the untreated fish (Fig 3.3A).

FIGURE 3.1

Chloride cell (CC) morphometry including (A) area of individual CCs (B) CC density, and (C) CC fractional area in untreated (clear histograms) and cortisol-treated (shaded histograms) catfish during normocapnia and after 6 h hypercapnia. + indicates significant difference between untreated and cortisol-treated catfish. * indicates significant difference from the normocapnic value within each group. ($P < 0.05$, $n = 6$)

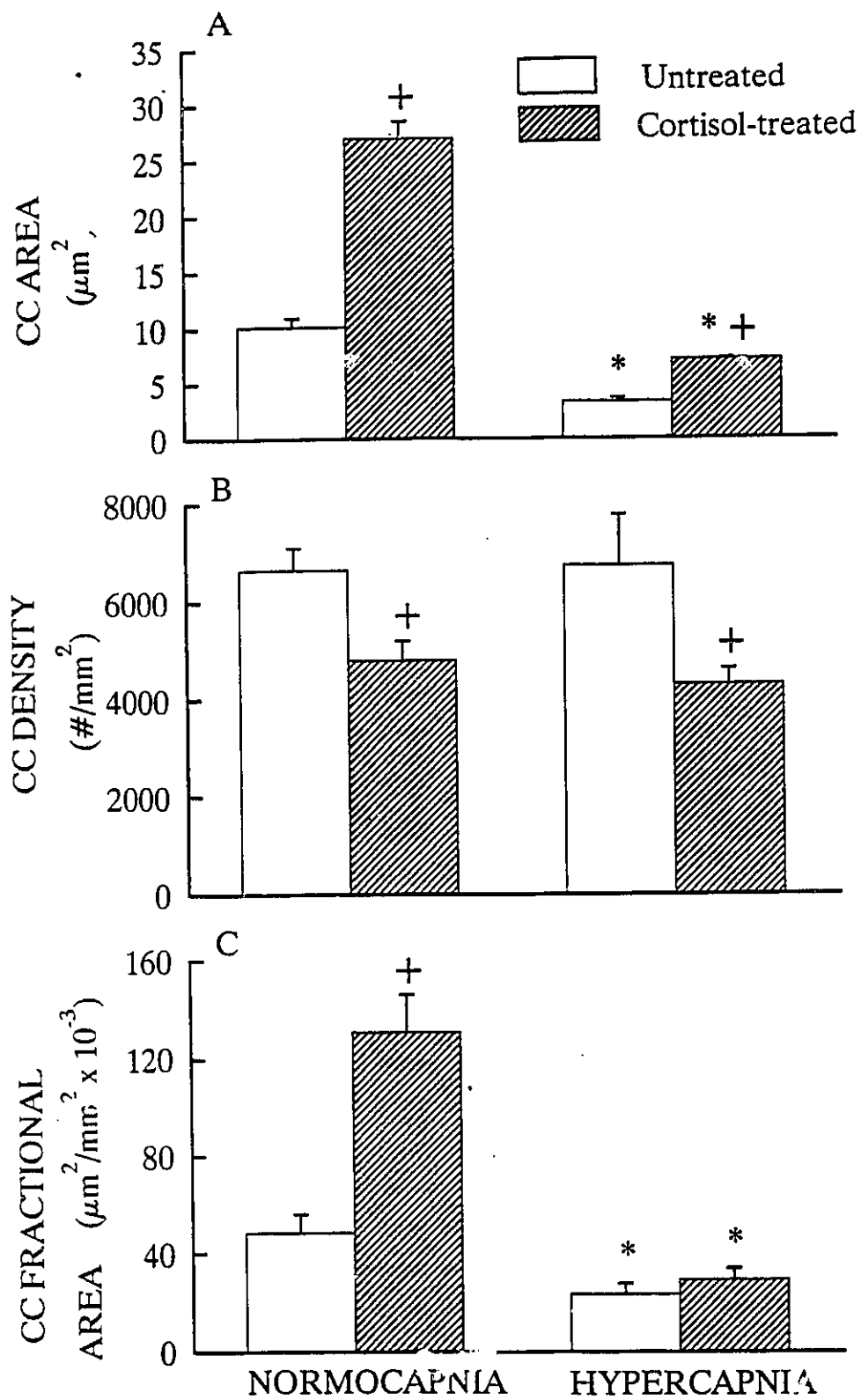


FIGURE 3.2

Representative low magnification SEM photographs of the filamental epithelium (interlamellar area) of untreated and cortisol-treated catfish under normocapnic conditions (Figs. 3.2a; $J_{in}^{Cl^-} = 317 \mu\text{mol/kg/h}$) and after 6 h of exposure to environmental hypercapnia (Figs. 3.2b; $J_{in}^{Cl^-} = 21.4 \mu\text{mol/kg/h}$) are shown in the top panels. Note the reduction in the CC apical surface area and change in the appearance of the CCs (compare cells indicated by black arrowheads). Representative SEM photographs of cortisol-treated fish under normocapnic conditions (Fig 3.2c; $J_{in}^{Cl^-} = 585 \mu\text{mol/kg/h}$) and after 6 h of hypercapnia (Figs 3.2d; $J_{in}^{Cl^-} = 45.4 \mu\text{mol/kg/h}$) are shown in the bottom panels. Note the huge increase in size of the CC apical surface area (white arrows) and its spongy organization under normocapnic conditions (Fig 3.3c). After 6 h of hypercapnia cortisol treated fish showed a large reduction in the CC apical surface area similar to that found in the untreated group. Scale bar = 20 μm .

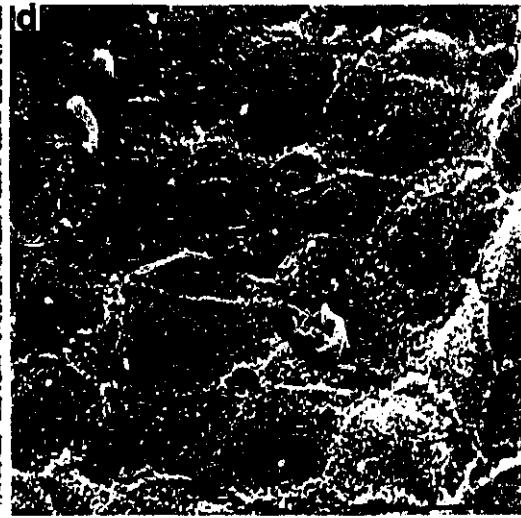
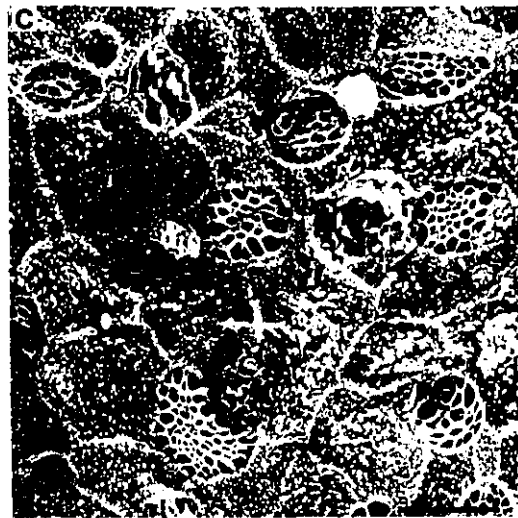
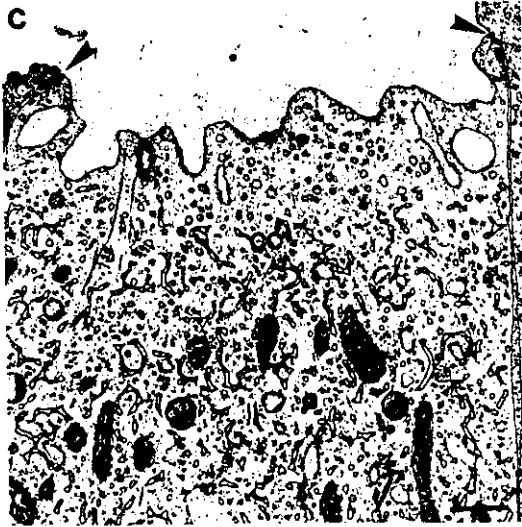
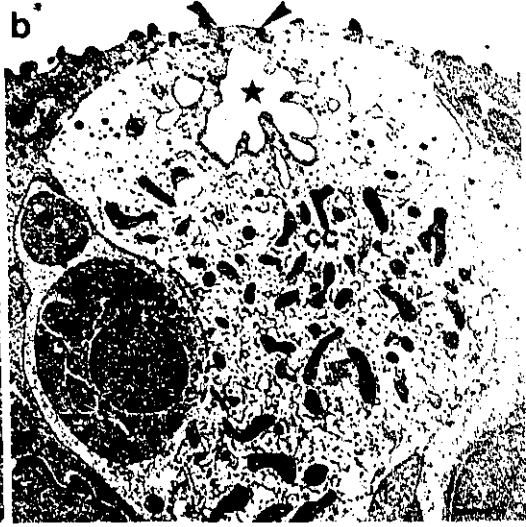


FIGURE 3.3

Representative high magnification TEM photographs of chloride cells from the filamental epithelium (interlamellar area) of untreated and cortisol-treated catfish under normocapnic conditions (Figs. 3.2a; $J_{in}^{Cl^-} = 317 \mu\text{mol/kg/h}$) and after 6 h of exposure to environmental hypercapnia (Figs. 3.2b; $J_{in}^{Cl^-} = 21.4 \mu\text{mol/kg/h}$) are shown in the top panels. Cortisol treatment (Fig 3.2c) markedly increased the CC apical surface area associated with an elevated rate of chloride uptake ($J_{in}^{Cl^-} = 612.4 \mu\text{mol/kg/h}$). After 6 h hypercapnia, the apical surface of the chloride cell was reduced in both treatment groups combined with a reduction in chloride uptake (Fig 3.3b $J_{in}^{Cl^-} = 20.1 \mu\text{mol/kg/h}$; Fig 3.3d $J_{in}^{Cl^-} = 27 \mu\text{mol/kg/h}$). In cortisol-treated fish (Fig 3.3d), note the appearance of membrane processes (arrows) separating the outer portion of the CC from the inner portion showing normal ultrastructural organization. Black arrowheads indicate cell-cell junctions. Bar = 1 μm .



Under normocapnic conditions, the CCs of cortisol-treated fish showed a large apical surface area (Fig 3.3c) compared to the untreated fish (Fig 3.3a) and this corresponded with the large increase in $J_{in}^{Cl^-}$ measured at this time (see below). After 6 h of hypercapnia, the apical surface area of CCs in the cortisol-treated fish was markedly reduced in a similar manner to that seen in the untreated fish. Figure 3.3b shows a CC from an untreated fish that is characterized by a reduced apical exposure corresponding to the physiological adjustments noted (see below). Fig 3.3d shows a CC from a cortisol-treated fish with an apical area having few organelles; the basal portion of the cell retained a typical appearance suggesting rapid removal of the organelles from the apical portion of the cell, perhaps as a means of reducing the transport capacity of the cell.

B) Pavement cell morphology

Under normocapnic conditions, cortisol treatment significantly increased the filamental PVC microvilli density from 41 ± 2.3 to 51 ± 2.5 intercepts/grid (Fig 3.4). After 6 h of hypercapnia, microvilli density significantly increased in both the untreated (62 ± 2.5 intercepts/grid) and the cortisol-treated (76 ± 2.2 intercepts/grid) groups (Fig 3.4). Representative SEM micrographs of the gill filament in untreated and cortisol-treated fish before and after exposure to hypercapnia are shown in Fig 3.5. Under normocapnic conditions there were more PVC microvilli present on the filament in cortisol-treated fish (Fig 3.5c) compared to untreated fish (Fig 3.5a). Ultrastructurally, the PVCs in untreated and cortisol-treated normocapnic fish were characterized by sparse mitochondria and relatively few microvilli on the apical membrane (Fig 3.6a, c). In both treatment groups, exposure to hypercapnia resulted in PVCs with greater numbers of mitochondria, especially in the sub-apical regions of the cell (Fig 3.6b, d). In both groups, the apical membrane was ornamented with dense arrays of microvilli after 6 h exposure to

FIGURE 3.4

The effects of hypercapnia on the surface microvilli density of pavement cells (PVC) in untreated (open histograms) and cortisol-treated fish (shaded histograms). + indicates significant difference between untreated and cortisol-treated catfish. * Indicates significant difference from the normocapnic value within each group. ($P < 0.05$, $n = 6$). See text for further details.

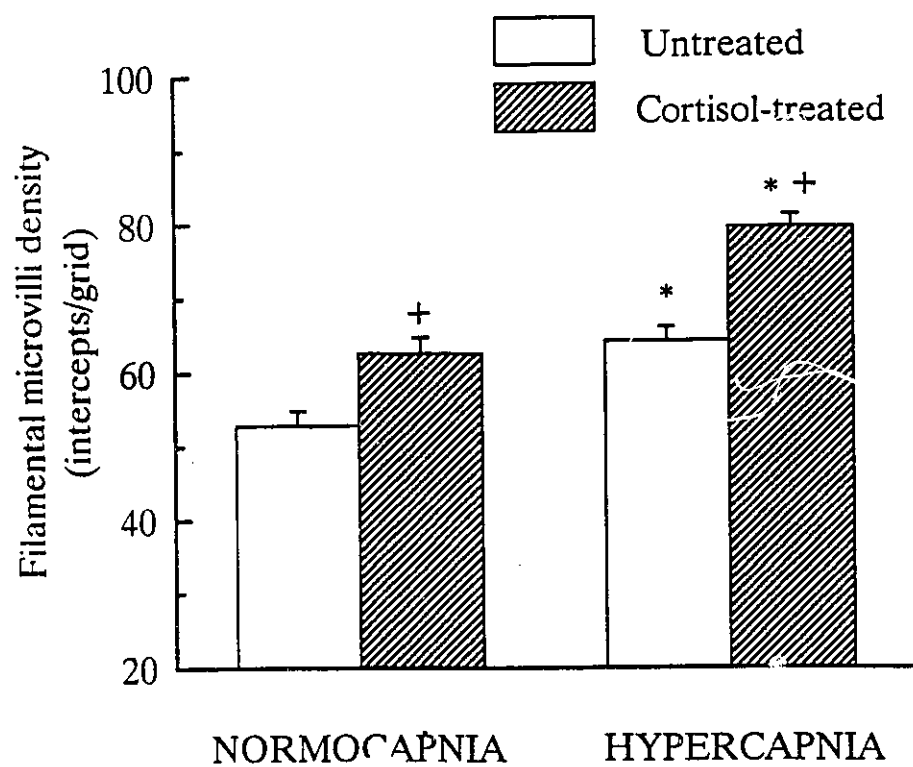


FIGURE 3.5

Representative SEM photographs from untreated (Fig 3.6 a, b) and cortisol-treated (Fig 3.6c, d) fish under normocapnic conditions (Fig 3.6a, c) and after 6 h exposure to hypercapnia (Fig 3.6 b, d). Note that cortisol treatment increased PVC microvilli density under normocapnic conditions (Fig 3.6c) while after 6 h of hypercapnic exposure, there was an increase in PVC microvilli density in both treatment groups (Fig 3.6b, d). Bar = 2 μ m.

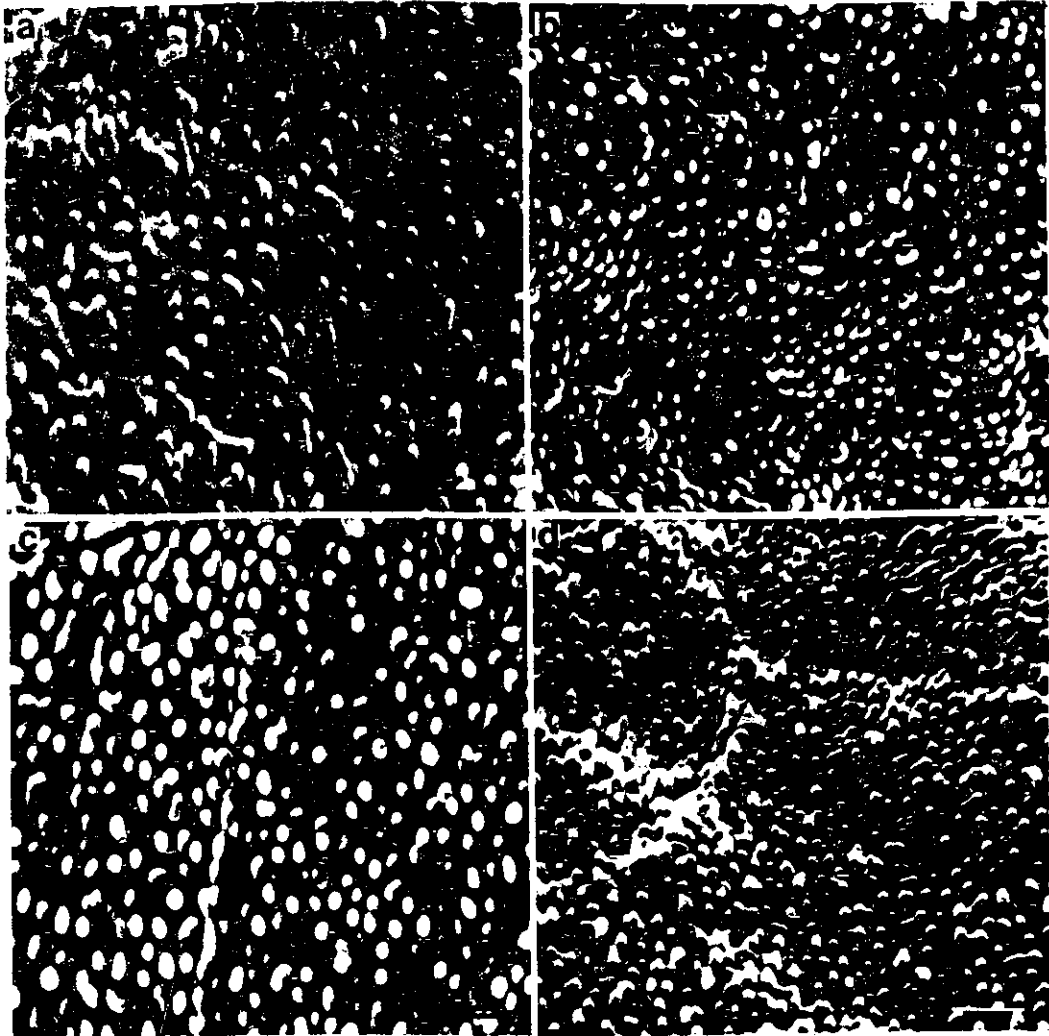
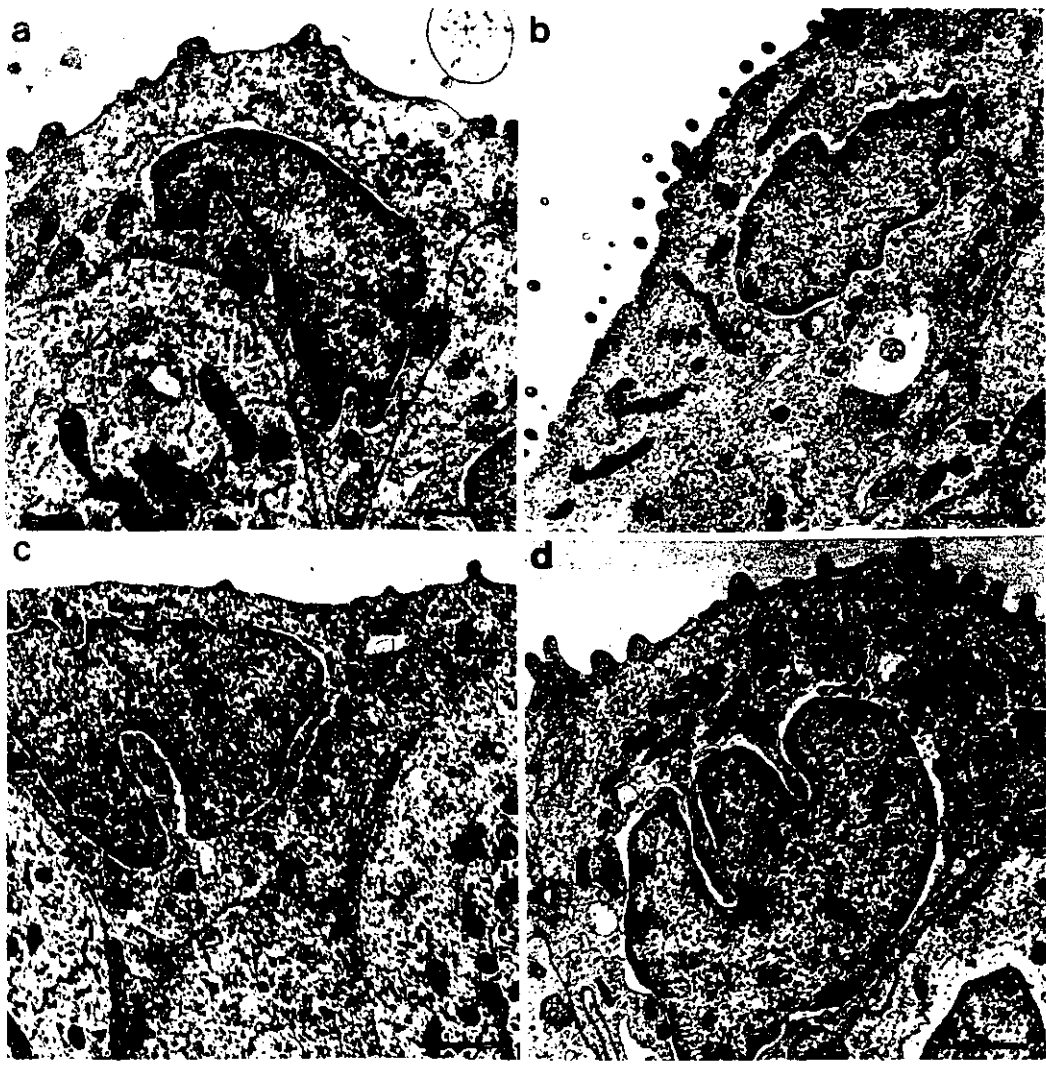


FIGURE 3.6

Representative high magnification TEM photographs of PVC ultrastructure from untreated (Fig 3.6 a, b) and cortisol-treated (Fig 3.6c, d) fish under normocapnic conditions (Fig 3.6a, c) and after 6 h exposure to hypercapnia (Fig 3.6 b, d). Under normocapnic conditions in both untreated and cortisol-treated fish, mitochondria were scarce and did not display any particular localization. In addition, the PVCs possessed few apical villi. In both treatment groups, hypercapnia caused an increase in apical villi and mitochondria that appeared to be localized to the sub-apical regions of the cell. Bar = 2 μm .



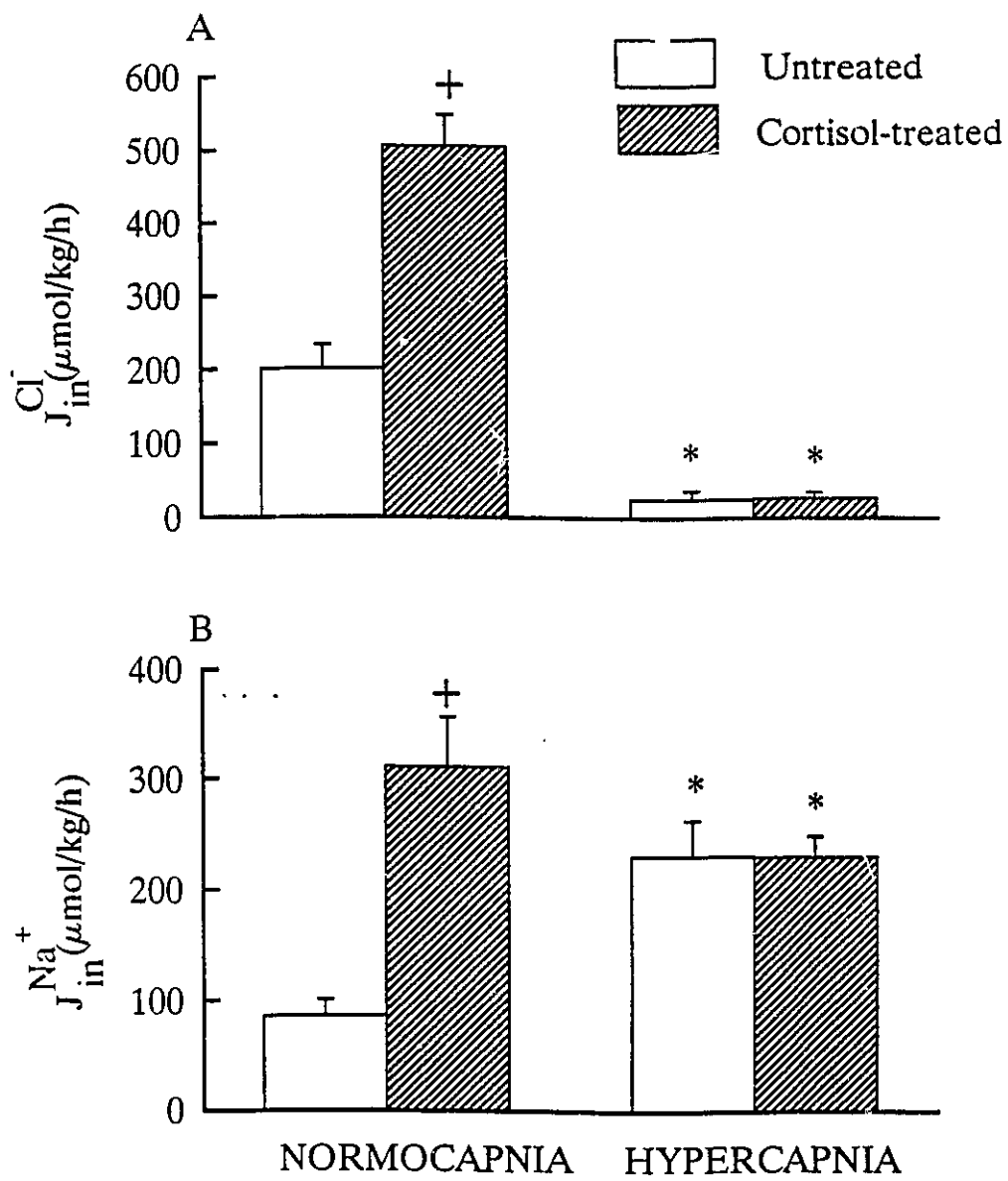
hypercapnia (Fig 3.5b, d; Fig 3.6b, d).

C) Physiology

The effects of cortisol on $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$ are shown in Fig 3.7. Under normocapnic conditions (Pre), cortisol caused large increases in the influx rates for both Cl^- and Na^+ . $J_{in}^{Cl^-}$ was increased from 201 ± 32 to 505 ± 42 $\mu\text{mol/kg/h}$ while $J_{in}^{Na^+}$ was increased from 86 ± 14 to 311 ± 45 $\mu\text{mol/kg/h}$. After 6 h of exposure to hypercapnia, $J_{in}^{Cl^-}$ was reduced in both the untreated and the cortisol-treated groups to the same level (25 and 27 $\mu\text{mol/kg/h}$, respectively). $J_{in}^{Na^+}$, on the other hand, was increased in the untreated group from 86 $\mu\text{mol/kg/h}$ to 230 $\mu\text{mol/kg/h}$ while in the cortisol-treated group, $J_{in}^{Na^+}$ was decreased from 310 $\mu\text{mol/kg/h}$ to 230 $\mu\text{mol/kg/h}$ (Fig 3.7).

FIGURE 3.7

Whole body influx of (A) chloride ($J_{in}^{Cl^-}$) and (B) sodium ($J_{in}^{Na^+}$) in untreated (clear histograms) and cortisol-treated (shaded histograms) catfish during normocapnia and after 6 h hypercapnia. + indicates significant difference between untreated and cortisol-treated catfish. * indicates significant difference from the normocapnic value within each group. ($P < 0.05$, $n = 6$).



DISCUSSION

The effects of long-term treatment of fish with cortisol on ion fluxes and gill morphology under normocapnic conditions have been demonstrated previously (Perry and Wood, 1985; Laurent and Perry, 1990; Perry *et al*, 1992a, b) and the results of the present study confirm their effects for bullhead catfish. Daily intramuscular injections of cortisol for 10 days caused a significant increase in CCFA and simultaneous increases in both $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$. Laurent and Perry (1990) reported a similar relationship between gill ionic uptake rates and the fractional area of CCs on the gill filament epithelium of trout and consequently concluded that the CC is an important site of both Na^+ and Cl^- uptake. However, their study was not performed during an acid-base disturbance. Under normocapnic conditions where acid-base status is unchanging, there is an obligate relationship between the Na^+ uptake and Cl^- uptake (as long as the ratio between the efflux of these two ions remains constant) owing to the relationship between net strong ion difference fluxes (approximated by $J_{net}^{Cl^-} - J_{net}^{Na^+}$) and blood acid-base status (Stewart, 1978, 1983; McDonald *et al*, 1989a; see General Introduction). Thus, the stimulatory effect of cortisol on both $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$ is expected even if the CC is **not** the site of both uptake mechanisms. In other words, under control conditions, the uptake rates of Cl^- and Na^+ must co-vary if blood pH is to remain constant. During acid-base disturbances, however, this relationship does not hold and therefore permits a reliable determination of the relationships between ion uptake and gill morphology. The correspondence between the rates of Cl^- influx and CC surface areas during hypercapnia reinforce the view stated in Chapter 2 that the Cl^- uptake mechanism (Cl^-/HCO_3^- exchange) is probably associated with the CC while the lack of correspondence between Na^+ uptake and CC surface area does not support the view that the Na^+ uptake mechanism (Na^+/H^+ exchange - Na^+ channel/ H^+ pump) is associated with the CC.

The results of this study indicate that the increase in the fractional area of chloride cells (CCFA) and the corresponding direct or indirect increases in both $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$ in the cortisol-treated fish did **not** impart an increased capacity to regulate hypercapnic acidosis based on similar compensatory adjustments of ionic uptake rates. Although there were increases in CCFA, $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$ during normocapnia, after 6 h of hypercapnia, the absolute values of their variables were similar in both the untreated and cortisol-treated groups. During hypercapnia, the CCFA decreased dramatically and corresponded to a greatly decreased $J_{in}^{Cl^-}$ (Cl^-/HCO_3^- exchange). In the control fish, $J_{in}^{Na^+}$ increased concomitantly with the reduction of CCFA during the hypercapnia. In cortisol-treated fish, there was a perplexing decrease in $J_{in}^{Na^+}$ during hypercapnia. The reduction in $J_{in}^{Na^+}$ is inappropriate during hypercapnic acidosis because it would reduce the amount of acid excretion at this time and actually impair the ability of cortisol-treated fish to regulate acid-base status compared to the untreated fish.

Taken together, these results do not support the results or interpretation put forth by Cameron and Iwama (1987) and Cameron (1989) in which an increase in CCs was reported in catfish during exposure to hypercapnia. In the present study, cortisol-injected fish displaying elevated CCFA, $J_{in}^{Cl^-}$ and $J_{in}^{Na^+}$ reached almost exactly the same values after 6 h of hypercapnia as in the untreated fish. The arguments put forward by Cameron and Iwama (1987) were based on the belief that the Na^+ uptake/acid excretion mechanism (Na^+/H^+ exchange - Na^+ channel/ H^+ -ATPase) was located on the CC. These results, and those of Chapter 2, indicate that the CC is not the site of Na^+ uptake (at least during hypercapnia when CCs are removed from the epithelial surface) but that it is the predominant site of Cl^-/HCO_3^- exchange. Assuming that base excretion is accomplished, at least in part, *via* the CC associated Cl^-/HCO_3^- exchanger, then perhaps increases in CCFA and $J_{in}^{Cl^-}$ through cortisol pre-treatment would impart an increased ability to

regulate an alkalosis owing to the requirement of enhanced $\text{Cl}^-/\text{HCO}_3^-$ exchange to excrete base. This hypothesis will be examined in Chapters 6 and 7.

The present study confirms the result of Chapter 2 that demonstrated morphological alteration of the PVCs during an acid-base disturbance. During normocapnia, cortisol treatment caused an increase in the PVC microvilli density. There were further increases in microvilli density in both the untreated and cortisol-treated fish during hypercapnia. These changes were associated with ultrastructural changes in PVCs including more numerous mitochondria that were polarized in the sub-apical regions of the cell, characteristics usually associated with more actively transporting cells (Madsen and Tisher, 1983). These morphological alterations may be associated with alterations in $J_{\text{in}}^{\text{Na}^+}$ during the hypercapnic exposure and suggest that the site of Na^+ uptake is the PVC, at least during hypercapnia when the CC become largely uncoupled from the environment. As was found in Chapter 2, the correspondence between $J_{\text{in}}^{\text{Na}^+}$ and the alterations in PVC structure was not ideal. The possible reasons for lack of correspondence between PVC microvilli density and $J_{\text{in}}^{\text{Na}^+}$ were discussed in Chapter 2 and will not be further elaborated on here.

Although the results of the present study do not indicate an increased ability to compensate hypercapnic acidosis in fish possessing experimentally increased gill CC surface area, further experiments employing a longer exposure period utilizing simultaneous measurements of blood acid-base (arterial pH, blood P_{CO_2} , $[\text{HCO}_3^-]$) variables, ion fluxes and morphological parameters would be helpful to clarify further the effects of alteration in CCFA on acid-base regulation. Nevertheless, this experimental series strongly reinforces the results and interpretation of Chapter 2 that covering of the CCs during hypercapnic acidosis is an important mechanism for the reduction in $J_{\text{in}}^{\text{Cl}^-}$ (*i.e.* $\text{Cl}^-/\text{HCO}_3^-$ exchange) and hence, acid-base regulation in brown bullhead catfish.

CHAPTER 4

PHYSIOLOGICAL AND MORPHOLOGICAL REGULATION OF ACID-BASE STATUS

DURING HYPERCAPNIA IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

INTRODUCTION

The results of Chapter 2 showed that catfish undergo significant alterations in gill epithelial morphology during environmental hypercapnia. Specifically, hypercapnia was associated with a 95% reduction of the surface area of CCs exposed to the water. These alterations mirrored the changes in the rates of Cl^- uptake but did not correspond to alterations in the rates of Na^+ uptake. This suggested that i) the $\text{Cl}^-/\text{HCO}_3^-$ exchange system is associated with the CC while the Na^+/H^+ (alternatively Na^+ channel/ H^+ pump) exchange system is not and ii) an important mechanism underlying the reduction of $\text{Cl}^-/\text{HCO}_3^-$ exchange during hypercapnia is uncoupling of the exchange sites from the water by covering/removal of the CCs. The response noted in Chapter 2 was rapid with the CCs acting as a homogeneous group. However, the possibility exists that this response is a species specific adaptation related to life history and/or lifestyle. The brown bullhead catfish is a benthic species which inhabits ponds and slowly flowing rivers. This environment may be subject to large and rapid fluctuations in the P_{CO_2} , P_{O_2} , and temperature (Truchot, 1987). Change in any of these parameters may cause alterations in the acid-base status of the fish (see General Introduction). Freshwater rainbow trout are a pelagic euryhaline species which inhabit fast flowing rivers and oligotrophic lakes. As a result, they would rarely encounter similar rapid fluctuations in environmental conditions. A primary goal of the present study, therefore, was to determine whether morphological modification of the gill is also utilized by rainbow trout to regulate Cl^- uptake during hypercapnic acidosis. This would aid in determining whether morphological adjustments of the fish gill is a general strategy for acid-base balance or simply a unique feature of the brown bullhead catfish and perhaps other benthic species.

A fundamental technique for understanding the function of transport systems is to analyze their transport kinetics (Shaw, 1959). This involves measuring the transport rate

at a variety of substrate (external ion) concentrations. The resulting relationship yields estimates of the kinetic parameters, affinity ($1/K_m$) and maximal transport rate (J_{max}). This model has successfully described branchial Na^+ and Cl^- transport in several studies on freshwater fish and long term acclimation to their environment (De Renzis and Maetz, 1973; Wood and Randall; 1973; Maetz, 1974; Avella *et al*, 1987; Avella and Bornancin, 1989). Typically, acclimation to ion-poor water results in lowered K_m (increased affinity) and an increased J_{max} for both the Na^+ and Cl^- transporters. Perry and Laurent (1989) showed that acclimation of freshwater rainbow trout to lowered environmental $[NaCl]$ caused increased rates of Cl^- and Na^+ uptake as well as increases in the fractional area of CCs on the gill filamental epithelium. Avella *et al* (1987) have associated alterations in $J_{max}^{Na^+}$ resulting from differing environmental salinity with alterations in the abundance of CCs on the lamellae of rainbow trout and suggested that the increase in $J_{max}^{Na^+}$ was the result of an increase in the number of transport sites available.

Evidence that J_{max} is manipulated by fish to compensate acid-base disturbances was recently provided (Goss and Wood, 1990a, b). These studies showed that during respiratory acidosis induced by hyperoxia, J_{max} for both the Na^+ and Cl^- uptake mechanisms was unaltered and that reductions in the rates of ion uptake noted during this period were accomplished by elevations in K_m (reduced affinity). During the ensuing post-hyperoxic alkalosis, a reduction in Na^+ uptake and elevation in Cl^- uptake were accomplished by reduced $J_{max}^{Na^+}$ and elevated $J_{max}^{Cl^-}$, respectively. Classically, these alterations have been interpreted as an increase/decrease in the number of available sites (Shaw, 1959). These increases/decreases in the number of transport sites may reflect alteration in the gill morphology caused by increases/decreases in CC surface area (Avella *et al*, 1987; Avella and Bornancin, 1990). However, in addition to the number of transport sites, J_{max} may be affected by the availability of internal substrate (see General

Introduction). If the concentration of the internal counter-ions (HCO_3^- , H^+) are limiting the rate of ionic transport as are the external ions (Cl^- , Na^+), then increases/decreases in the internal counter-ion concentration would be expected to directly increase/decrease J_{max} . In light of these variable responses, a second goal of the present study was to examine the relative roles that morphological alteration and alterations in substrate availability play in the compensation from acid-base disturbances.

METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) [mean weight = 236 ± 6 (SE) n=64] were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and held in large (500 l) fibreglass tanks (Living Stream, Toledo, Ohio) supplied with flowing dechlorinated Ottawa tap water ($\text{Na}^+ = 0.15$ mM; $\text{Cl}^- = 0.18$ mM; $\text{Ca}^{2+} = 0.42$ mM, pH=7.70; temp= 5-14°C). All fish were maintained in the lab for a period of three months prior to use in experiments. Fish were fed three times weekly but not in the 7 days prior to experimentation to negate the effect of feeding on ion and acid-base transfers (Wood and Caldwell, 1978). Photoperiod was kept constant at 12h light: 12h dark.

Experimental Protocol

Experimental Water

Analysis of the kinetic parameters (K_m , J_{max}) was performed using a NaCl-free media designed to duplicate the Ca^{2+} , and titratable alkalinity (TA) composition of Ottawa tapwater using the protocol of Goss and Wood (1990a, b). Water Ca^{2+} was maintained at normal levels because of its well known role in regulating membrane permeability and transepithelial potential (TEP) (Potts, 1984; McDonald and Rogano, 1986) and TA was maintained to provide water buffer capacity, HCO_3^- availability, and the correct pH. This NaCl-free water was made by passing dechlorinated Ottawa tapwater through a deionizing cartridge (Culligan Corp.) and then adding back appropriate amounts of CaCO_3 to bring Ca^{2+} to normal levels. Owing to the relative insolubility of CaCO_3 in water at neutral pH, the water was gassed with 100% CO_2 to achieve complete dissolution. The water was then vigorously aerated for at least 12 h to remove the CO_2 . The resulting media had appropriate levels of Ca^{2+} , Mg^{2+} , and TA (titratable alkalinity to fixed endpoint pH= 4.00), but undetectable levels of Na^+ or Cl^- .

Exposure Regime

Fish were placed in opaque acrylic boxes at least 48 h prior to experimentation. The experiments were designed to measure simultaneously the influx kinetics of Na^+ and Cl^- over an increasing external $[\text{NaCl}]$ ($[\text{NaCl}]_e$). At the beginning of the experimental series, the water flow to the boxes was provided by a high surface area gas exchange column that was equilibrated with varying amounts of CO_2 /air mixtures (as described by Perry *et al*, 1987a) to achieve a final $P_w\text{CO}_2$ of 1% (7.5 torr). A $P_w\text{CO}_2$ of 1% was used for trout instead of the 2% used for bullhead catfish (Chapter 2) because the latter level was toxic to trout. However, in both treatments, the degree of disturbance of blood pH is similar. Measurements were then made over several periods during the next 96 h of maintained hypercapnia ($P_w\text{CO}_2 = 7.52 \pm 0.06$ torr, $N=110$ measurements). The periods chosen were: Control (Pre-hypercapnia), 6, 12, 18, 24, 48, and 96 h. At 96 h, the CO_2 was replaced with air, quickly returning $P_w\text{CO}_2$ to normocapnic levels and a further series of measurements were made (102, 108 h = 6, 12 h recovery).

Experimental Series

In each period, flow was stopped and the boxes were flushed with NaCl-free water so that $[\text{NaCl}]_e$ was below $10 \mu\text{mol/l}$; again, pH, Ca^{2+} , and TA remained at normal levels. At this point, NaCl was added to the boxes from a common 1 mol/l NaCl stock solution containing approximately 4.0-12.0 $\mu\text{Ci/ml}$ of $^{24}\text{Na}^+$ (varied due to decay) and 2.0 $\mu\text{Ci/ml}$ $^{36}\text{Cl}^-$. After the stock was added, 10 min was allowed for mixing and then a sample was withdrawn to begin the flux period. After 0.5 h, another sample was taken to end the flux and more stock was added to the box to further increase the water $[\text{NaCl}]_e$. Six flux periods of increasing $[\text{NaCl}]_e$ (nominally 20, 50, 120, 500, 1000, 2000 $\mu\text{mol/l}$) were measured. Each lasted 0.5 h except for the final flux period lasting 0.67 h (to increase count separation). Samples (40 ml) were analyzed for $[\text{Na}^+]_e$, $[\text{Cl}^-]_e$, and total radioactivity

(cpms) ($^{24}\text{Na}^+$ and $^{36}\text{Cl}^-$ cpm combined), water total ammonia (Amm) and titratable alkalinity (TA). Net acidic equivalent flux ($J_{\text{net}}^{\text{H}^+}$) was calculated as the arithmetic sum of the net flux of Amm (J^{Amm}) and the net flux of titratable alkalinity (J^{TA}).

The $^{24}\text{Na}^+$ isotope was allowed to decay for at least 50 half-lives (half-life = 14.96 h) and the sample was recounted assuming that only $^{36}\text{Cl}^-$ cpm (half-life = 300,000 years) remained. The $^{24}\text{Na}^+$ counts were obtained by subtraction and corrected for decay during counting by:

$$A_0 = \frac{A}{e^{(-\ln(2) \cdot t/T(1/2))}}$$

(Wang *et al*, 1975) where A_0 is the value corrected to time t_0 , A is the value obtained by subtraction of the original counts, t is the time elapsed in hours and $T_{1/2}$ is the half life of the $^{24}\text{Na}^+$ (14.96 h).

After the final flux period, the fish were removed from the boxes and a caudal blood sample was taken (300 μl). The plasma was removed after centrifugation (1 min, 10000g) for immediate analysis of plasma total CO_2 content. A portion of the second right gill arch was then excised and prepared for examination in the scanning electron microscope as described in Chapter 2. Each fish was examined for estimates of the average CC area, CC surface density, and the fractional area of CCs exposed using a digitizing table (Numonics 2201) and a morphometry program (Sigmascan, Jandel Scientific) in a similar manner to that described in Chapter 2.

Analytical Techniques and Calculations

Water $[\text{Na}^+]_e$ was measured by flame emission spectroscopy (Varian AA1275) and water $[\text{Cl}^-]_e$ by the mercuric thiocyanate method (Zall *et al*, 1956). Duplicate 5 ml $^{36}\text{Cl}^-$ / $^{24}\text{Na}^+$ water samples were measured directly by scintillation counting (LKB Rackbeta).

Water [Amm] was determined by the salicylate-hypochlorite method (Verdouw *et al*, 1978)

and TA was determined as described by McDonald and Wood (1981). Plasma total CO_2 was determined on 50 μl samples using a CO_2 analyzer (Corning Model 905).

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. The effect of increasing the $[\text{NaCl}]_e$ on the influx of either Na^+ or Cl^- showed distinctive saturation kinetic curves that were characterized by Michaelis-Menten analysis. The curves (not shown) were used to yield estimates of the uptake kinetic parameters (K_m , J_{max} for both Na^+ and Cl^- uptake mechanisms) during the hypercapnic 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.

Statistical Analyses

All values are presented as means \pm 1 SE. Comparisons between multiple means were performed by a one-way analysis of variance (ANOVA), followed by Duncan's new multiple range test to determine individual differences in cases where the F-value of the ANOVA indicated significance. Comparisons with the control value are indicated with an asterisk (*). Comparisons between 96 h and recovery periods are indicated with a plus symbol (+). 5% was accepted as the fiducial limit of significance in all cases.

RESULTS

To ensure that the fish were compensating from the hypercapnic acidosis in a similar manner to that shown in previous studies (e.g. Perry *et al*, 1987a), total CO_2 of the extracellular compartment was measured. Blood pH was not measured in this experiment because it is highly skewed by caudal blood sampling. Although total CO_2 taken by caudal puncture is not an ideal method for assessing the acid-base status of the fish, at physiological pH, >95% of C_{CO_2} is present as HCO_3^- . Therefore, C_{CO_2} does provide a relative measure of the degree of acid-base compensation and the relative levels of HCO_3^- (internal substrate for the $\text{Cl}^-/\text{HCO}_3^-$ exchanger) in the blood. Exposure to hypercapnia (Fig 4.1) resulted in a rapid increase in C_{CO_2} (from 8.4 ± 0.5 mmol/l in the pre-hypercapnic period to 14.0 ± 0.2 mmol/l at 6 h) which continued to rise until 24 h (20.0 ± 0.5 mmol/l). After this point, no further increases in C_{CO_2} were found. During the post-hypercapnic period, C_{CO_2} was reduced from 20.7 ± 0.4 mmol/l at 96 h to 13.3 ± 0.6 mmol/l after 6 h of recovery.

Net acidic equivalent fluxes

Exposure to hypercapnia resulted in a significant loss of acidic equivalents from the animal (negative $J_{\text{net}}^{\text{H}^+}$; Fig 4.2) while during the post-hypercapnic recovery period, this trend was reversed to yield a net acidic equivalent uptake or basic equivalent loss (positive $J_{\text{net}}^{\text{H}^+}$). These alterations in net acidic equivalent loss or gain were due wholly to reductions or elevations in J^{TA} ; J^{Amm} was invariant throughout the hypercapnic and post-hypercapnic periods.

Kinetic Analysis - Ionic Fluxes

Sequentially increasing the $[\text{NaCl}]_e$ resulted in increases in $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ which followed typical first order Michaelis-Menten curves (data not shown). These data could then be linearized by Eadie-Hofstee regression analysis to obtain estimates of K_m and J_{max}

FIGURE 4.1

The effects of exposure to 96 h of environmental hypercapnia ($P_w\text{CO}_2 = 7.5$ torr) and the subsequent 12 h post-hypercapnic period on plasma total carbon dioxide content (C_{CO_2}) in rainbow trout. * indicates significant difference from Pre (control) period. Means \pm SE (n=6).

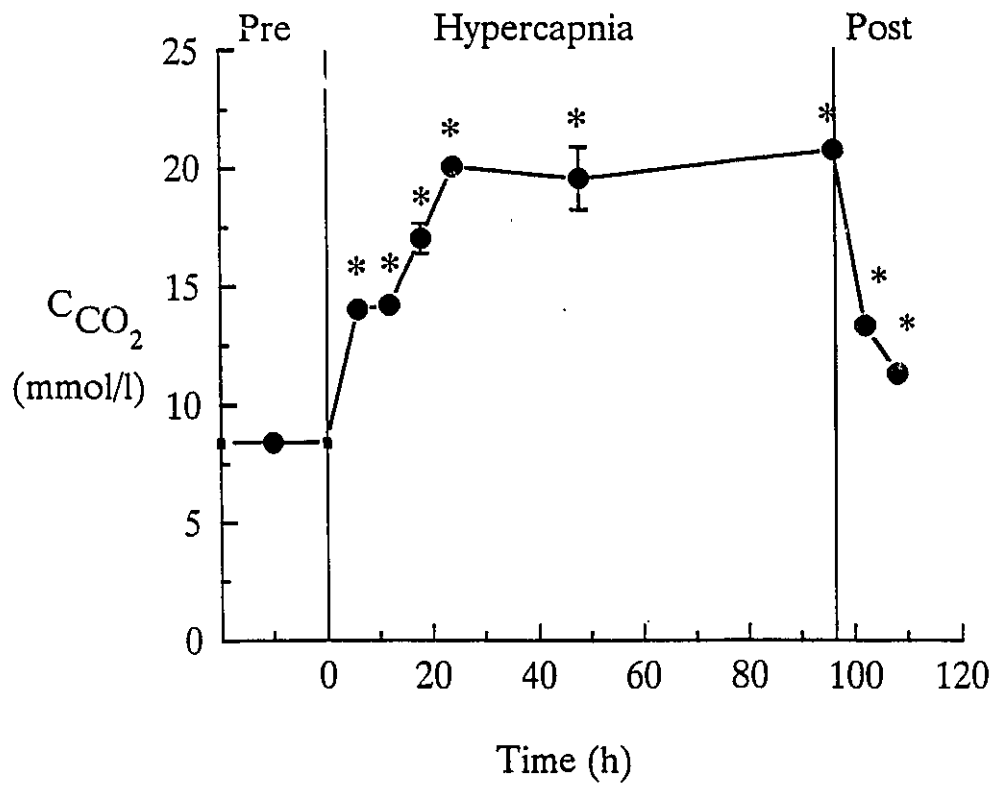
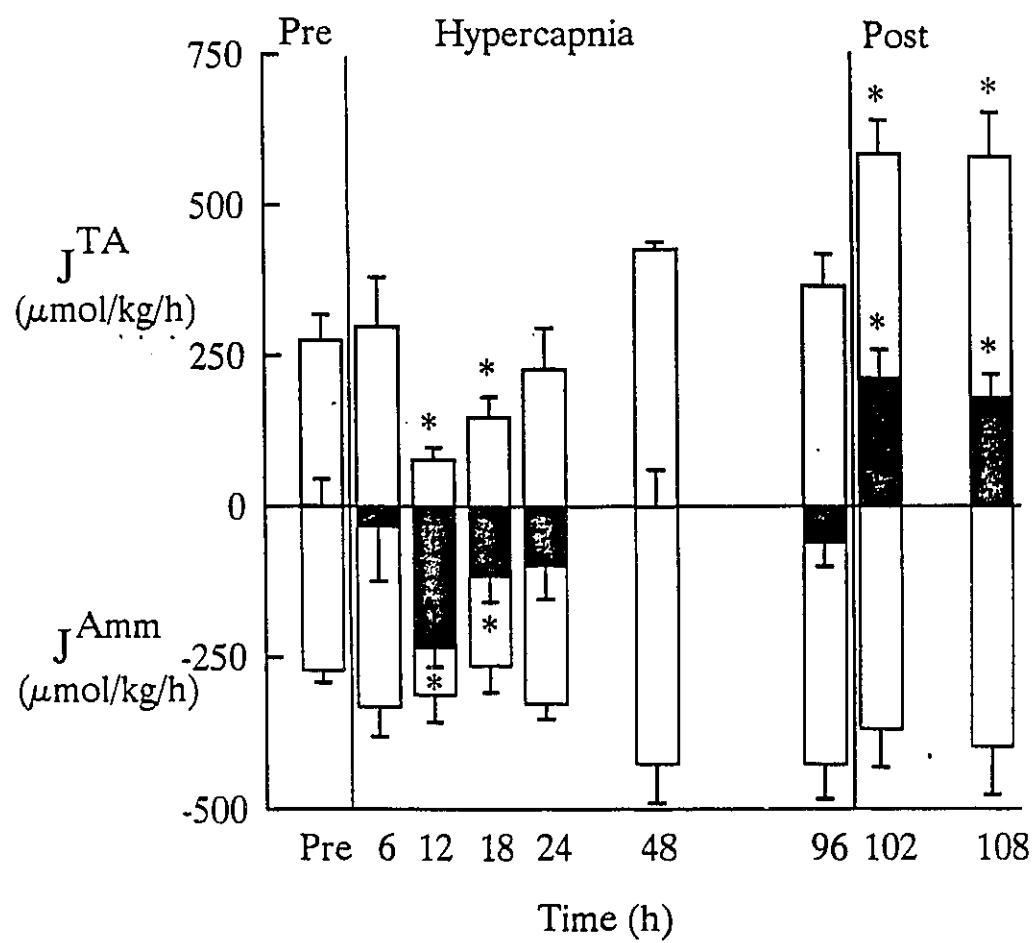


FIGURE 4.2

The effects of exposure to 96 h of environmental hypercapnia ($P_{wCO_2} = 7.5$ torr) and the subsequent 12 h post-hypercapnic period on whole body flux rates of titratable alkalinity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents ($J_{net}^{H^+}$). Positive values indicate acidic equivalent uptake (or base excretion) while negative values indicate acidic equivalent excretion. Solid areas indicate net acidic equivalent flux ($J_{net}^{H^+}$) as the sum of the two components, J^{TA} and J^{Amm} , signs considered. * indicates significant difference from Pre (control) values. + indicate significant difference from 96 h value. Means \pm SE (n=6).



for individual fish. The mean values obtained by this analysis are shown in Fig 4.3. Exposure to hypercapnia did not affect the kinetic parameters (K_m , J_{max}) for the Na^+ transport mechanism. However, this regimen did result in a significant increase in $K_m^{\text{Cl}^-}$ (decrease in affinity) from a pre-hypercapnia value of $41 \pm 16 \mu\text{mol/l}$ to $115 \pm 29 \mu\text{mol/l}$ at the 48 h period. There was no change in $J_{max}^{\text{Cl}^-}$ throughout the hypercapnic period despite the marked increase in $[\text{HCO}_3^-]$ (as indicated by the adjustment in C_{CO_2}) that otherwise would be expected to increase $J_{max}^{\text{Cl}^-}$ (Fig 4.1).

During the post-hypercapnic recovery period, $K_m^{\text{Na}^+}$ and $K_m^{\text{Cl}^-}$ were not significantly altered compared to their initial pre-hypercapnic value (Fig 4.3). However, after 6 hours of removal of the hypercapnic stimulus (102 h), $J_{max}^{\text{Cl}^-}$ ($560 \pm 66 \mu\text{mol/kg/h}$) was significantly elevated above the initial pre-hypercapnic value ($224 \pm 21 \mu\text{mol/kg/h}$). This elevation of $J_{max}^{\text{Cl}^-}$ was maintained over the 12 h recovery period. Concomitantly, $J_{max}^{\text{Na}^+}$ was reduced in the recovery period from $585 \pm 93 \mu\text{mol/kg/h}$ during pre-hypercapnia to $374 \pm 89 \mu\text{mol/kg/h}$ after 6 h of recovery (Fig 4.3).

Gill Morphology

Hypercapnia caused a 30% reduction in the fractional area of exposed CCs on the gill filamental surface (Fig 4.4c). This reduction took 24 h to occur and there was a further 10% reduction in CC fractional area by 96 h. The reductions in CC fractional area were accomplished initially by reductions in the average area of individual CCs (Fig 4.4a), CC density was unaffected initially by the exposure to hypercapnia. By 96 h, there were significant reductions in the density of exposed filamental CCs (Fig 4.4b). During the post-hypercapnia recovery period, there was a rapid and significant increase in CC fractional area by the 102 h point (6 h post-hypercapnia; Fig 4.4c). This increase in CC fractional area resulted from an increase in the CC density which was significant when

FIGURE 4.3

The effects of exposure to 96 h of environmental hypercapnia ($P_{wCO_2} = 7.5$ torr) and the subsequent 12 h post-hypercapnic period on mean estimates of the affinity (K_m) and maximal transport rate (J_{max}) for both the Na^+ and Cl^- uptake mechanisms. * indicates significant difference from Pre (control) values. + indicates significant difference from 96 h value. Means \pm SE (n=6).

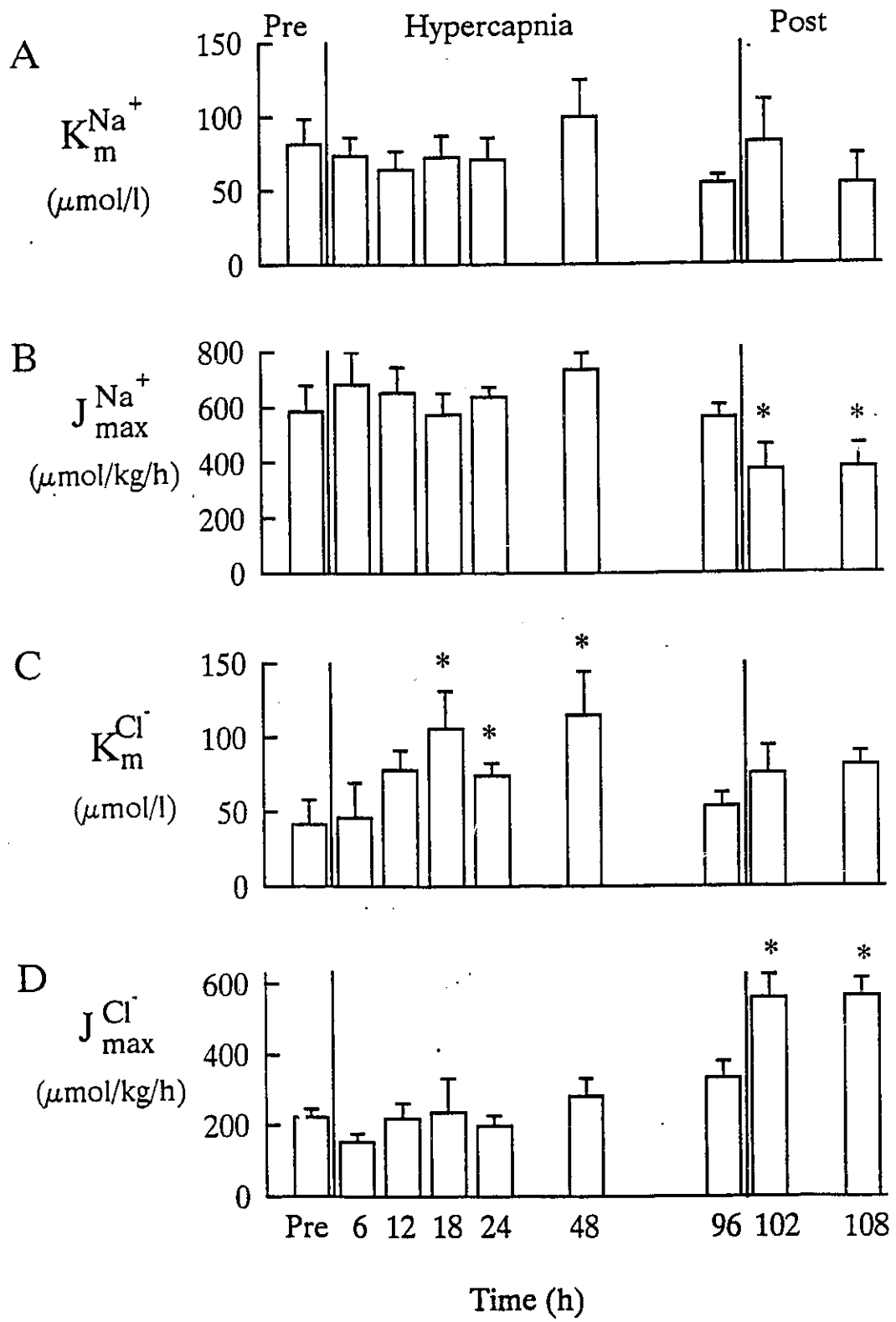
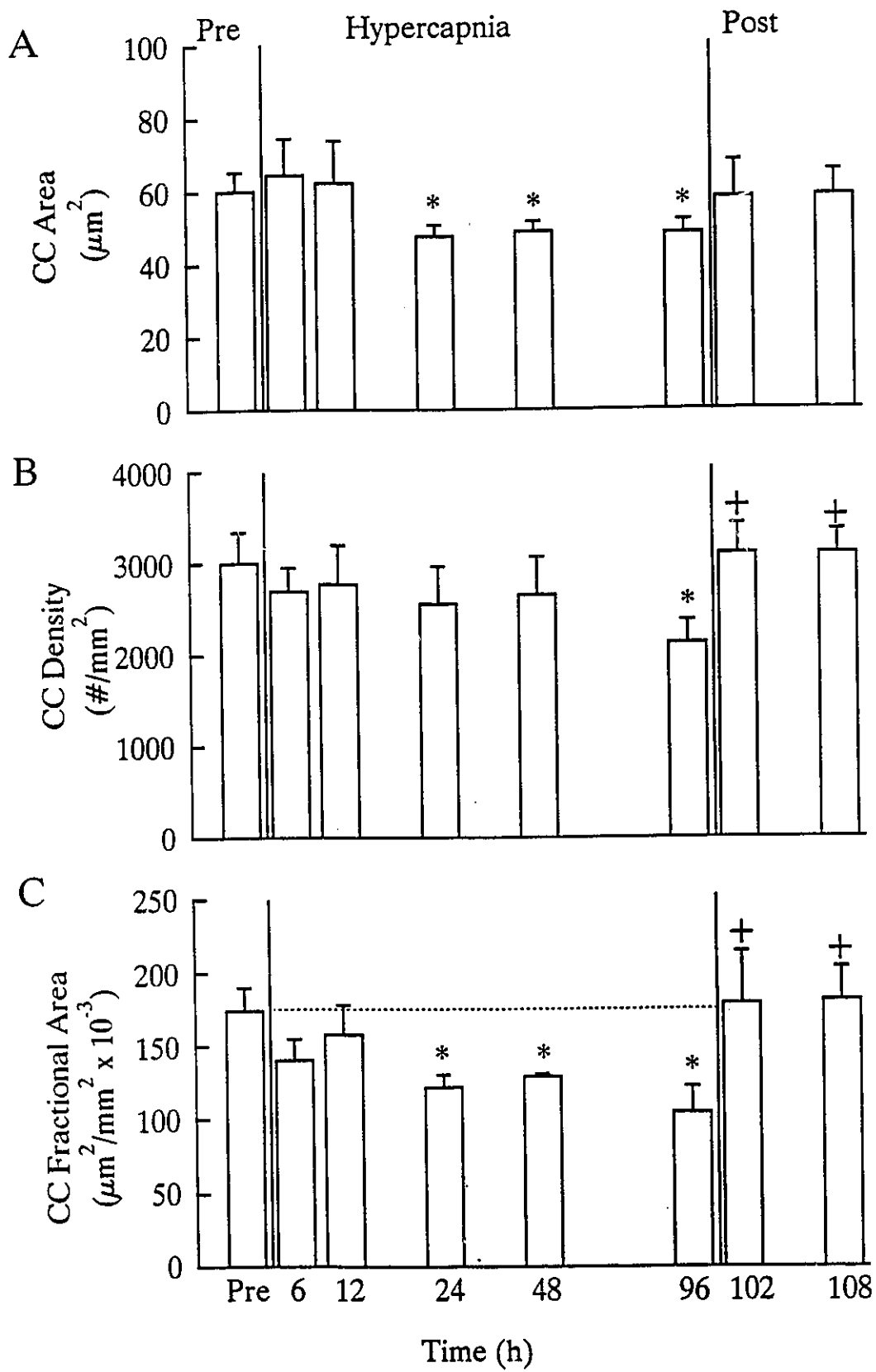


FIGURE 4.4

The effects of exposure to 96 h of environmental hypercapnia ($P_{wCO_2} = 7.5$ torr) and the subsequent 12 h post-hypercapnic 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 Pre (control) value. + indicates significant difference from 96 h value. Means \pm SE (n=6).



compared to the 96 h sample but not significantly different from the pre-hypercapnia value (Fig 4.4b). Average CC area also increased during the post-hypercapnic period (Fig 4.4a). Representative SEM photographs from the control, 96 h period and the 6 h recovery period are shown in Fig 4.5.

FIGURE 4.5

Representative low magnification scanning electron microscopy (SEM) photographs of the filament epithelium during normocapnic conditions (Fig 4.5a), after 96 h of exposure to environmental hypercapnia ($P_{wCO_2} = 7.5$ torr; Fig 4.5b), and during the subsequent recovery period (Figs 4.5c). Note the reduction in the CC apical surface area with exposure to hypercapnia. Arrowheads indicate chloride cells, * indicate mucous cells. Scale bar = 10 μ .



DISCUSSION

Morphology of the Gill Epithelium during Hypercapnic Acidosis

The results of the present study demonstrate that morphological adjustments of the fractional surface area of CC's is a significant mechanism utilized by rainbow trout to compensate the respiratory acidosis associated with environmental hypercapnia. As previously suggested in Chapter 2, the reduction of CC surface area exposed to the water serves to limit the availability of the $\text{Cl}^-/\text{HCO}_3^-$ exchange sites. Thus, the predominant mechanism of acid-base regulation during hypercapnia, reduction of $\text{Cl}^-/\text{HCO}_3^-$ exchange (Heisler, 1984, 1986; Wood *et al.*, 1984, Perry *et al.*, 1987a; Goss and Wood, 1990a, b) can in part, be explained by reduced numbers of functional $\text{Cl}^-/\text{HCO}_3^-$ exchangers.

In this study, there was a 40% reduction in CCFA over the 96 h of hypercapnia. This response however, is considerably less pronounced than the more rapid (50% by 6 h) and nearly complete (95%) reduction noted for the brown bullhead catfish (Chapters 2 and 3). The reasons for the differences in the magnitude of the morphological responses are not readily apparent. It is feasible that the response is amplified in the brown bullhead catfish owing to environmental factors to which it might normally be exposed. In contrast to trout, which are an active pelagic species, brown bullhead catfish live in slowly moving rivers, lakes and ponds; areas which are subjected to rapid alterations in temperature, P_{O_2} and P_{CO_2} (Truchot, 1987). Each of these factors may act to induce an acid-base disturbance and therefore, the development of a rapid mechanism to combat these acid-base disturbances would certainly be advantageous. In addition, it is known that catfish have far lower rates of Ca^{2+} uptake compared to that of trout (Höbe *et al.*, 1984a; Perry and Wood, 1985; Perry *et al.*, 1992a) and since the CC is presumed to be the site of Ca^{2+} uptake (Perry and Wood, 1985; Ishihara and Mugiya, 1987; Perry and Flik,

1988), this may constrain the maintenance of a higher CCFA so as not to impair Ca^{2+} balance. Another possible reason for the attenuated response in trout is the higher inherent leakiness to branchial ion loss in trout compared to catfish (Pierre Laurent, Steve Perry, - unpublished observations) which may necessitate the maintenance of a higher CCFA. The differences in ion loss rates are reflected in the fact that under control conditions, the rate of ionic uptake in trout is far higher than in bullhead and, similarly, CCFA in trout is higher than in bullhead (Perry *et al.*, 1992b).

It should be noted that the CCs of trout and catfish possess markedly different surface morphologies. These differences have been noted in other studies (Laurent and Perry, 1991; Perry *et al.*, 1992a, b). Under control conditions, catfish CCs have a characteristic spongy appearance, small average CC areas and microvilli that are arranged laterally. In trout, the CCs do not appear spongy, have larger average CC areas and possess varying numbers of vertically projecting microvilli on the cell surface. The combination of SEM and TEM in this and previous studies confirms that although the two species have widely varying surface morphologies, their identity in each case is indeed the CC.

Combined Effects of Alterations in Morphology and Internal Substrate on the Kinetic Parameters, K_m and J_{\max} during Hypercapnia.

In this study and the previous one on brown bullhead catfish (Chapter 2), the initial response to hypercapnic acidosis was a reduction in the average CC area followed by a secondary reduction in CC density. According to the classical interpretation for one-substrate kinetics, this should result in a reduced $J_{\max}^{\text{Cl}^-}$ owing to a general reduction in the number of transporters. These reductions in CCFA, however, were not associated with reductions in J_{\max} for either the Na^+ or Cl^- transport mechanisms. In a previous

study by Goss and Wood (1990a) on the physiological responses to environmental hyperoxia, there were also no alterations in J_{\max} noted for either Na^+ or Cl^- uptake. Thus, the reductions in Cl^- and Na^+ uptake which occur during hyperoxia (Wood *et al*, 1984, Goss and Wood, 1990a) and hypercapnia (Perry *et al*, 1987a) are the result of increases in K_m (decreases in affinity) with no reduction in J_{\max} (Goss and Wood, 1990a). This alone would act to reduce the uptake of both Cl^- and Na^+ without affecting J_{\max} . The mechanisms involved in the regulation of K_m are unknown but likely reflect a direct modulation of the active site rather than a non-specific effect such as alteration of external Ca^{2+} which has been suggested as a mechanism for altering K_m (Maetz, 1972; Avella *et al*, 1987).

The reason for the lack of a decrease in $J_{\max}^{\text{Cl}^-}$ accompanying the reduction in CCFA was likely due to the confounding increase in internal substrate availability acting to elevate $J_{\max}^{\text{Cl}^-}$ according to the two-substrate model put forward by Wood and Goss (1990) and Goss and Wood (1991). Compensation of respiratory acidosis in trout is usually accomplished through elevations of plasma $[\text{HCO}_3^-]$ (Cameron, 1976; Heisler *et al*, 1978; Perry, 1982; Höbe *et al*, 1984c; Heisler, 1986; Cameron and Iwama, 1987, Perry *et al*, 1987a; Iwama and Heisler, 1991) and net acid excretion *via* alteration of the titratable alkalinity component (J^{TA}) to net H^+ transfer (Fig 4.2). The fish in the present study were compensating the hypercapnic acidosis as indicated by the increases in C_{CO_2} (Fig 4.1) and negative $J_{\text{net}}^{\text{H}^+}$ (Fig 4.2) during hypercapnia. The elevations in plasma $[\text{HCO}_3^-]$ would invariably result in an increase in the availability of internal counter-ion for the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. According to the model of Goss and Wood (1991), this would serve to increase $J_{\max}^{\text{Cl}^-}$ and actually impede the retention of HCO_3^- and compensation of the acidosis. Consequently, the reduction of CCFA during hypercapnia is essential to

minimize the effects of inappropriately increasing substrate availability or $J_{\max}^{\text{Cl}^-}$.

Therefore, the maintenance of $J_{\max}^{\text{Cl}^-}$ during hypercapnia is likely the result of opposing mechanisms (increased internal HCO_3^- levels *versus* decreased numbers of $\text{Cl}^-/\text{HCO}_3^-$ exchangers).

The post-hypercapnic recovery period is associated with a rapid reduction in P_{CO_2} and the development of a metabolic alkalosis owing to the persistent elevation of $[\text{HCO}_3^-]$ (Höbe *et al.*, 1984c). At this time, the relative contributions of substrate availability and epithelial morphology to $J_{\max}^{\text{Cl}^-}$ were more apparent. By the 6 h recovery period (102 h), the CCFA had returned to control levels (Fig 4.4c). Concomitantly, $J_{\max}^{\text{Cl}^-}$ was elevated (Fig 4.3d) suggesting that increases in $J_{\max}^{\text{Cl}^-}$ during recovery were the result of elevation in both the internal substrate (HCO_3^-) concentration and CCFA. These two mechanisms would act in concert to help clear the elevated $[\text{HCO}_3^-]$ from the extracellular compartment. Under these conditions, the relative contributions of morphology and substrate availability can be discerned. Since CCFA is similar in both the control and recovery period, the additional elevation of $J_{\max}^{\text{Cl}^-}$ above those found under control conditions is presumably due to the increase in substrate availability. These two mechanisms cause an elevation in $J_{\text{in}}^{\text{Cl}^-}$ in the post-hypercapnic fish, a response previously noted in both post-hypercapnic (Perry *et al.*, 1987a) and post-hyperoxic (Goss and Wood, 1990a) fish. In addition to the increase in $J_{\max}^{\text{Cl}^-}$, $J_{\max}^{\text{Na}^+}$ is reduced during the post-hypercapnic recovery. The elevation in pH would result in a reduction in $J_{\max}^{\text{Na}^+}$ through a reduced substrate (H^+) availability, providing a third mechanism to compensate the acid-base disturbance. Unfortunately, the contributions of morphological alteration to alterations in $J_{\max}^{\text{Na}^+}$ cannot be discerned until a precise localization and characterization of the Na^+ uptake mechanism (Na^+/H^+ exchange - Na^+ channel/ H^+ -ATPase) is

established.

In summary, the alterations in $J_{\max}^{\text{Cl}^-}$ through variations of counter-ion substrate availability and morphological adjustments of the fractional surface area of gill CCs appear to be key mechanisms utilized by freshwater fish to regulate hypercapnic acidosis and the subsequent recovery period. Furthermore, a change in the affinity of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger ($1/K_m$) also is an integral component of the overall compensatory response. Future studies should focus on the mechanisms involved in Na^+ uptake (Na^+/H^+ exchange - Na^+ channel/ H^+ -ATPase) to determine whether morphological modification is an important mechanism utilized for the regulation of Na^+ uptake.

CHAPTER 5

MORPHOLOGICAL RESPONSES OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) GILL TO HYPEROXIA, BASE (NAHCO_3) AND ACID (HCL) INFUSIONS

INTRODUCTION

The results of Chapters 2-4 indicate that a response of both catfish (Chapters 2 and 3) and trout (Chapter 4) to respiratory acidosis induced by hypercapnia is a reduction of the fractional area of exposed branchial CC, thereby reducing the numbers of functional $\text{Cl}^-/\text{HCO}_3^-$ exchangers. This response contributes to the lowering of $\text{Cl}^-/\text{HCO}_3^-$ exchange and thus aids in the compensatory elevations in plasma $[\text{HCO}_3^-]$. During the subsequent metabolic alkalosis that is known to accompany the removal of a hypercapnic stimulus (Cameron, 1976; Claiborne and Heisler, 1984, 1986; Cameron and Iwama, 1987; Perry *et al.*, 1987a), $J_{\text{in}}^{\text{Cl}^-}$ is stimulated by an increase in CCFA and an increased internal substrate (HCO_3^-) availability for the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Both these mechanisms act in concert to clear the metabolic base load.

In contrast to environmental hypercapnia, hyperoxia (elevated P_{wO_2}) results in an *endogenously* induced respiratory acidosis (Wood and Jackson, 1980; Wood *et al.*, 1984; Goss and Wood, 1990a, Wood, 1991b) owing to hypo-ventilation 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 $[\text{H}^+]$ (decreasing pH) and elevated circulating catecholamine levels (Perry *et al.*, 1987a, 1989) on moderating net acid transfer (McDonald and Wood, 1981; McDonald, 1983; McDonald *et al.*, 1983; Boutilier *et al.*, 1986; Tang and Boutilier, 1988a, b; McDonald and Milligan, 1988; McDonald *et al.*, 1989a, b; Tang *et al.*, 1989b).

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.*, 1991a; Milligan *et al.*, 1991) and bases (e.g. NaHCO_3 ; Claiborne and Heisler, 1986; Heisler *et al.*, 1988; McDonald and Prior, 1988; Goss and Wood, 1990b) induce metabolic alterations in the acid-base status of

the fish (*i.e.* minimal alteration in P_{CO_2}) 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). In addition, the possibility of direct effects of elevated P_{O_2} and P_{CO_2} on gill morphology should not be overlooked.

The primary goal of the present study was to examine simultaneously alterations in whole body ionic fluxes and gill epithelial morphology during and after prolonged hyperoxia (70 h) and after 19 h continuous infusion of base (as NaHCO_3) or acid (as HCl). The experiments were designed to determine whether morphological regulation of blood acid-base status, as suggested in Chapters 2-4, is a generalized and coordinated response to acid-base disturbances and not simply a peculiarity of hypercapnic acidosis. A second goal of the present study was to examine the relative contributions of modifications of the gill epithelial morphology and alterations of internal substrate availability to acid-base balance in fish experiencing diverse acid-base disturbances.

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 ($\text{Na}^+ = 0.6 \text{ mmol/l}$, $\text{Cl}^- = 0.8 \text{ mmol/l}$, $\text{Ca}^{2+} = 0.9 \text{ mmol/l}$, $\text{pH} = 7.8$). The fish were not fed for 1 week prior to experimentation to minimize any variations in acid output caused by feeding (Wood and Caldwell, 1978). 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 72 h prior to experimentation. Vigorous aeration thoroughly mixed the water. In series II (infusion series), the trout were first anaesthetized (MS-222 1:10,000; Sigma), fitted with indwelling dorsal aortic cannulae (Soivio *et al*, 1973) and placed in the boxes where they were allowed to recover for 72 h prior to experimentation. Temperature was maintained $14 \pm 1 \text{ }^\circ\text{C}$.

Exposure Regime

Series 1 - Exposure to hyperoxia

Series 1 was designed to examine the effects of exposure to hyperoxia on the gill epithelial morphology of rainbow trout and to relate these changes to alterations in branchial ion fluxes that are known to occur during such a treatment. After 72 h of recovery from surgery, the water flowing to the boxes was gassed with O_2 , which elevated the water P_{O_2} within 0.33 h from 140 torr to > 500 torr. After 72 h of exposure to continuous hyperoxia, the O_2 was replaced with air, quickly returning P_{O_2} to normoxic levels. Fish were killed by a cephalic blow after exposure to control conditions ($n=11$), 72 h of continuous hyperoxia ($n=11$), or 6 h of recovery from hyperoxia ($n=11$). The second

right gill arch was removed and prepared for electron microscopic analysis as described in Chapter 2.

Series 2 - Acid/base infusions

(i) Series 2 was designed to examine the effects of infusion (19 h) of either A) 140 mmol/l NaCl (sham control, n=10; 2.81 ± 0.10 ml/kg/h) B) 140 mmol/l NaHCO₃ (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.*, 1984c; Goss and Wood, 1990a), 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 NaHCO₃ (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.

In order to generate a relationship between the maximal transport rates for Cl⁻ ($J_{\max}^{\text{Cl}^-}$) and the fractional area of CCs on the filament epithelium, the radioisotopic influx of Cl⁻ ($J_{\text{in}}^{\text{Cl}^-}$, n=8) was examined in control over increasing [NaCl]_e in the water as in Chapter 3. Six flux periods of increasing [NaCl]_e (nominally 50, 150, 300, 600, 1200, 2400 μM/l) were measured. Each lasted 0.5 h except for the final flux period lasting 0.67 h. To determine $J_{\max}^{\text{Cl}^-}$, samples were analyzed as in Chapter 4. At the end of the kinetic study in control (non-infused) fish or after 19 h of infusion with either solution A, B or C, the fish were killed by a cephalic blow and the second right gill arch was removed and prepared for scanning electron microscopic analysis as described in Chapter 2.

Analytical Techniques and Calculations

The analytical techniques were identical to those described in Chapter 4.

Morphological methods

Preparation of samples for morphological and morphometrical analysis was identical to the procedure outlined in Chapter 2.

Statistical Analyses

All values are presented as means \pm 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

The effects of hyperoxia on gill CC morphology are shown in Fig 5.1. After 70 h, CCFA was decreased by 33% (from 91771 ± 15415 to $61624 \pm 13113 \mu\text{m}^2/\text{mm}^2$; Fig 5.1C). The decrease in CCFA was caused entirely by a decrease in the average area (Fig 5.1A) of exposed CCs on the filament epithelium; CC density was unaffected (Fig 5.1B). In the post-hyperoxic period, there was a large increase in the density of filamental CCs displayed on the filament epithelium from $841 \pm 133 /\text{mm}^2$ at the end of hyperoxia to $2746 \pm 432 /\text{mm}^2$ only 6 h after the removal of the hyperoxic stimulus (Fig 5.1B). This resulted in a large increase in the CCFA at this time (Fig 5.1C); the increase was significantly greater than both the control and the 70 h hyperoxia periods. Representative SEM and TEM photographs showing the effects of exposure to environmental hyperoxia are presented in Fig 5.2. The CCs in control fish displayed few apical membrane microvilli and were frequently raised above adjacent PVCs. The ultrastructural characteristics of the CC included numerous mitochondria and a well developed apical tubular network (Fig 5.2D). The changes which occurred during hyperoxia included a decrease in CCFA owing to a small decrease in average cell area (Fig 5.2B). The return to normoxia was marked by a large increase in the density of filament CCs (Fig 5.2C) in accordance with the morphometric analysis (Fig 5.1B).

Morphological Responses to Base (NaHCO_3) or Acid (HCl) Infusion

Infusion of NaHCO_3 resulted in a significant 45% increase in the density of filament CCs from $1613 \pm 262 /\text{mm}^2$ in NaCl -infused fish to $2364 \pm 222 /\text{mm}^2$ in NaHCO_3 infused fish (Fig 5.3B). In addition there was a trend (not significant) toward an increase in the average CC area (Fig 5.3A). The net result was a significant 70% increase

FIGURE 5.1

The effects of exposure to 70 h of environmental hyperoxia ($P_{O_2} > 500$ torr in water) and the subsequent 6 h post-hyperoxic 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 Pre (control) value. + indicates significant difference from 70 h value. Means \pm SE.

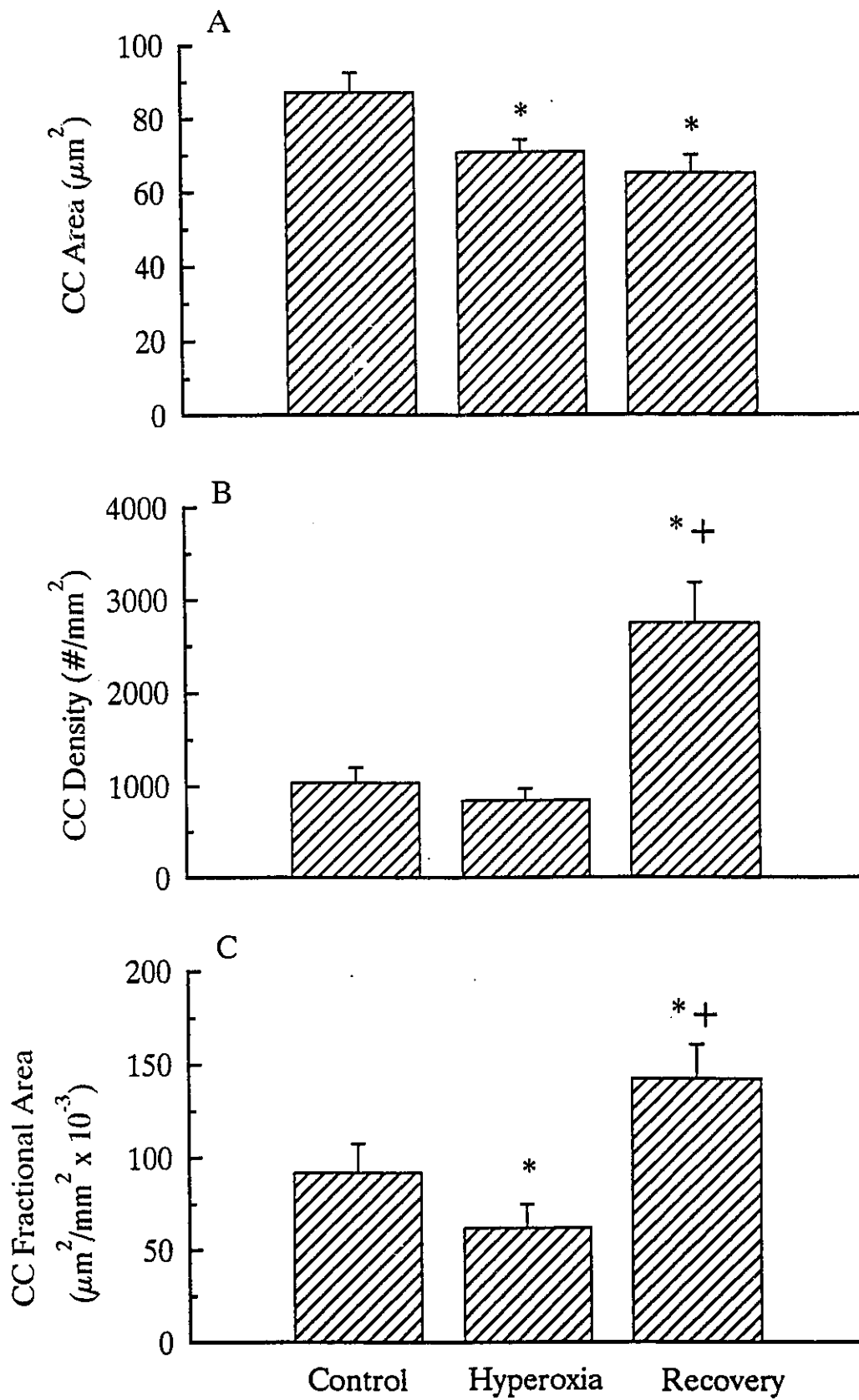


FIGURE 5.2

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 5.2A, D), after 70 h (Fig 5.2B, E) and the subsequent post-hyperoxic recovery period (Figs 5.2C, F). Note the reduction in the CC fractional area during exposure to hyperoxia and subsequent increase in the post-hyperoxic recovery period. Figs 5.2A-C scale bar = 10 μ . Figs 5.2D-F scale bar = 1 μ m.

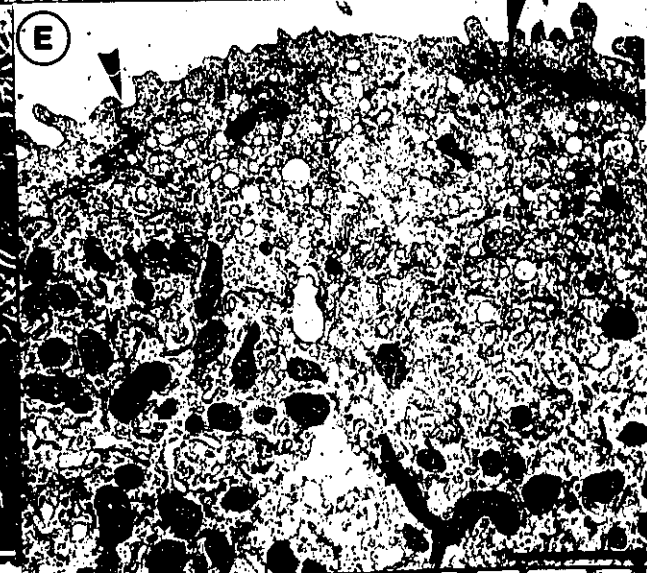
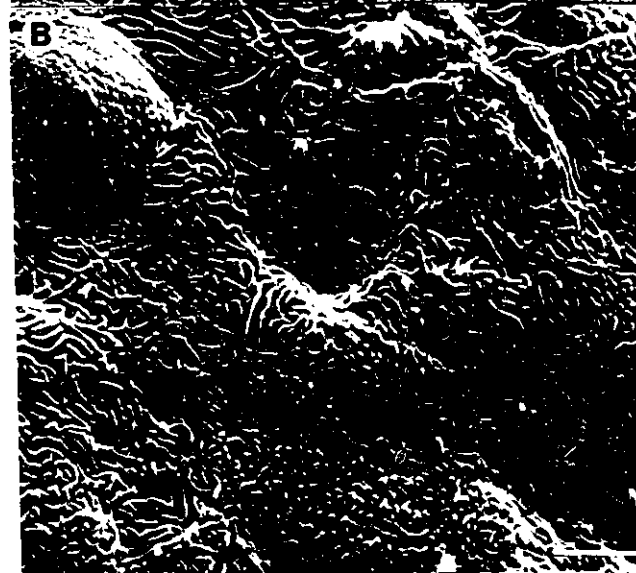
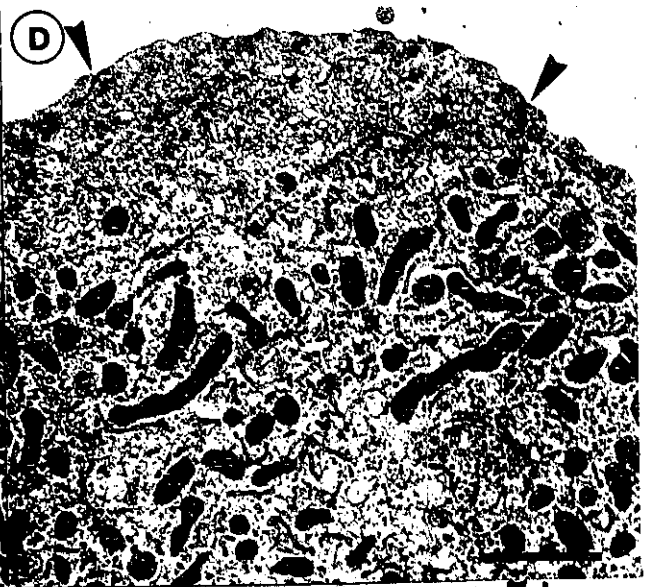
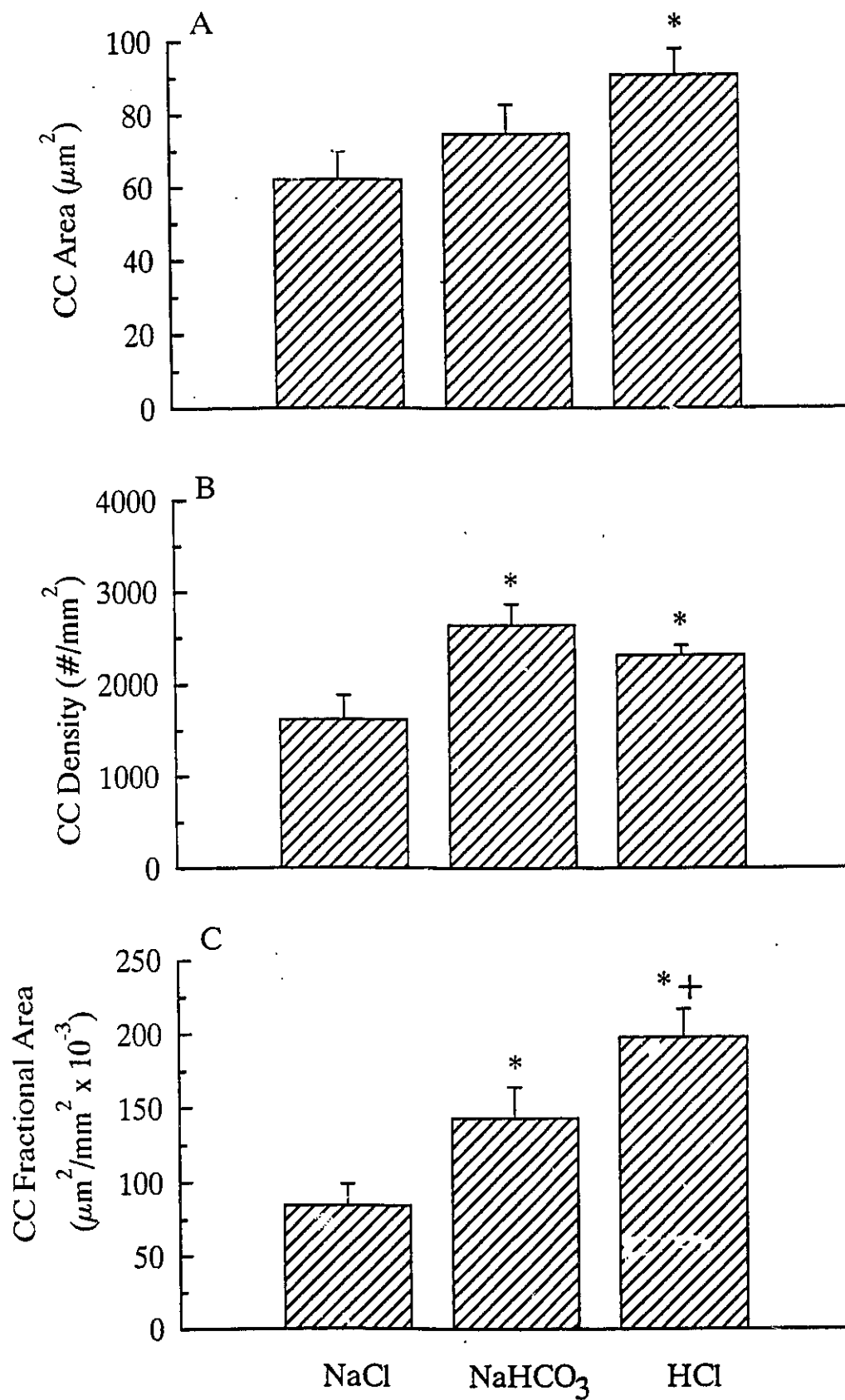


FIGURE 5.3

The effects of infusion of 140 mmol NaCl, 140 mmol NaHCO₃, 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 Pre (control) value. + indicates significant difference from NaHCO₃-infused value. Means \pm SE.



in the CCFA from $83959 \pm 14693 \mu\text{m}^2/\text{mm}^2$ in NaCl-infused fish to $143098 \pm 20633 \mu\text{m}^2/\text{mm}^2$ in NaHCO_3 infused fish (Fig 5.3C; cf. recovery from hyperoxia: Fig 5.1). Infusion of acid (HCl) caused a significant increase in both the area ($90.6 \pm 7.2 \mu\text{m}^2$; Fig 5.3A) and density ($2304 \pm 108 /\text{mm}^2$; Fig 5.3B) of filament CCs and together these two alterations resulted in a large 135% increase in the CCFA in the HCl-infused fish.

Representative SEM (Figs 5.4A-C) and TEM (Figs 5.4D-F) photographs showing the effects of base (NaHCO_3) or acid (HCl) infusion are presented in Fig 5.4. In the base-infused group, the CCs had a very characteristic smooth apical surface presenting few apical microvilli while in the acid-infused group, the CCs appeared to have increased numbers of apical microvilli (Fig 5.4C). Occasionally in the acid-infused group the CCs protruded from the filamental epithelium (Fig 5.4E). 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 5.4F).

Relationship between $J_{\text{max}}^{\text{Cl}^-}$ and CCFA during Acid-Base Disturbances

Table 5.1 shows the effects of various acid-base disturbances on the kinetic parameters K_m and J_{max} for the Cl^- transport systems as generated by one-substrate kinetic analysis. These values are taken from the previous publications (Goss and Wood, 1990a, b; Wood and Goss, 1991). Acidosis, arising from either respiratory (hyperoxia) or metabolic (HCl infusion) perturbations, was associated with increases in K_m (decreases in affinity) of Cl^- ($\text{Cl}^-/\text{HCO}_3^-$ exchanger). $J_{\text{max}}^{\text{Cl}^-}$ was not significantly altered by acidosis. Metabolic alkalosis, arising from either post-hyperoxia or NaHCO_3 infusion, on the other hand, was associated with significant increases in $J_{\text{max}}^{\text{Cl}^-}$ while $K_m^{\text{Cl}^-}$ was not significantly altered.

Figure 5.5 shows the relationship between CCFA and $J_{\text{max}}^{\text{Cl}^-}$. The regression line

TABLE 5.1

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).

Treatment	$K_m^{Cl^-}$ ($\mu\text{mol/l}$)	$J_{max}^{Cl^-}$ ($\mu\text{mol/kg/h}$)	Reference
Control	127 ± 20	286 ± 27	Goss & Wood, 1990a
Hyperoxia (70h)	$248 \pm 42^*$	272 ± 49	"
Post-hyperoxia (6h)	137 ± 13	$445 \pm 54^*$	"
NaCl infusion	121 ± 13	303 ± 68	Goss & Wood, 1990b
NaHCO ₃ infusion	135 ± 12	$674 \pm 89^+$	"
HCl infusion	178 ± 53	336 ± 39	Goss and Wood, 1991

is the coupling between the two variables when acid-base status is not changing (*i.e.* $[\text{HCO}_3^-]$ is unchanged) and can be used to predict the effect of changing CCFA on $J_{\text{max}}^{\text{Cl}^-}$ under control conditions. Superimposed on this graph are the measured values of $J_{\text{max}}^{\text{Cl}^-}$ for each treatment group from Table 5.1 and the measured values of CCFA from the present study. Those values that follow this relationship indicate that alterations in $J_{\text{max}}^{\text{Cl}^-}$ 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 alteration in internal counter-ion concentration ($[\text{HCO}_3^-]$).

There was a significant positive relationship between $J_{\text{max}}^{\text{Cl}^-}$ and CCFA under control conditions ($y = 0.0015x + 118$, $r = 0.69$, $n = 8$). $J_{\text{max}}^{\text{Cl}^-}$ for the hyperoxic (70 h) and NaCl-infused fish fell directly in line with the relationship generated between CCFA and $J_{\text{max}}^{\text{Cl}^-}$ under control conditions. However, metabolic alkalosis as a result of removal of the hyperoxic stimulus resulted in $J_{\text{max}}^{\text{Cl}^-}$ being slightly higher than predicted from the relationship while NaHCO_3 infusion caused an even greater deviation from the predicted relationship. HCl infusion, which does not change internal $[\text{HCO}_3^-]$ appreciably (Goss and Wood, 1991), adhered to the predicted relationship.

FIGURE 5.4

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 5.4A, D), NaHCO₃ infusion (Fig 5.4B, E) and HCl infusion (Figs 5.4C, F). Note the increase in the CC fractional area during infusion of NaHCO₃ or HCl. Figs 5.3A-C, arrowheads indicate CCs, Figs 5.3 D-F, arrowheads indicate cell-cell junctions. Figs 5.2A-C scale bar = 10 μ . Figs 5.2D-F scale bar = 1 μ m.

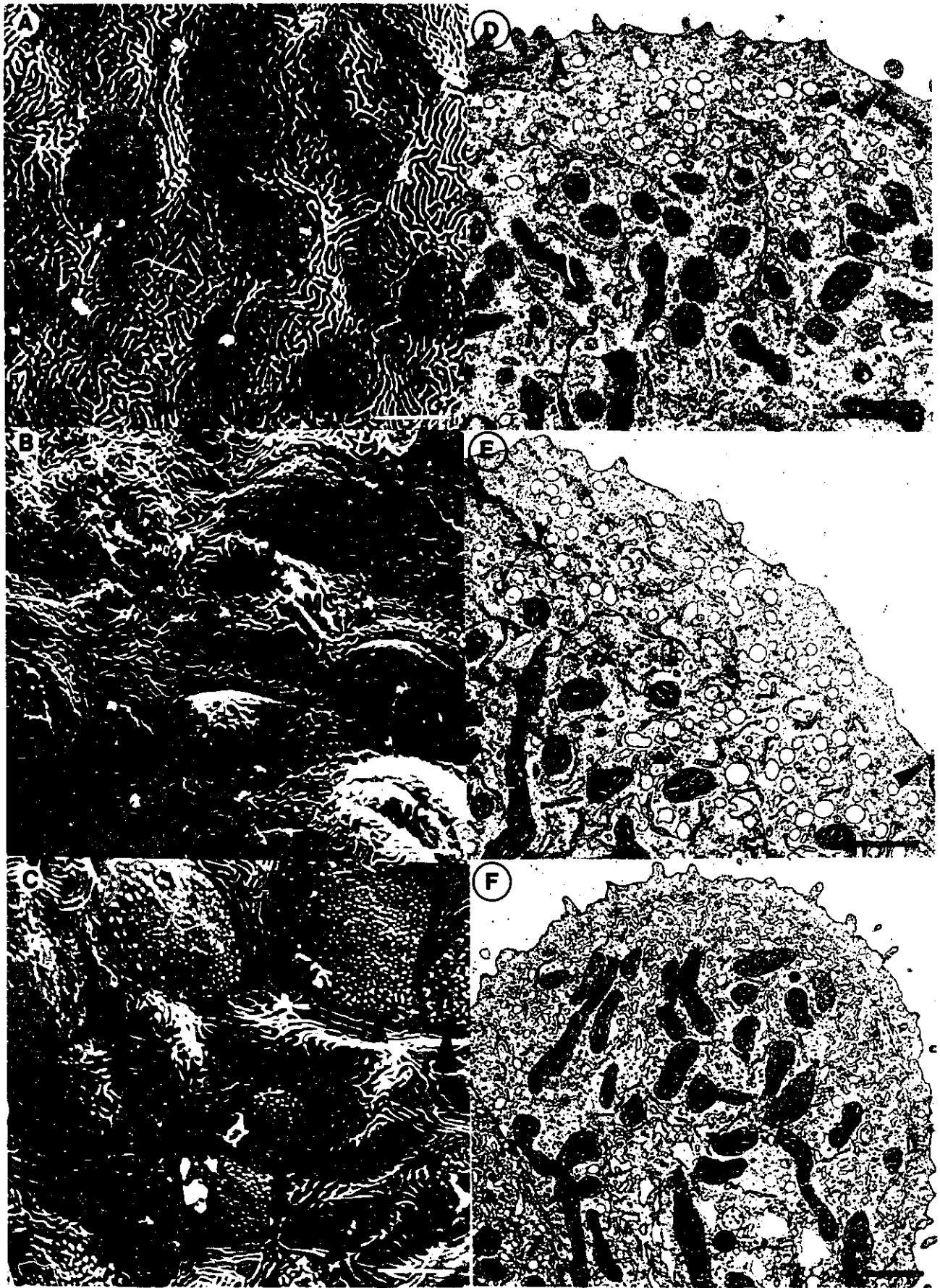
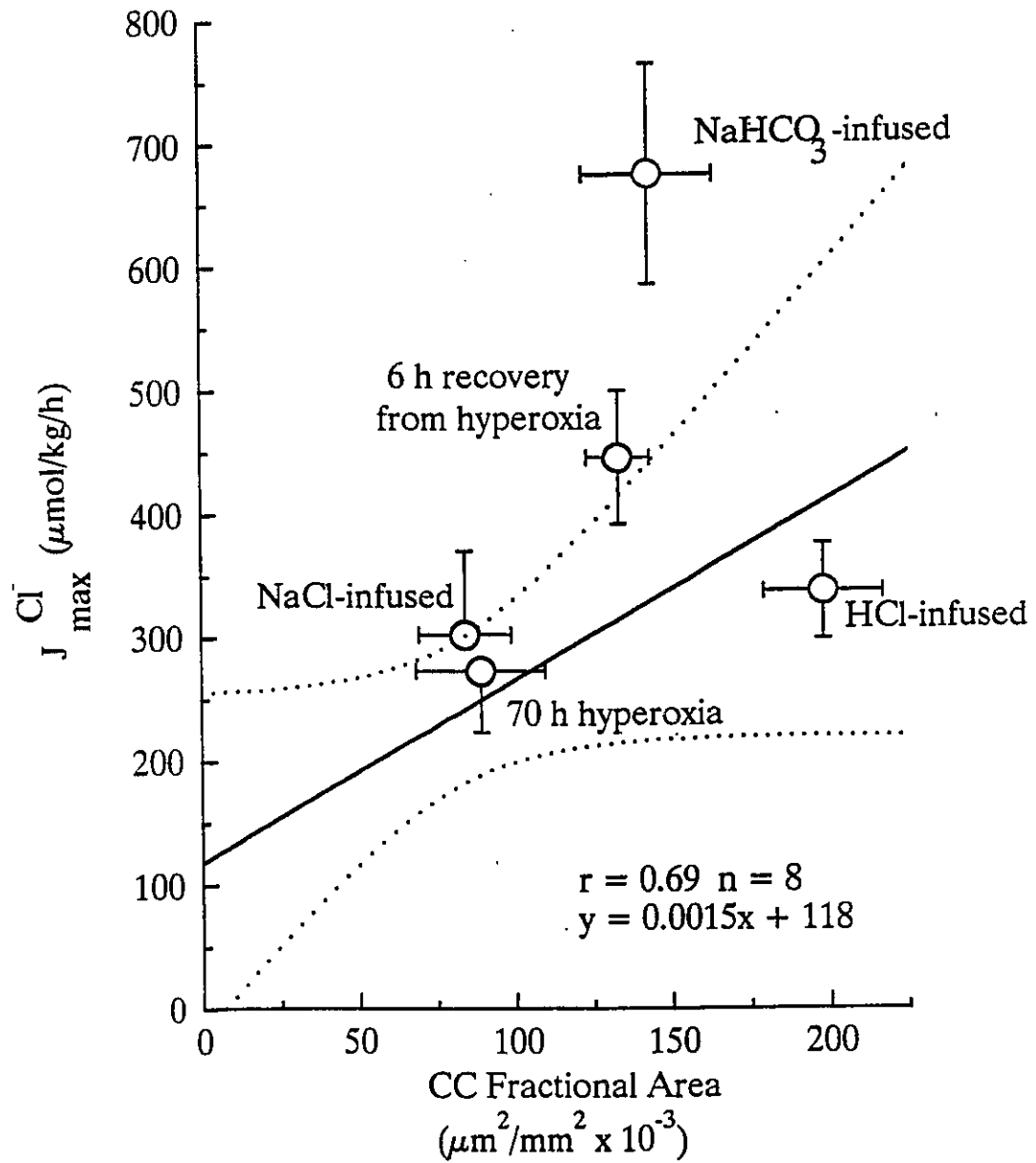


FIGURE 5.5

Correlation and 95% confidence intervals for the relationship between maximal transport rate for chloride ($J_{\max}^{\text{Cl}^-}$) with gill filament CC fractional area under control conditions (internal counter-ion is unchanged). In addition, the measured $J_{\max}^{\text{Cl}^-}$ values from Table 5.1 are plotted at the CC fractional area for each treatment group listed above (mean \pm SE). Fig 5.5, $y = 0.0015x + 118$, $r = 0.690$.



DISCUSSION

Morphological Modification of the Gill CCFA in Response to Acid-Base Disturbances.

The present study confirms and extends the results of previous Chapters showing that morphological modification of branchial CCFA is associated with regulation of blood acid-base status. CCFA was reduced significantly during hyperoxia-induced respiratory acidosis, a condition similar to the hypercapnic acidosis utilized in previous studies. The means used to decrease CCFA during hyperoxia were through 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 both brown bullhead catfish (Chapters 2, 3) and rainbow trout (Chapter 4). 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 fish (Chapters 2-4). However, in the present study, 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 filament 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 (Chapter 2). This is further supported by the study of Bartels and Potter (1991) in which rapid covering/uncovering of the CC by adjacent PVCs was shown to be a mechanism for regulating ionic uptake during freshwater-seawater transfer in lamprey (*Geotria australis*). Alternatively, the increases/decreases 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*, 1982a; Stetson and Steinmetz, 1985; Kniaz and Arruda, 1990). However, the β -type of MR cell, the cell type responsible for HCO_3^- secretion in the turtle bladder and mammalian kidney cortical collecting duct and 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 internalization of apical membrane components 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 of surface area) associated with increases in HCO_3^- secretion. Future experiments should focus on determinations of the exact mechanisms responsible for the increases/decreases in trout CC area during acid-base disturbances.

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* $\text{Cl}^-/\text{HCO}_3^-$ exchange. Clearly, an increase in the CCFA would act to increase $\text{Cl}^-/\text{HCO}_3^-$ exchange capacity which would be detrimental to the regulation of the metabolic acidosis. However, in a similar study by Goss and Wood (1991), 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,

1990a). 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*, 1992a, b) may represent 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 is that the differences in staining represent variations in the life cycle of the cell (Wendelaar-Bonga and van der Meij, 1989).

Effect of Variations in CCFA and Substrate Availability on $J_{\max}^{\text{Cl}^-}$.

Although there was a significant positive relationship between $J_{\max}^{\text{Cl}^-}$ and CCFA under control conditions, alteration of the acid-base status of the fish produced significant deviations from this relationship. These deviations were apparent during metabolic alkalosis resulting from 6 h post-hyperoxic recovery and NaHCO_3 -infusion. The deviations from the predicted relationship between $J_{\max}^{\text{Cl}^-}$ and CCFA are likely caused by alterations in internal substrate availability. Based on previous studies of hyperoxic recovery and NaHCO_3 infusion (Höbe *et al*, 1984c; Goss and Wood, 1990a, b), plasma $[\text{HCO}_3^-]$ would be significantly elevated. According to the two-substrate model (Wood and Goss, 1990; Goss and Wood, 1991), the internal counter-ion (HCO_3^-) for the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (HCO_3^-) limits the rate of Cl^- transport. This occurs because $K_m^{\text{HCO}_3^-}$ is greater than

physiological levels ($K_m^{\text{HCO}_3^-} = 33.3 \text{ mmol/l}$; Goss and Wood, 1991; see section ix, General Introduction). Therefore, elevations in $[\text{HCO}_3^-]$ result in increases in $J_{\text{max}}^{\text{Cl}^-}$ independent of, or additive to, alterations in CC morphology. Compared to NaCl-infusion, when no acid-base disturbance is present, $J_{\text{max}}^{\text{Cl}^-}$ 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 the excess $[\text{HCO}_3^-]$ 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, the increase in $J_{\text{max}}^{\text{Cl}^-}$ falls within the predicted relationship between $J_{\text{max}}^{\text{Cl}^-}$ and CCFA. This helps to explain the increase in $J_{\text{max}}^{\text{Cl}^-}$ during acid infusion (Table 5.1; Goss and Wood, 1991) that has hitherto been unexplained.

The present study confirms that morphological alteration of the gill epithelium is an important response utilized by fish during acid-base disturbances. Therefore, experimental manipulation of these variables may act to alter the ability of freshwater fish to compensate an acid-base disturbance. This is examined in Chapter 6.

CHAPTER 6

INTER-SPECIFIC VARIATIONS IN THE RATES OF ACID-BASE COMPENSATION

INTRODUCTION

The previous Chapters (2-5) have provided evidence that the CC is the gill cell responsible for $\text{Cl}^-/\text{HCO}_3^-$ exchange. The dynamic manipulation of $\text{Cl}^-/\text{HCO}_3^-$ exchange for the purposes of acid-base regulation is accomplished, at least in part, by morphological alterations of the gill CCFA (Chapter 2). This mechanism acts in conjunction with alterations in maximal transport (J_{max}) caused by changes in internal substrate (HCO_3^-) availability. Specifically, the reduction of $J_{\text{in}}^{\text{Cl}^-}$, and hence base excretion during periods of acidosis, is accomplished by decreasing CCFA, thereby limiting the availability, and hence the functioning, of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Conversely, the increase of $J_{\text{in}}^{\text{Cl}^-}$ during periods of alkalosis can be accomplished through increasing CCFA in concert with elevated levels of substrate (HCO_3^-) for the exchanger.

Most species of freshwater fish that have been examined appear to rely predominantly on the branchial $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism to regulate acid-base disturbances with the branchial Na^+/H^+ exchange (Na^+ channel/ H^+ -ATPase) mechanism playing a lesser, but significant role (Claiborne and Heisler, 1984, 1986; Wood *et al*, 1984; Perry *et al*, 1987a). The eel, however, is unusual among freshwater teleosts because it lacks appreciable Cl^- uptake (Kirsch, 1972, Bornancin *et al*, 1987, Hyde and Perry, 1987, 1989). Although dynamic manipulation of the branchial $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism has been reported for eels (Bornancin *et al*, 1977), the absolute values are so small as to have a negligible effect on whole body acid-base regulation. Thus, the eel must rely on other mechanisms to regulate blood pH such as adjustments of the branchial Na^+ uptake mechanism (Na^+/H^+ exchange - Na^+ channel/ H^+ -ATPase) (Hyde and Perry, 1987, 1989).

Morphologically, there are significant differences in the surface area of CCs exposed to the water in trout and eel. Eels display few, small CCs on the gill filament

epithelium and hence, CCFA is much lower than in trout (Perry *et al.*, 1992a). These differences may contribute to differing capacities to regulate acid-base disturbances. Thus, the naturally occurring variability in CC morphology and Cl^- uptake between trout and eel presents an opportunity to examine the role of the CC in the correction of a metabolic alkalosis.

Another possible mechanism for acid-base regulation that is often overlooked is the manipulation of the efflux component to net ion balance thereby altering net fluxes of strong ions from the fish to the water (Stewart, 1978, 1983). By allowing a greater efflux of Na^+ over Cl^- , the extracellular SID will increase, constraining a decrease in the extracellular pH. This would be an appropriate response to correct an alkalosis. The converse would be true for an acidosis (Goss and Wood, 1990a, b). In the absence of an appreciable influx component to net Cl^- balance, manipulation of the efflux component could serve as an important mode of acid-base regulation in the eel.

As mentioned in Chapter 5, infusion of NaHCO_3 induces a metabolic alkalosis similar to that seen in the post-hypercapnic (Chapters 2, 4) and post-hyperoxic (Chapter 5) periods without causing confounding effects of gill perfusion or ventilation that are known to accompany these treatments (Dejours, 1973; Wood and Jackson, 1980). In the present study, chronic infusion of NaHCO_3 was used to examine the morphological and physiological responses of eel and trout to metabolic alkalosis. The goals were i) to determine whether variations in gill morphology and the rates of Cl^- uptake between trout and eel result in differing abilities to compensate metabolic alkalosis, and ii) to examine whether modulation of the efflux component to net ion transfer is utilized as a means of regulating alkalosis.

METHODS

Experimental animals

Rainbow trout, *Oncorhynchus mykiss* [mean weight 272 ± 3 g, $n = 152$] were obtained from Linwood Acres trout farm, (Cambellcroft, Ontario) and held as described in Chapter 4. American eels, *Anguilla rostrata*, [mean weight 175 ± 11 g, $n = 25$] were obtained from an eel ladder associated with the Saunders Hydroelectric dam in Cornwall, Ontario and were transported on ice to the University of Ottawa and held in acclimation conditions as described in Chapter 4. Eels were not fed throughout the period of study. All fish were maintained at ambient water temperature ($8-13$ °C) and on a 12L:12D photoperiod.

Animal Surgical Preparation

To allow repetitive blood sampling (Series 1 only) and infusion in trout, fish were anaesthetized (MS222 1:10000; Sigma adjusted to pH 7.0 with NaHCO_3) and fitted with indwelling dorsal aortic cannulae filled with heparinized Cortland saline (Wolf, 1963) according to the method of Soivio *et al* (1973). The fish were allowed to recover for at least 48 h prior to experimentation. To permit blood sampling (Series 1 only) and infusion in eels, fish were anaesthetized (MS222 1:500; Sigma, adjusted to pH 7.0 with NaHCO_3) and indwelling cannulae were implanted into the pneumogastric artery (e.g. Hyde and Perry, 1989). Briefly, an incision was made 3 cm caudal to the heart and lateroventrally. The pneumogastric artery was isolated, a small incision was made and polyethylene tubing (PE 50, Clay Adams) was inserted. A suture was used to clamp the vessel wall to the cannulae. The wound was then sutured and the fish transferred to the individual acrylic boxes supplied with flowing dechlorinated tapwater. The fish were allowed to recover for 48 h prior to experimentation.

Infusion Regime

To elicit a chronic metabolic alkalosis, fish were infused *via* the arterial cannulae (Sage syringe pump) with 140 mmol/l NaHCO₃ at a nominal infusion rate of 800 μmol/kg/h (trout actual infusion rate = 932 ± 24 μmol/kg/h, n = 64; eel actual infusion rate = 957 ± 121 μmol/kg/h, n = 14) for a period of 24 h. Infusion of 140 mmol/l NaCl (trout: 884 ± 44 μmol/kg/h, n = 60; eel: 1019 ± 163 μmol/kg/h, n = 11) served as a control. The flow rate of the syringe pump was matched to the weight of the fish to yield an infusion rate equal to the nominal infusion rate. The solution concentrations (140 mmol/l) were chosen so as to minimize changes in plasma osmolarity. After 24 h, the infusion was stopped and the subsequent 24 h post-infusion period was examined.

Series 1 - Blood Acid-base Status during Infusion

This series was designed to characterize the alterations in blood acid-base status associated with chronic infusion of either 140 mmol/l NaCl (trout n = 5; eel n = 6) or 140 mmol/l NaHCO₃ (trout n = 6; eel n = 5). In addition, a non-infusion control experiment was performed for trout only (n=6). Repetitive blood samples (300 μl) were withdrawn from the arterial cannulae at the following times: 0 (Pre), 1, 4, 8, 12, and 24 h after the start of infusion. In the post-infusion period, blood samples were withdrawn at 25, 28, 32, 36, and 48 h (1, 4, 8, 12, 24 h post-infusion). Blood samples were analyzed immediately for pH. The blood was then centrifuged at 13000g for 15 seconds after which the plasma was drawn off and analyzed for total CO₂. After the initial (Pre) blood sample, the fish were infused as described above with either NaCl or NaHCO₃ and blood was sampled at the times indicated.

Series 2 - Ion-Acidic/Basic Equivalent Fluxes and Gill Morphology during Infusion.

This series of experiments was designed to characterize the unidirectional and net

ion and acidic/basic equivalent fluxes and to relate them to the gill morphology at various periods throughout the infusion and post-infusion periods. In all experiments, whole body fluxes were determined. However, because of the relative impermeability of fish skin (Kirsch, 1972) and the negligible contribution of renal fluxes in both trout (Perry *et al*, 1987a, b) and eel (Hyde and Perry, 1987), whole body fluxes are assumed to represent branchial fluxes in this experiment. From the blood acid-base data, the following periods were chosen for analysis of the ionic-acidic/basic equivalent fluxes and gill morphology. In addition to a non-infused control run simultaneously with each time period ($n = 22$), the 4, 12, and 24 h infusion, and the 28, 36 and 48 h periods (4, 12, 24 h post-infusion) were examined ($n = 6-9$ at each period).

For rainbow trout, measurements of whole body ionic fluxes were accomplished by monitoring the disappearance of $^{24}\text{Na}^+$ and $^{36}\text{Cl}^-$ from the water over a 2 h flux period ending at the times noted above. At the appropriate time, flow to the boxes was stopped and both $^{24}\text{Na}^+$ ($\sim 10 \mu\text{Ci}$) and $^{36}\text{Cl}^-$ ($1 \mu\text{Ci}$) were added. After a 0.5 h mixing period, water samples (40 ml) were taken and again 2 h later for determination of $[\text{Na}^+]$, $[\text{Cl}^-]$, total ammonia (Amm), titratable alkalinity and $^{24}\text{Na}^+ / ^{36}\text{Cl}^-$ activity. At the end of the flux period, the trout were killed by spinal section, the left gill arch removed and fixed for examination in the scanning electron microscope (SEM) as described in Chapter 2.

For eel, the protocol was modified slightly; to measure accurately ionic and acidic/basic equivalent fluxes in the eel, the flux period was extended to 3 h and the specific activity of Cl^- was increased by the addition of $3.0 \mu\text{Ci}$ of $^{36}\text{Cl}^-$ instead of $1.0 \mu\text{Ci}$. These modifications were necessary owing to the low rates of $J_{\text{in}}^{\text{Cl}^-}$ in eels. In addition, instead of measuring the ionic and acidic/basic equivalent fluxes in separate fish for each time period as was performed in trout, the measurements were performed sequentially for

each period due to lack of animals. Separate groups of fish were a) infused with either NaCl or NaHCO₃ at the appropriate rate for 24 h or b) maintained under control (Pre) conditions. These fish were then killed, the gills removed and fixed for examination in the SEM as described in Chapter 2.

Analytical techniques

Blood pH was determined with a microcapillary electrode (Radiometer G299A) in conjunction with a Radiometer PHM 71 acid-base analyzer and a BMS3 MK2 blood microsystem thermostatted to experimental temperature. Total CO₂ was analyzed on 50 µl plasma samples using a commercial carbon dioxide analyzer (Corning Model 905). Plasma P_{CO₂} and bicarbonate concentrations ([HCO₃⁻]) were calculated from the measured total CO₂ and pH values according to the Henderson-Hasselbalch equation. Values for the appropriate dissociation constants and solubility coefficients were obtained from Boutilier *et al* (1984). Water [Na⁺], [Cl⁻], [Ammonia], and TA and ²⁴Na⁺/³⁶Cl⁻ were determined as described in Chapter 4. Calculation of J_{net}^{Na+}, J_{net}^{Cl-}, J_{in}^{Na+} and J_{in}^{Cl-} were determined according to Maetz (1956). Techniques for fixation of gills, examination in the SEM, and morphometric analysis are as described in Chapter 2.

Statistical analyses

All data are presented as means ± 1 standard error (SE). Data for Fig 6.1 were analyzed by an unpaired Student's two-tailed t-test. Data for Figures 6.3-6.8 and 6.10-6.14 were analyzed by two-way ANOVA followed by Fishers LSD test for multiple comparisons where the F-value of the ANOVA indicated significance. 5% was accepted as the fiducial limit of significance in all cases.

RESULTS

Comparison of the Gill Morphology in Trout and Eel

An examination of the gill epithelial morphology under control conditions showed that there were distinct differences between trout and eel. Eels displayed significantly smaller (Fig 6.1a) and fewer (Fig 6.1b) chloride cells on the filament surface compared to trout. The discrepancy was much greater for average CC area (trout - $34.62 \pm 2.01 \mu\text{m}^2$ versus eel - $10.23 \pm 0.48 \mu\text{m}^2$) compared to density (trout - $2368 \pm 200 /\text{mm}^2$ versus eel $1912 \pm 92 /\text{mm}^2$). These two variables combine to yield a smaller CCFA in eel ($21355 \pm 981 \mu\text{m}^2/\text{mm}^2$; Fig 6.1c) when compared to trout ($83951 \pm 9789 \mu\text{m}^2/\text{mm}^2$).

Representative SEM photographs that depict the differences in gill morphology between trout and eel are shown in Fig 6.2. In addition to the obvious differences in size, note that the eels display a much greater degree of microvilli elaboration on the surface of the CC (Fig 6.2d) compared to those of trout (Fig 6.2b). These extensive vertical projections allowed for easy identification of the CC from adjacent PVCs which displayed membrane microridges rather than microvilli.

Series 1 - Blood Acid-Base Status During Infusion

a) Rainbow trout (Fig 6.3)

There were no significant changes in the blood/plasma acid-base variables pH, $[\text{HCO}_3^-]$ or P_{CO_2} during NaCl (sham) infusion whereas infusion of NaHCO_3 caused a severe metabolic alkalosis coupled with an elevation in P_{CO_2} . Blood pH rose significantly from a control value of 8.05 ± 0.01 to 8.53 ± 0.04 by 8 h as a result of NaHCO_3 infusion (Fig 6.3a). The increase was significantly greater than control (Pre) by the 1 h period (indicated by an *) and significantly greater than the NaCl (sham) infused group at every point other than the Pre (control) and 24 h post-infusion periods (indicated by a +). The

FIGURE 6.1

Comparison of the gill filament chloride cell (CC) morphometry including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area for rainbow trout (*Oncorhynchus mykiss*) and American eel (*Anguilla rostrata*). Mean \pm SE.

* significantly different from trout, $P < 0.05$.

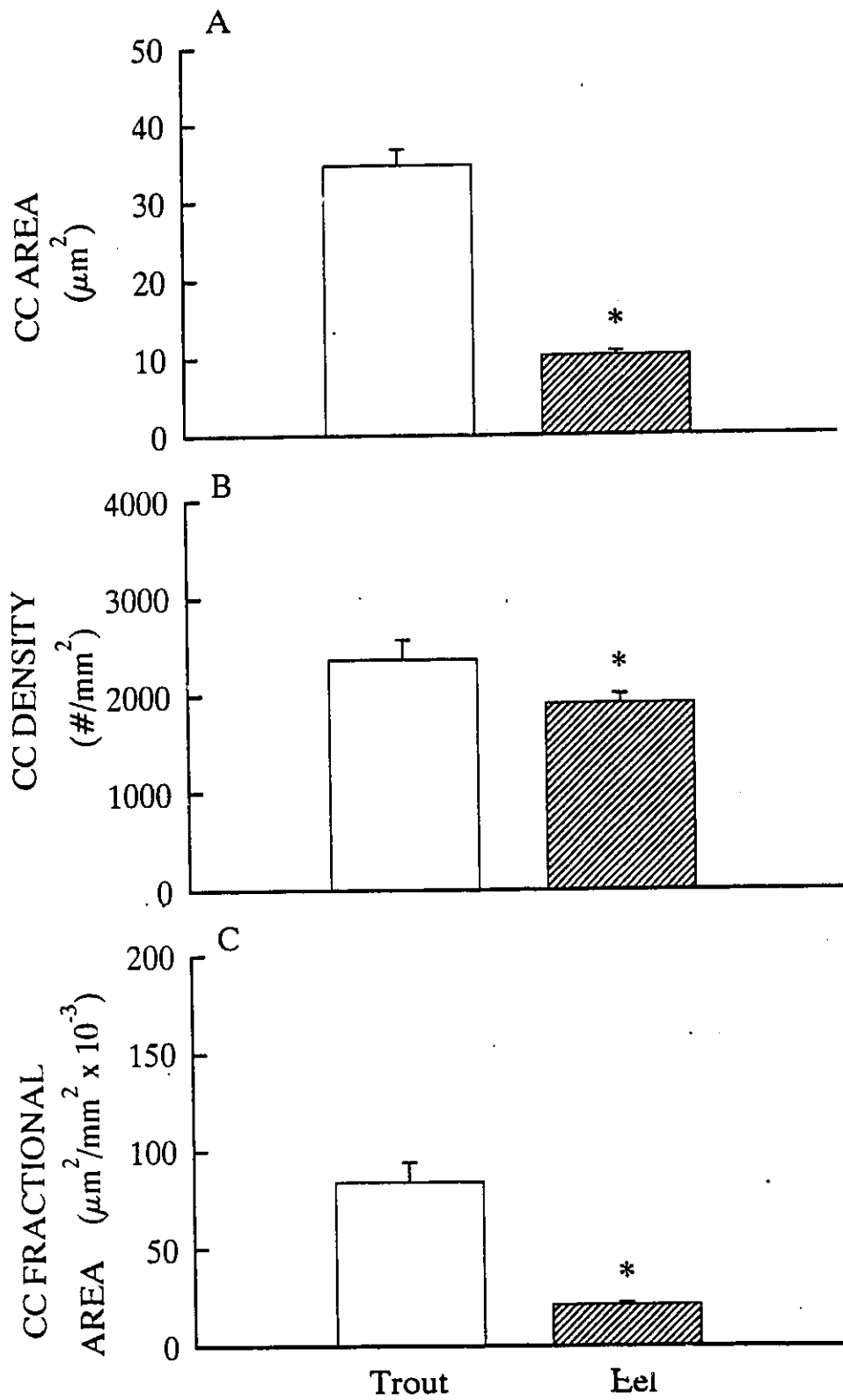


FIGURE 6.2

Representative low and high magnification scanning electron microscope (SEM) photographs of the filament epithelium of trout (Fig 6.2a, b) and eel (Fig 6.2c, d). Note the smaller average size of the chloride cell (CC) in the eel when compared to the trout. White arrowheads = CC, MC = mucous cells Fig 6.2 a, c scale bars = 10 μm , Fig 6.2b, b scale bars = 2 μm .

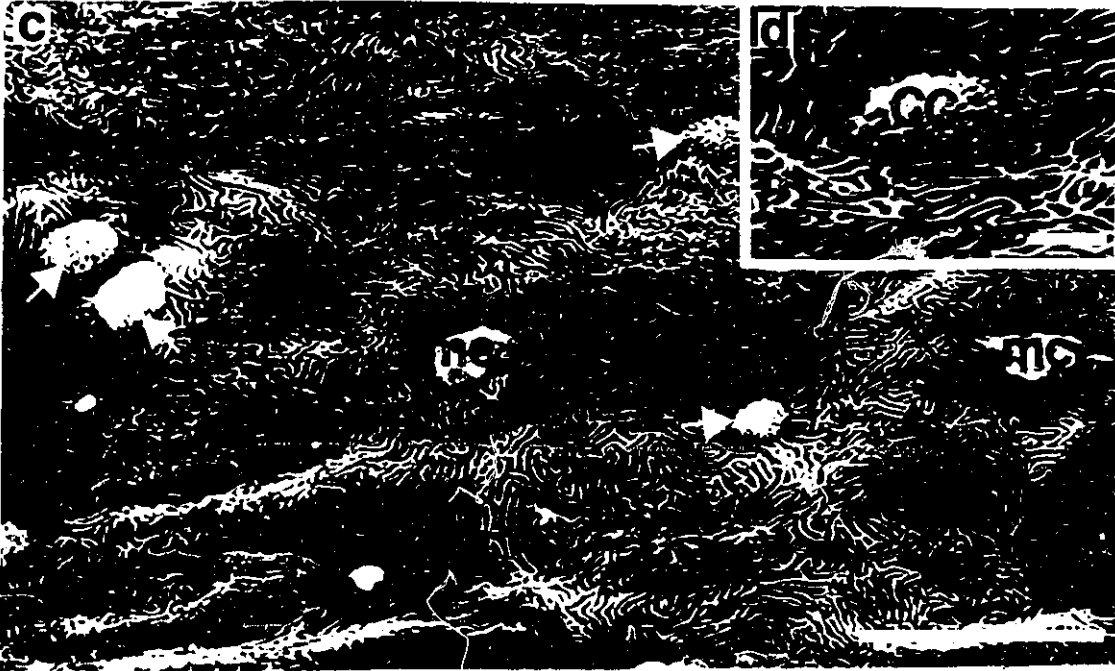
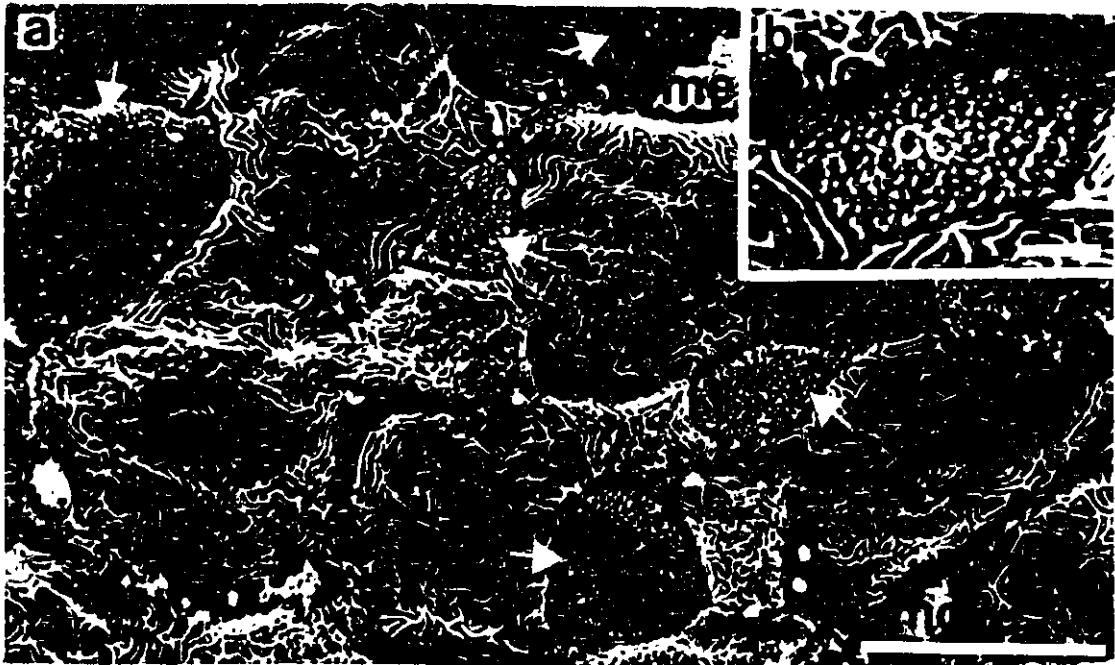
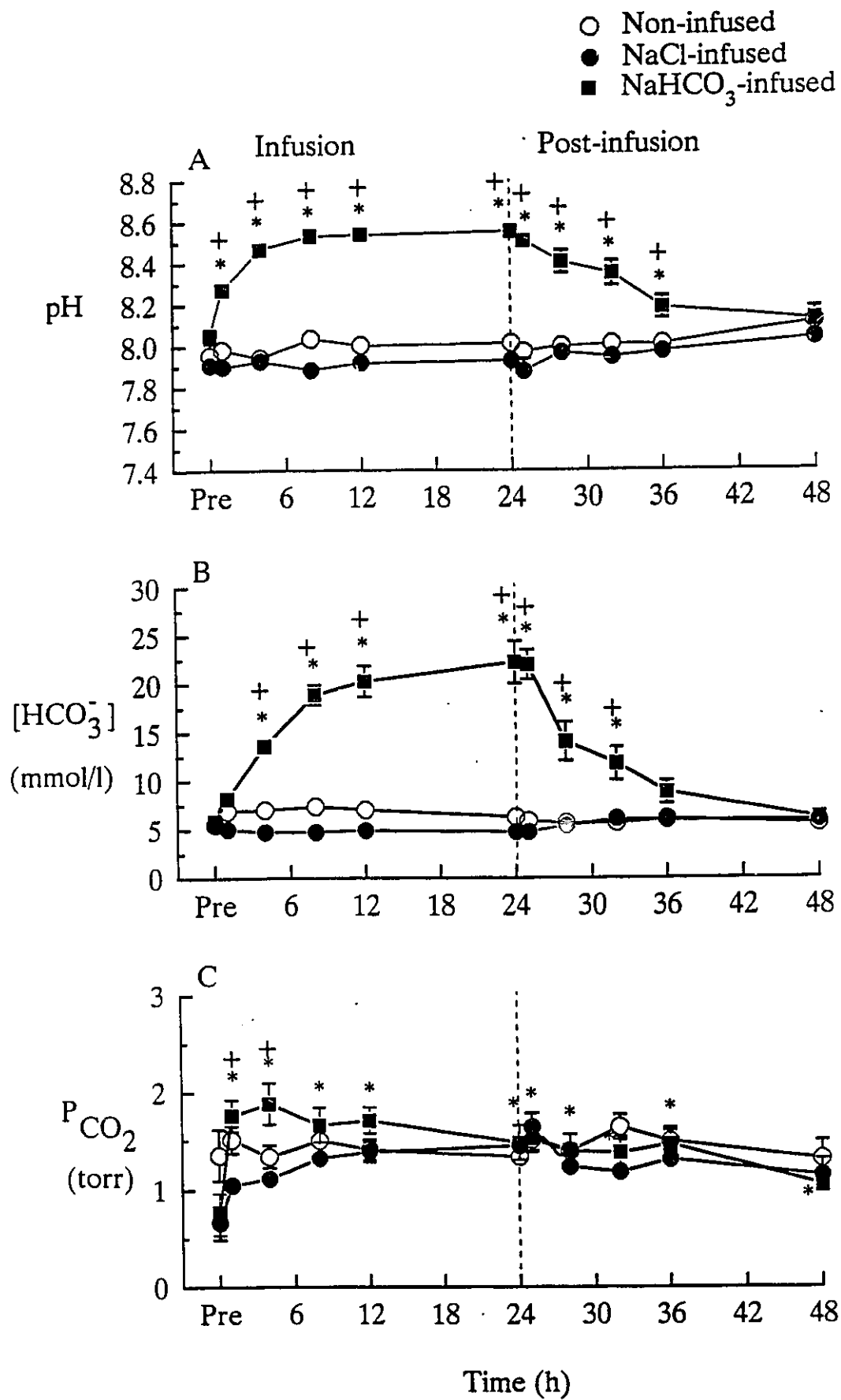


FIGURE 6.3

The temporal effects of 24 h of infusion of NaCl [filled circles (●)] or NaHCO₃ [filled squares (■)] and the subsequent 24 h post-infusion period on the acid-base parameters in rainbow trout (A) blood pH, (B) plasma [HCO₃⁻], and (C) blood P_{CO₂}. Non-infused controls are indicated by open circles (○). * indicates significant difference from the initial non infused control (Pre) value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean ± SE.



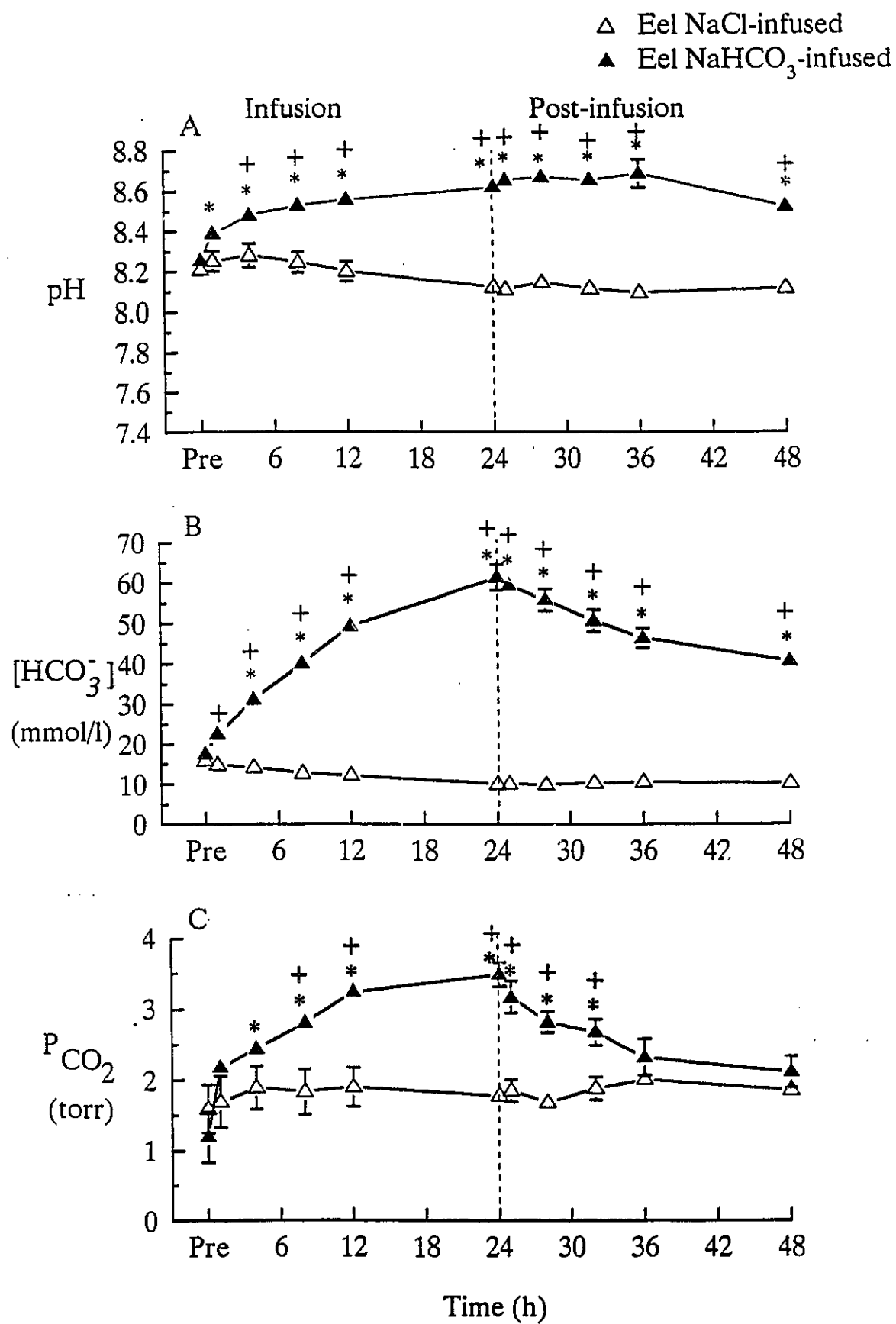
increases in blood pH in the NaHCO₃ infused trout were caused by large increase in plasma [HCO₃⁻] (Fig 6.3b). There were steady increases in plasma [HCO₃⁻] from a Pre value of 5.85 ± 0.44 to 19.0 ± 1.01 mmol/l by the 8 h period. There were only slight increases in plasma [HCO₃⁻] after the 8 h period and pH was not further elevated after the 8 h period. In the post-infusion period, blood pH had slowly returned to Pre values by the 48 h period (24 h recovery) through the removal of HCO₃⁻ from the extracellular compartment (Fig 6.3b).

b) American eel (Fig 6.4)

There were no significant changes in the blood/plasma acid-base status in NaCl (sham)-infused eels. As in trout, infusion of NaHCO₃ resulted in a severe metabolic alkalosis coupled with an elevated P_{CO₂}. Blood pH rose from a Pre value of 8.25 ± 0.01 to 8.56 ± 0.03 by 12 h; thereafter, there were only small increases in the pH. The elevation in pH was caused by a striking rise in the plasma [HCO₃⁻]. [HCO₃⁻] rose from a Pre value of 17.45 ± 1.02 mmol/l to 61.4 ± 3.22 mmol/l by the 24 h infusion period (compare with trout = ~21 mmol/l maximal value). Furthermore, the [HCO₃⁻] was still increasing after 24 h of infusion. During the post-infusion period, blood pH remained elevated in the NaHCO₃-infused eels (Fig 6.4a) even though plasma [HCO₃⁻] decreased significantly (Fig 6.4b). Despite the decline of plasma [HCO₃⁻] from 61.4 ± 3.22 (24 h) to 40.5 ± 1.74 mmol/l by the 48 h period, plasma [HCO₃⁻] remained elevated above Pre values at the conclusion of the 24 h post-infusion period. Plasma P_{CO₂} fell in the post-infusion period and by the 36 h period, it was not significantly different from that of NaCl-infused eels (Fig 6.4c).

FIGURE 6.4

The temporal effects of 24 h of chronic infusion of NaCl [open triangles (Δ)] or NaHCO₃ [filled triangles (\blacktriangle)] and the subsequent 24 h post-infusion period on the acid-base parameters in American eel (A) blood pH, (B) plasma [HCO₃⁻], and (C) blood P_{CO₂}. * indicates significant difference from the initial Pre (control) value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.



Series 2 - Ionic-Acidic/Basic Equivalent Fluxes and Gill Morphology During Infusion

a) Rainbow trout

Ionic-Acidic/Basic Equivalent Fluxes

Infusion of NaCl or NaHCO₃ resulted in a large, negative $J_{\text{net}}^{\text{Na}^+}$ (*i.e.* Na⁺ loss) reaching approximately -300 to -400 μmol/kg/h by the 24 h infusion period (Fig 6.5). There were no significant differences between the NaCl- and NaHCO₃-infused fish throughout the infusion period. In the post-infusion period, $J_{\text{net}}^{\text{Na}^+}$ remained highly negative in both groups. $J_{\text{net}}^{\text{Cl}^-}$ also was significantly altered in the NaCl-infused fish resulting in a large loss of Cl⁻ from the fish to the water of ~-200 μmol/kg/h by the 24 h infusion period (Fig 6.5); in NaHCO₃-infused trout, this trend was reversed. $J_{\text{net}}^{\text{Cl}^-}$ remained significantly positive (*i.e.* Cl⁻ gain) in the post-infusion period in NaHCO₃-infused fish and remained significantly negative in the NaCl-infused fish (Fig 6.5b).

The alterations in $J_{\text{net}}^{\text{Na}^+}$ and $J_{\text{net}}^{\text{Cl}^-}$ (Fig 6.5) were largely caused by manipulation of the influx (J_{in}) component to ion balance. Similar reductions in $J_{\text{in}}^{\text{Na}^+}$ occurred in both the NaCl- and NaHCO₃-infused trout during the infusion period from a Pre value of 158 ± 22 μmol/kg/h to <50 μmol/kg/h after 24 h. In addition, $J_{\text{in}}^{\text{Na}^+}$ remained reduced throughout the post-infusion period in both treatment groups (Fig 6.6). There were no significant differences in $J_{\text{in}}^{\text{Na}^+}$ between the two groups throughout the infusion/post-infusion regime. $J_{\text{in}}^{\text{Cl}^-}$ was reduced during NaCl-infusion (Fig 6.6) to account for the increase in net Cl⁻ loss observed (Fig 6.5). However, in NaHCO₃-infused trout, $J_{\text{in}}^{\text{Cl}^-}$ was nearly doubled from a Pre value of 64 ± 7 μmol/kg/h to 118 ± 13 μmol/kg/h by 12 h. The changes in $J_{\text{in}}^{\text{Cl}^-}$ caused by NaCl or NaHCO₃ infusion persisted throughout the post-infusion period (Fig 6.6).

There was a slight stimulation of net H⁺ loss (negative $J_{\text{net}}^{\text{H}^+}$) in NaCl-infused

FIGURE 6.5

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on the net fluxes of sodium ($J_{\text{net}}^{\text{Na}^+}$) and chloride ($J_{\text{net}}^{\text{Cl}^-}$) in rainbow trout. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.

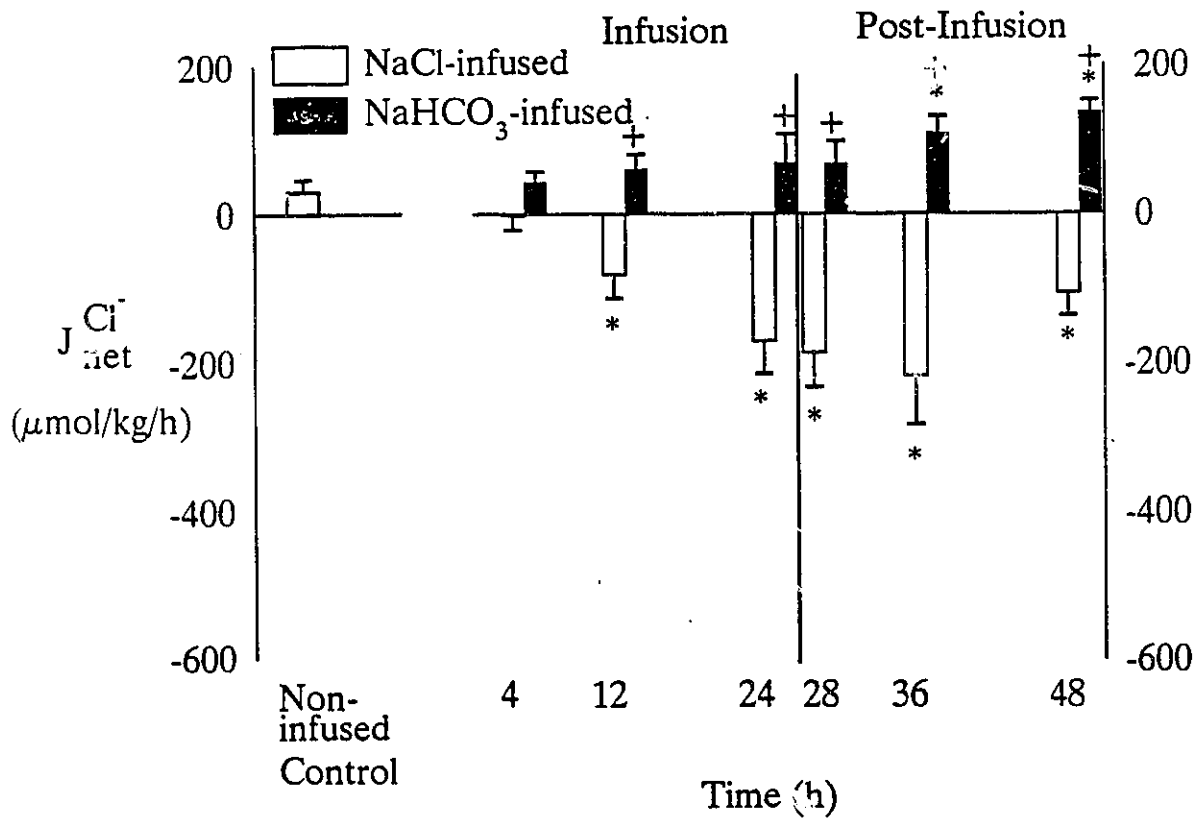
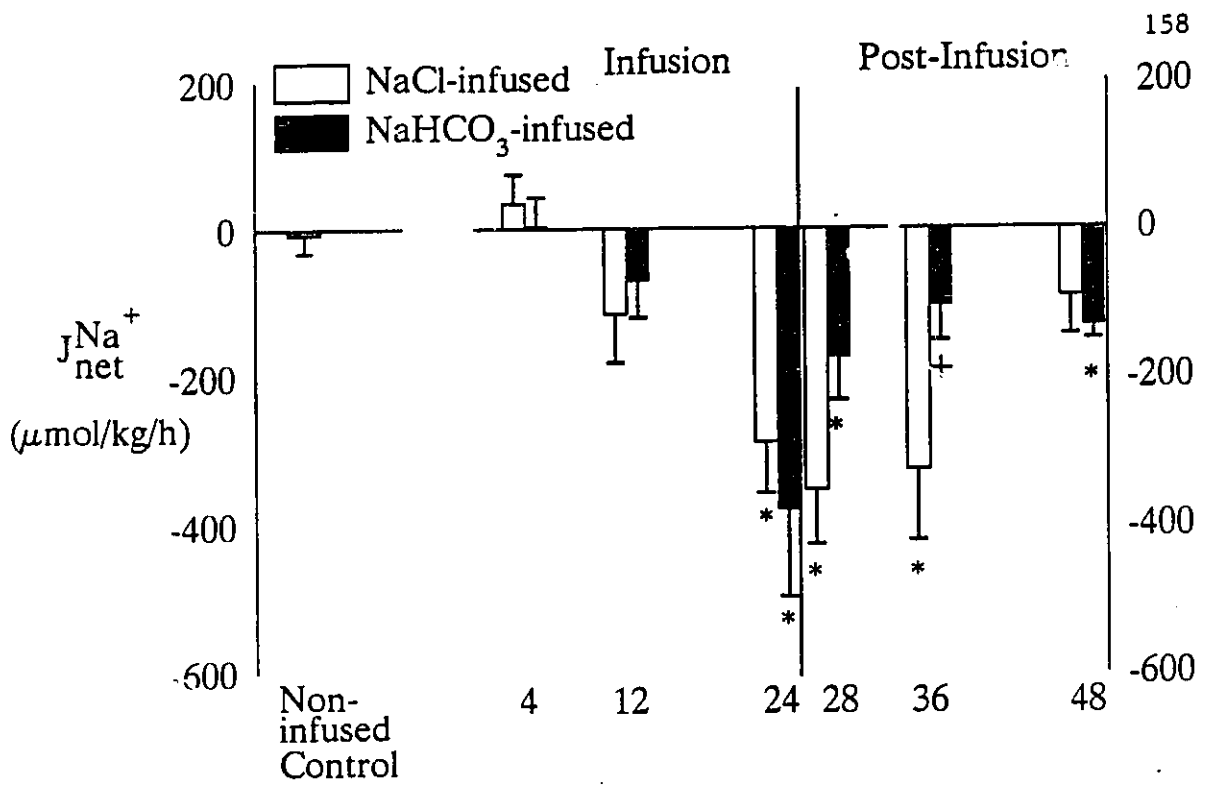


FIGURE 6.6

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on the influxes of sodium ($J_{in}^{Na^+}$) and chloride ($J_{in}^{Cl^-}$) in rainbow trout. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.

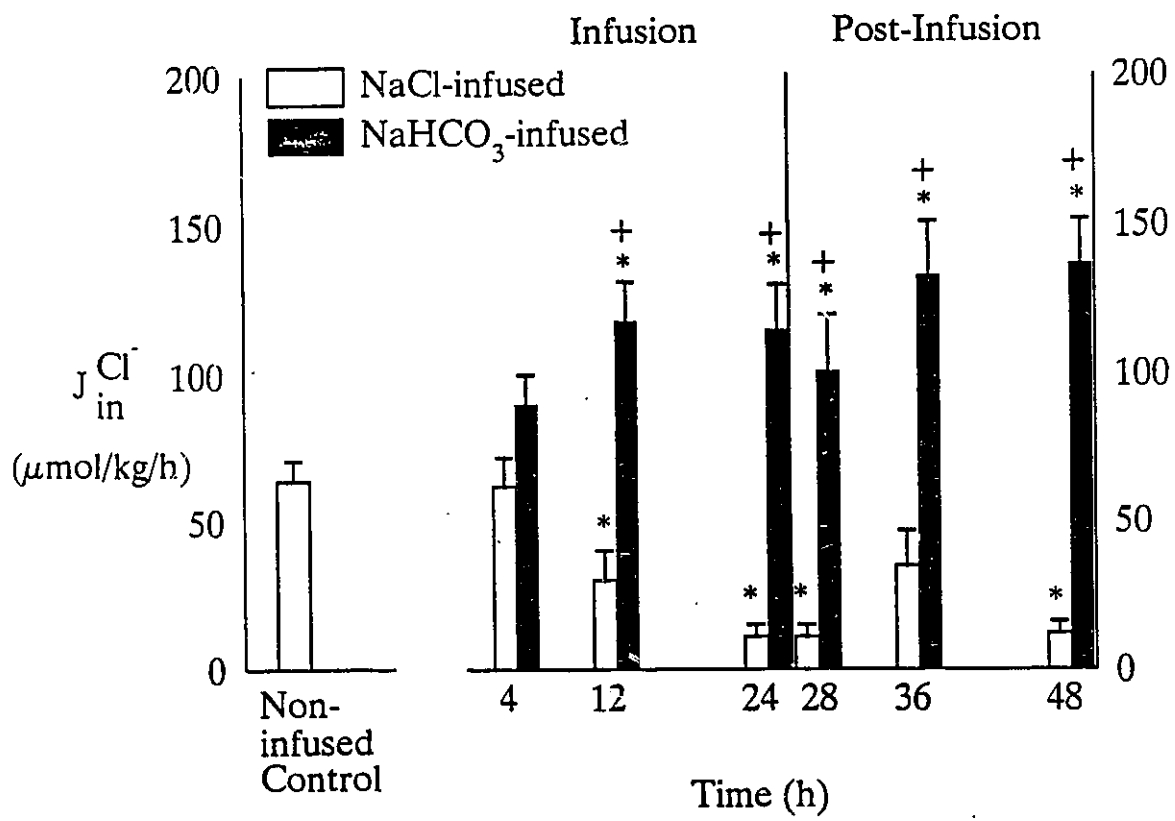
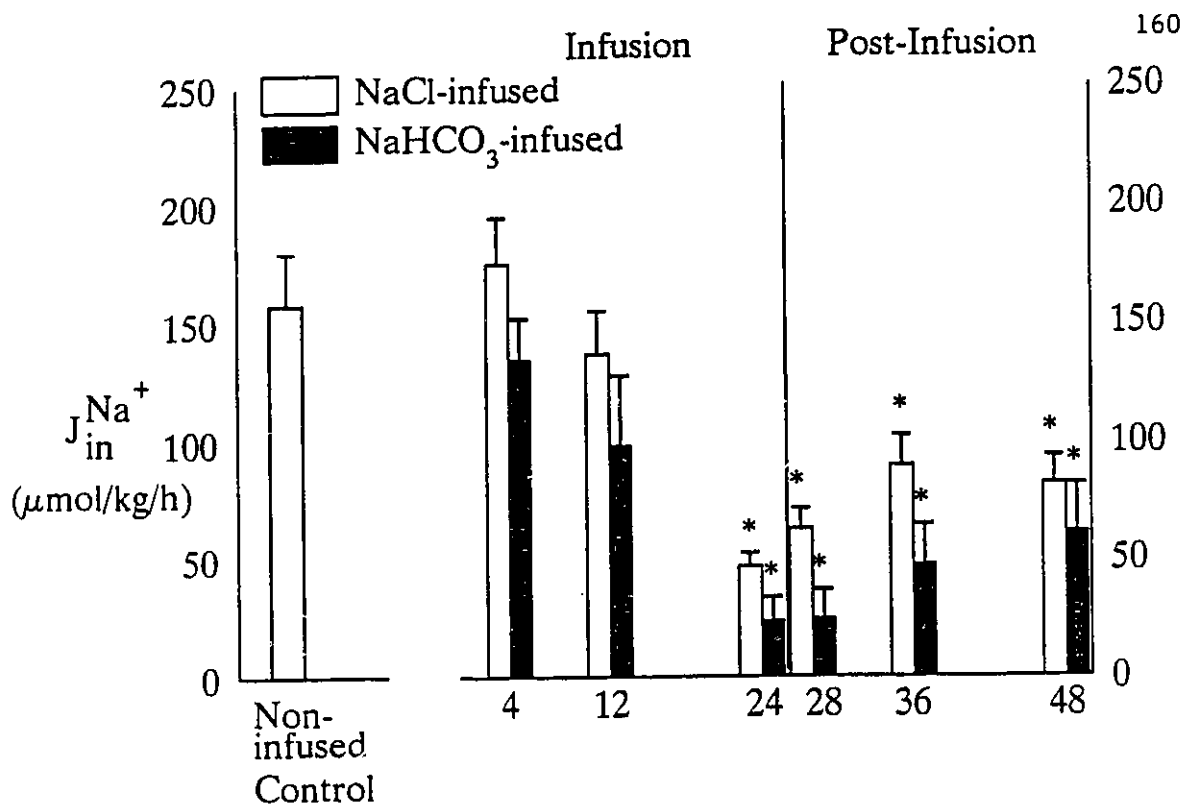
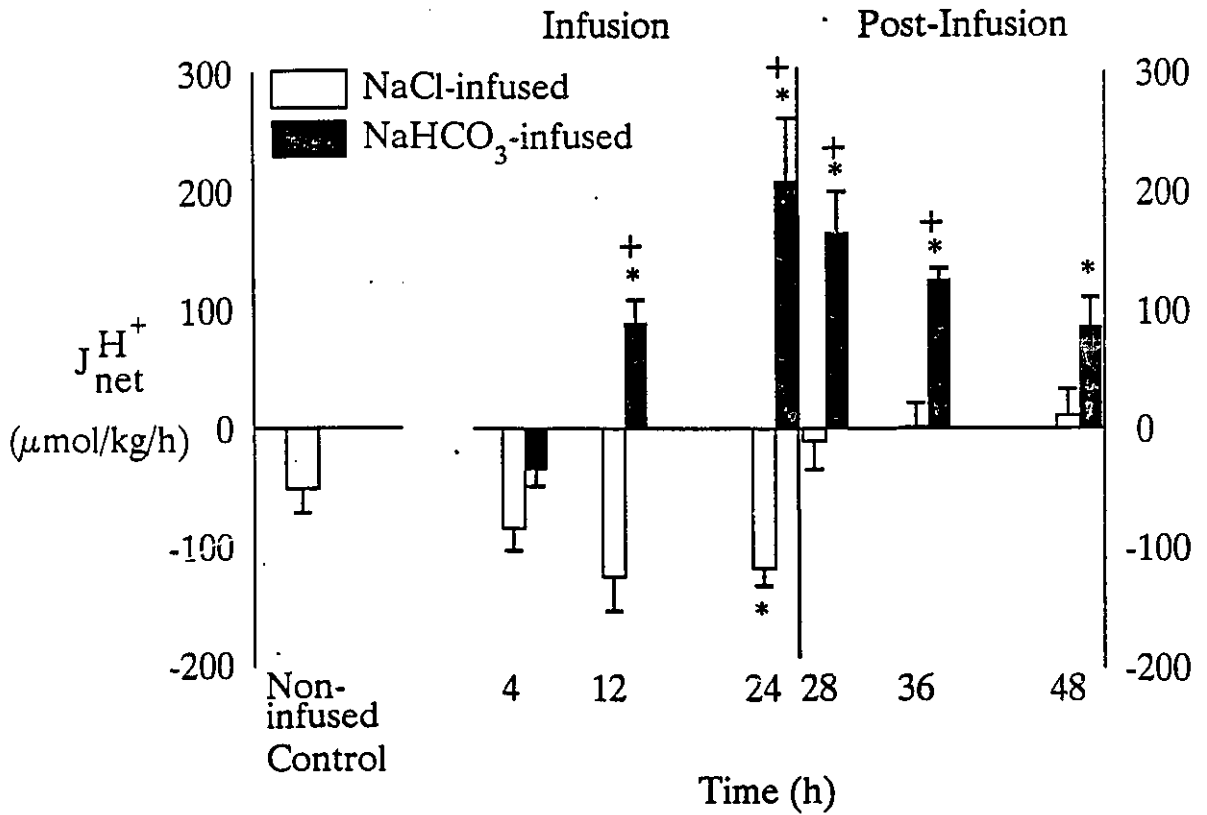
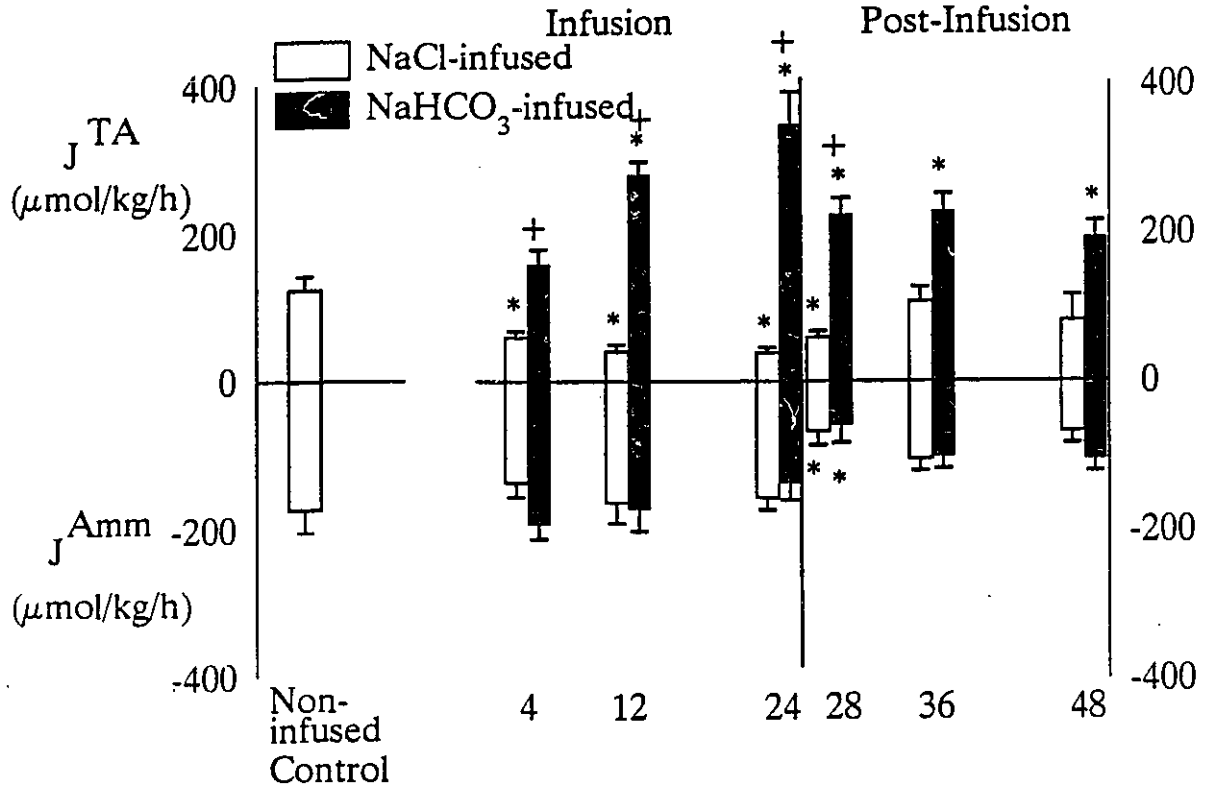


FIGURE 6.7

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on whole body flux rates of titratable alkalinity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents ($J_{net}^{H^+}$) in rainbow trout. Net acidic equivalent flux ($J_{net}^{H^+}$) is calculated as the sum of the two components, J^{TA} and J^{Amm} , signs considered. Positive values indicate acidic equivalent uptake (or base excretion) while negative values indicate acidic equivalent excretion. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.



trout (significant at 24 h) that resulted from a decrease in J^{TA} (Fig 6.7). On the other hand, NaHCO_3 infusion was associated with an increase in net H^+ gain (positive $J_{\text{net}}^{\text{H}^+}$) of approximately 100-300 $\mu\text{mol/kg/h}$ that resulted entirely from elevations in J^{TA} ; these effects persisted throughout the post-infusion period (Fig 6.7).

Gill Morphology

Infusion of NaCl resulted in a 25% decrease in CCFA from $83951 \pm 9789 \mu\text{m}^2/\text{mm}^2$ in the non-infused control to $64659 \pm 6480 \mu\text{m}^2/\text{mm}^2$ by the 24 h period (Fig 6.8c). The reductions in CCFA were the combined result of small, non-significant decreases in CC area (Fig 6.8a) and CC density (Fig 6.8c). In contrast, NaHCO_3 -infusion resulted in a 100% increase in CCFA by the 12 h infusion period (Fig 6.8c) owing exclusively to increases in average CC area (Fig 6.8a). The post-infusion period was marked by a persistent elevation of CCFA in the NaHCO_3 -infused fish while in NaCl -infused fish, there were further reductions in CCFA resulting entirely from decreases in the average CC area (Fig 6.8a).

Representative low and high magnification SEM photographs showing the effects of NaCl - or NaHCO_3 infusion on the gill external morphology are shown in Fig 6.9. The CCs in the NaCl -infused group were small and difficult to recognize but could be identified with confidence under close scrutiny (Fig 6.9b, e). In addition to the increases in CCFA noted during NaHCO_3 infusion (Fig 6.9c), there were pronounced differences in the surface morphologies of CCs displayed on the filament epithelium. The CCs after 24 h of infusion displayed a wide array of morphologies ranging from sparse small microvilli (Fig 6.9f) to densely packed vertical microridges (Fig 6.9g).

FIGURE 6.8

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on gill filament chloride cell (CC) morphometry in rainbow trout including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean ± SE.

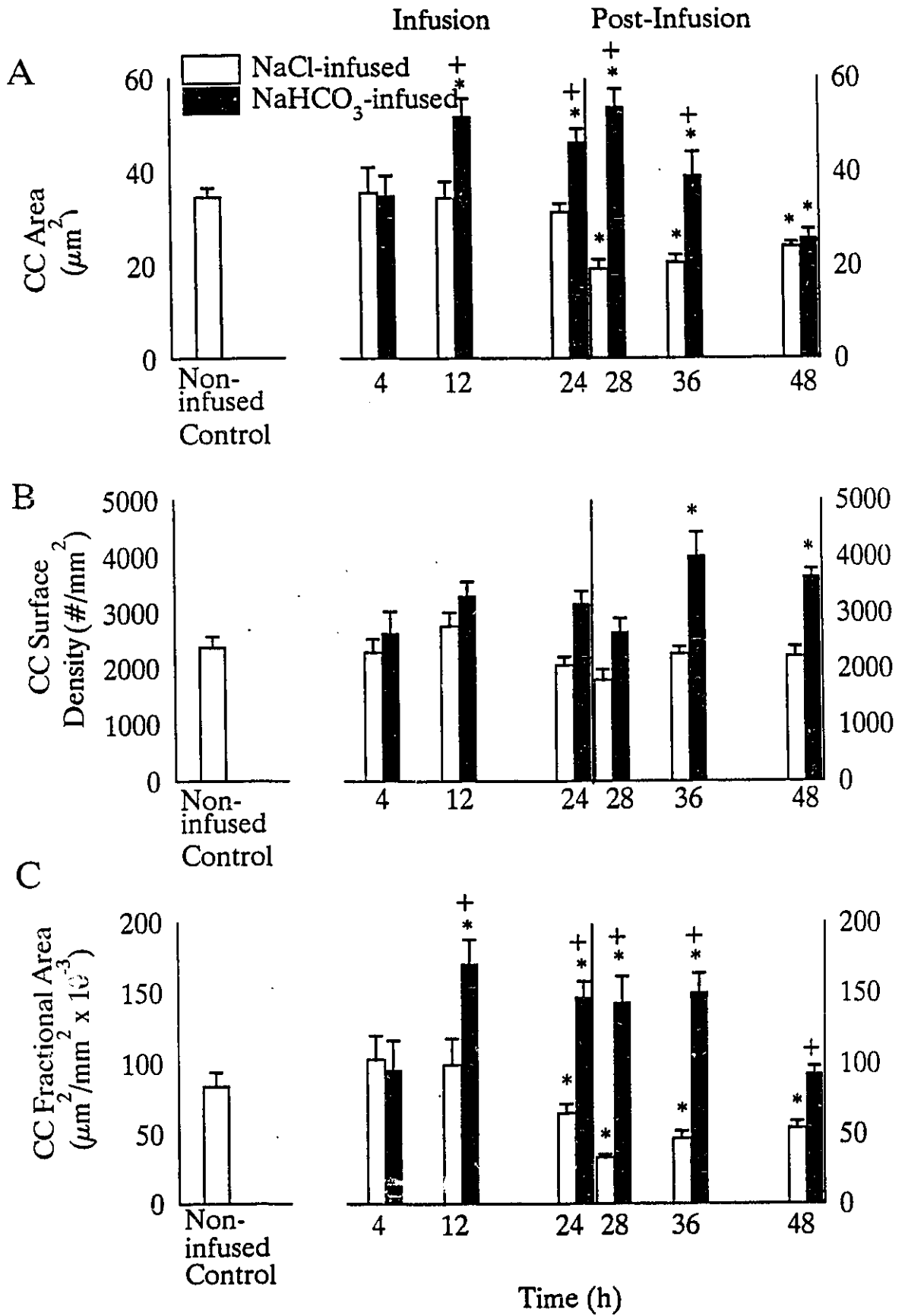
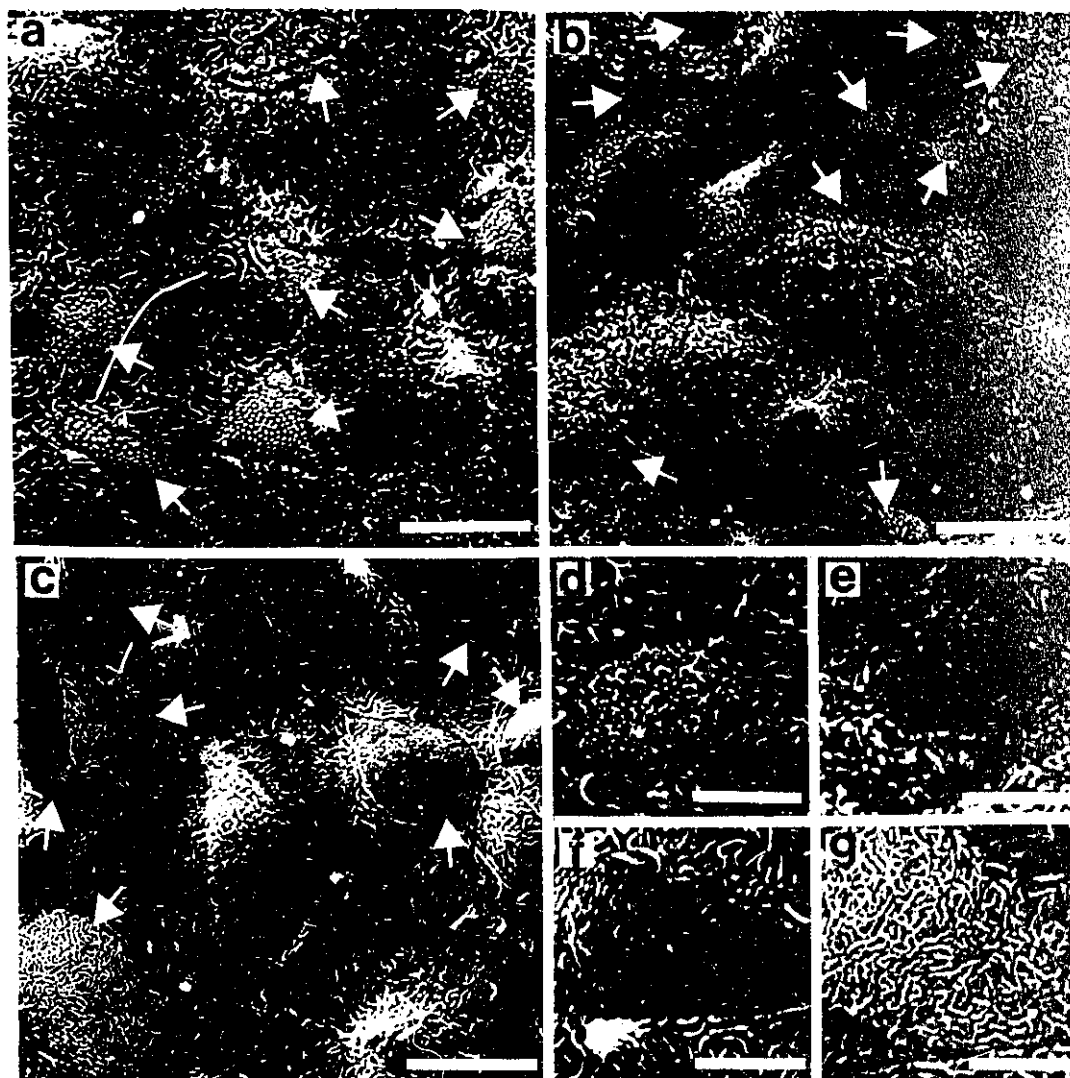


FIGURE 6.9

Representative low and high magnification scanning electron microscopy (SEM) photographs of the filament epithelium of rainbow trout under control (non-infused) conditions (Fig 6.8a, d), after 24 h NaCl infusion (Fig 6.8b, e), or after 24 h infusion of NaHCO₃ (Fig 6.8c, f, g). Note the reduction in the CC apical surface area in NaCl-infused fish corresponding to a reduced CC fractional area (CCFA) at this time while NaHCO₃ infusion resulted in large increases in the average area of the CCs and a corresponding increase in CCFA. In addition, during NaHCO₃ infusion, at least two distinct surface morphologies of CCs were noted. One had sparse microvilli present (Fig 6.8f) while the other displayed a dense network of apical microridges (Fig 6.8g). White arrowheads indicate chloride cells. Figs 6.8 a-c; scale bar = 10 μ . Figs 6.8 d-g, scale bar = 5 μ m.



b) American Eel

Ionic-Acidic/Basic Equivalent Fluxes

Infusion of NaCl resulted in large and equivalent Na^+ and Cl^- losses of approximately -800 to -900 $\mu\text{mol/kg/h}$ by the end of the infusion period (Fig 6.10). The net fluxes were returned to Pre values by 48 h (24 h post-infusion). In the NaHCO_3 -infused eels, $J_{\text{net}}^{\text{Na}^+}$ was not significantly different from NaCl-infused eels at any measurement period. On the other hand, net Cl^- loss ($-J_{\text{net}}^{\text{Cl}^-}$) was significantly lower in the NaHCO_3 -infused eels compared to NaCl-infused eels at every time period measured other than at 4 h infusion and 48 h post-infusion (Fig 6.10). The reduced losses of Cl^- in NaHCO_3 -infused eels resulted in a preferential net loss of Na^+ over Cl^- during infusion and recovery.

In contrast to trout, where manipulations of $J_{\text{net}}^{\text{Na}^+}$ and $J_{\text{net}}^{\text{Cl}^-}$ were mainly caused by adjustments of ionic influx, the changes in $J_{\text{net}}^{\text{Na}^+}$ and $J_{\text{net}}^{\text{Cl}^-}$ in eel were largely caused by adjustments of ionic efflux. Owing to the near absence of a significant influx component ($J_{\text{in}}^{\text{Cl}^-}$) for net Cl^- balance ($J_{\text{in}}^{\text{Cl}^-}$) and relatively low levels for Na^+ ($J_{\text{in}}^{\text{Na}^+}$), manipulation of $J_{\text{net}}^{\text{Cl}^-}$ and $J_{\text{net}}^{\text{Na}^+}$ in eels effectively represents the alterations in the efflux component to net ion balance. NaCl-infusion did not affect $J_{\text{in}}^{\text{Na}^+}$ in eels as in trout. NaHCO_3 infusion, however, caused a rapid and pronounced drop in $J_{\text{in}}^{\text{Na}^+}$ to a value near zero by the 24 h period; $J_{\text{in}}^{\text{Na}^+}$ remained low throughout the entire post-infusion period (Fig 6.11). $J_{\text{in}}^{\text{Cl}^-}$ was extremely low under control conditions (Fig 6.11: $2 \pm 2 \mu\text{mol/kg/h}$) compared to the values in trout (Fig 6.6: $64 \pm 7 \mu\text{mol/kg/h}$). In contrast to trout, $J_{\text{in}}^{\text{Cl}^-}$ remained at extremely low levels (Fig 6.11) throughout the infusion and post-infusion period in both the NaCl- and NaHCO_3 -infused eels.

Infusion of NaCl or NaHCO_3 produced markedly different responses in the rates

FIGURE 6.10

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on the net flux of sodium ($J_{\text{net}}^{\text{Na}^+}$) or chloride ($J_{\text{net}}^{\text{Cl}^-}$) in American eel. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.

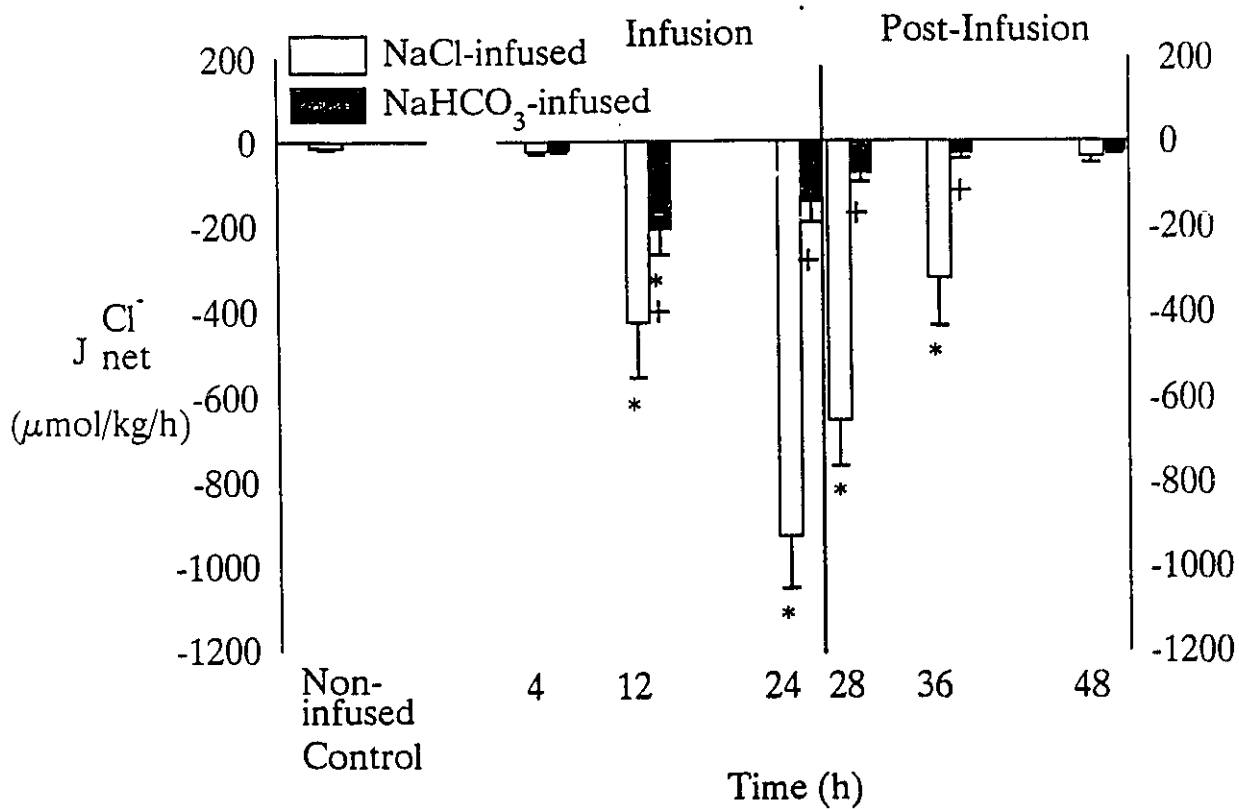
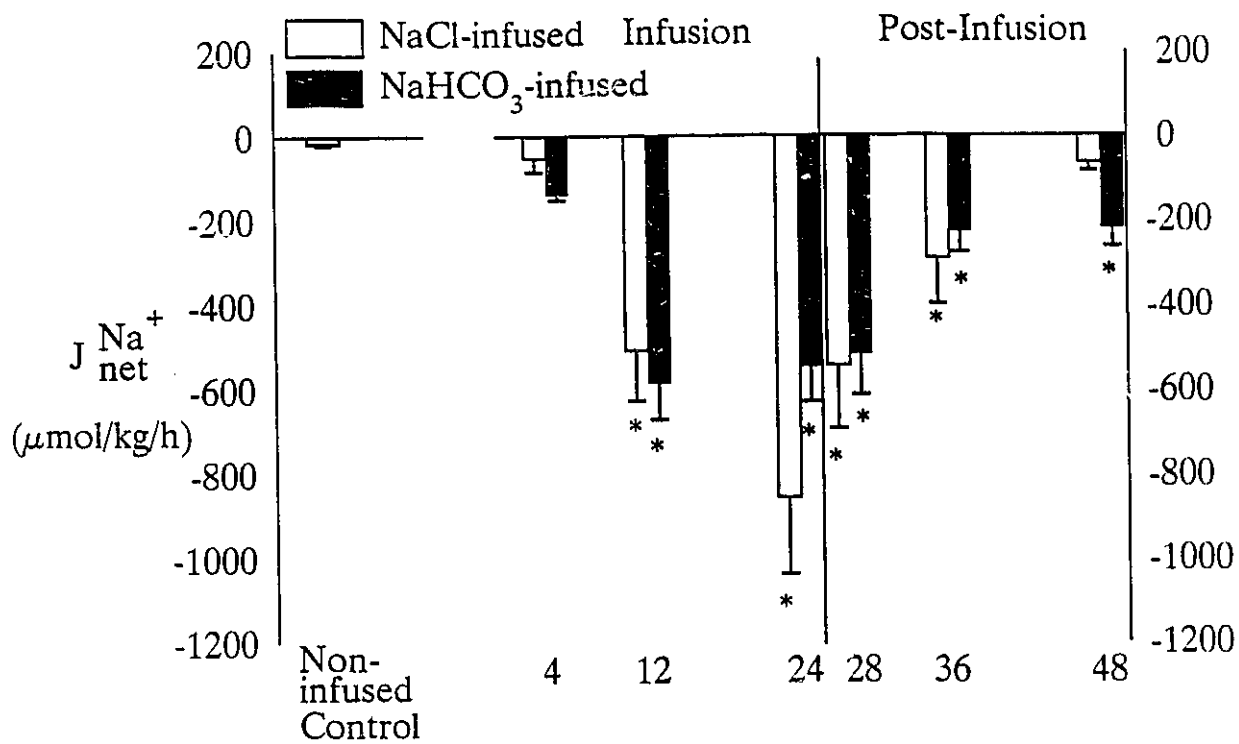
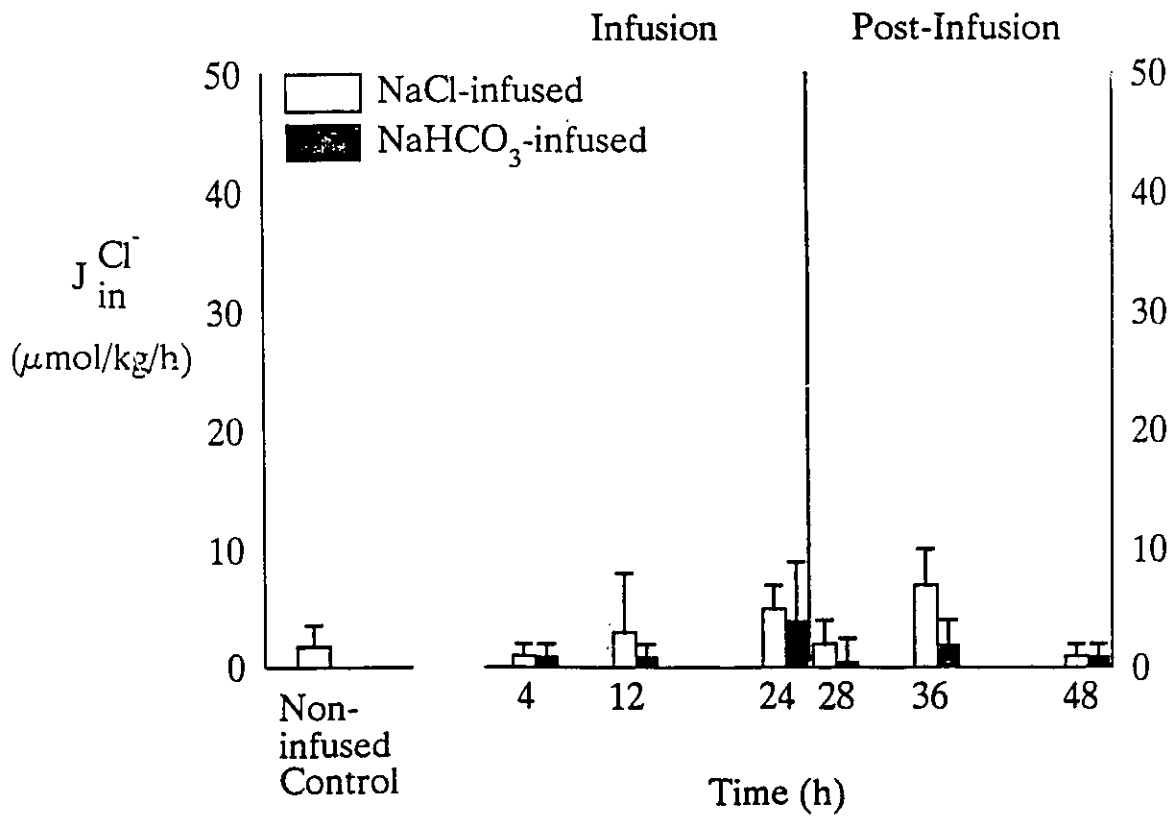
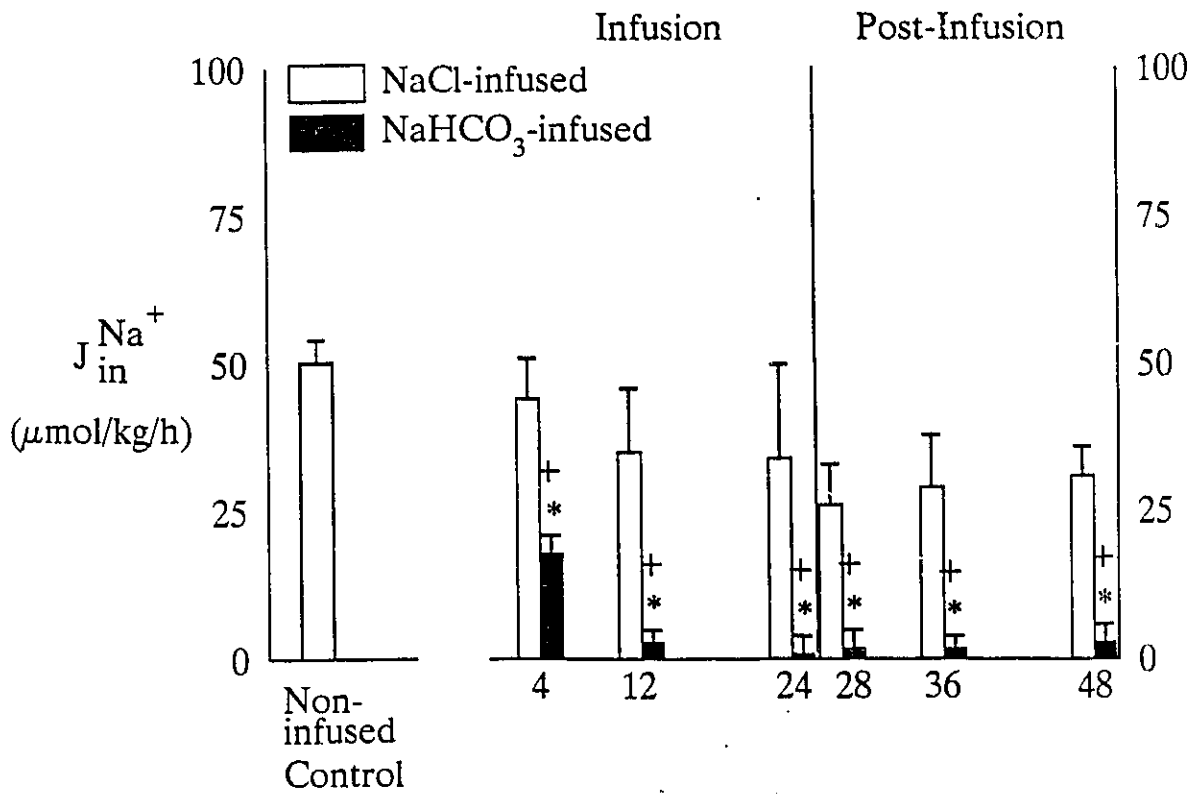


FIGURE 6.11

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on the influx of sodium ($J_{in}^{Na^+}$) or chloride ($J_{in}^{Cl^-}$) in American eel. * indicates significant difference from the initial non-infused control value. Note the extremely low values for $J_{in}^{Cl^-}$. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.



of net H^+ transfer. The NaCl-infused eels showed a maintained net H^+ loss that was not significantly different from non-infused controls (Fig 6.12). Correspondingly, there were no significant alterations in either J^{TA} or J^{Amm} , the components of net H^+ flux. $NaHCO_3$ infusion stimulated net H^+ uptake (positive $J_{net}^{H^+}$) reaching approximately $150 \mu\text{mol/kg/h}$ by 24 h. $J_{net}^{H^+}$ remained significantly elevated throughout the post-infusion period. These increases were almost entirely owing to changes in J^{TA} with a significant reduction in J^{Amm} noted only during the 4 h post-infusion period.

Gill Morphology

There were no significant changes in gill CCFA associated with either NaCl or $NaHCO_3$ infusion (Fig 6.13c) although there were significant increases in CC surface area in NaCl-infused eels (Fig 6.13a) and significant decreases in CC density in $NaHCO_3$ -infused eels (Fig 6.13b). Consequently, there was a significant difference in CCFA between NaCl- and $NaHCO_3$ -infused eels (Fig 6.13c). An important point, however, is that the absolute values of CCFA in eels ($21355 \pm 981 \mu\text{m}^2/\text{mm}^2$; Fig 6.13c) are far lower than those seen in trout ($83951 \pm 9789 \mu\text{m}^2/\text{mm}^2$; Fig 6.8c) and hence, the absolute value of the variations in CCFA in eels are negligible. Representative SEM photographs of the eel filamental epithelium in A) control B) NaCl-infused and C) $NaHCO_3$ infused eels are shown in Fig 6.14. The external appearance of the CCs in eels did not change as a result of either NaCl or $NaHCO_3$ infusion in contrast to the changes noted in trout (Fig 6.9).

FIGURE 6.12

The temporal effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) and the subsequent 24 h post-infusion period on whole body flux rates of titratable acidity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents ($J_{net}^{H^+}$) in American eel. Net acidic equivalent flux ($J_{net}^{H^+}$) is calculated as the sum of the two components, J^{TA} and J^{Amm} , signs considered. Positive values indicate acidic equivalent uptake (or base excretion) while negative values indicate acidic equivalent excretion. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.

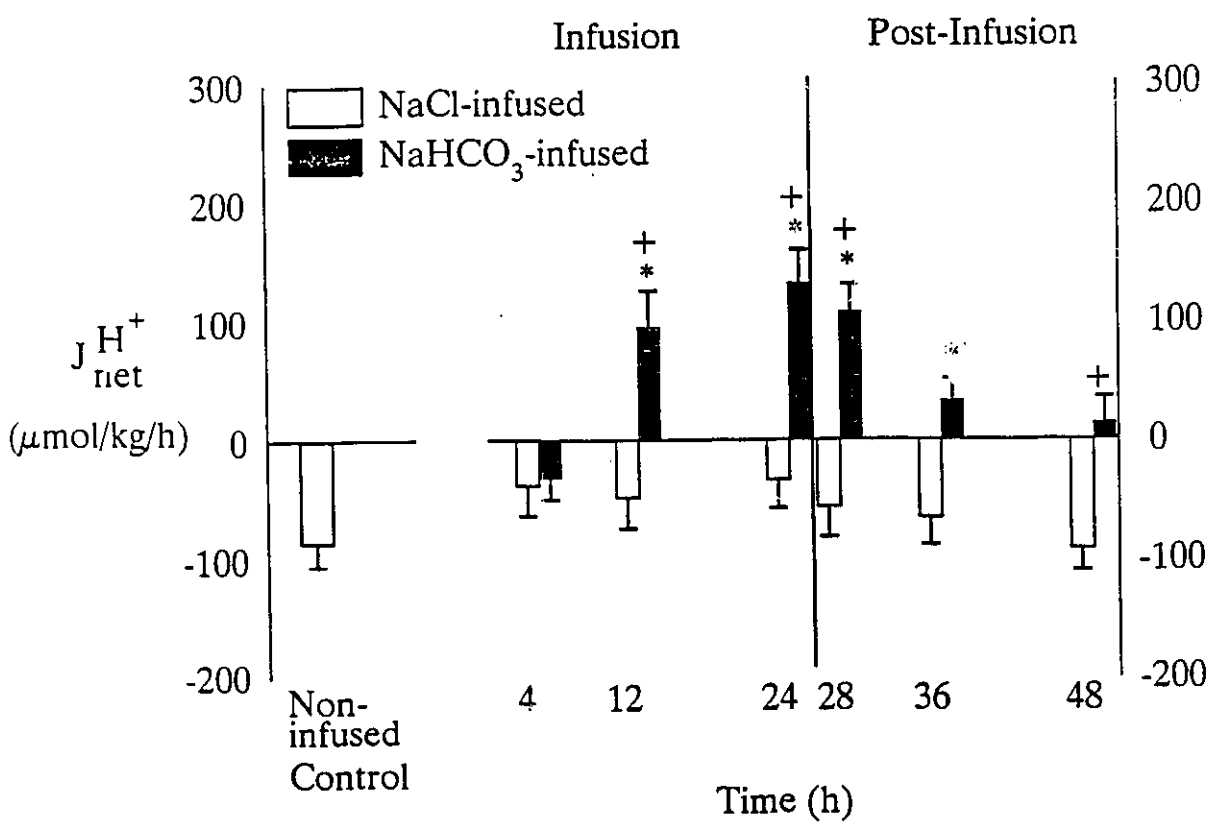
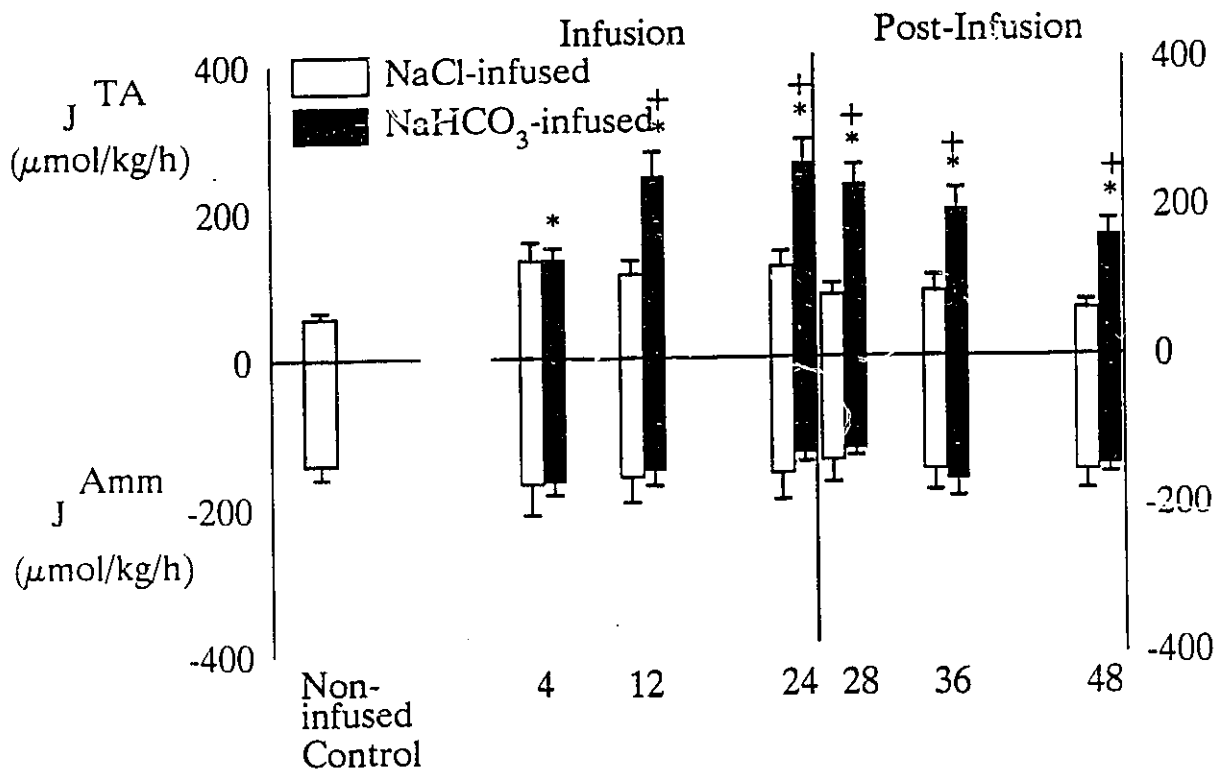


FIGURE 6.13

The effects of 24 h of chronic infusion of NaCl (open histograms) or NaHCO₃ (filled histograms) on gill filamental chloride cell (CC) morphometry in American eel including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO₃-infused only). Mean \pm SE.

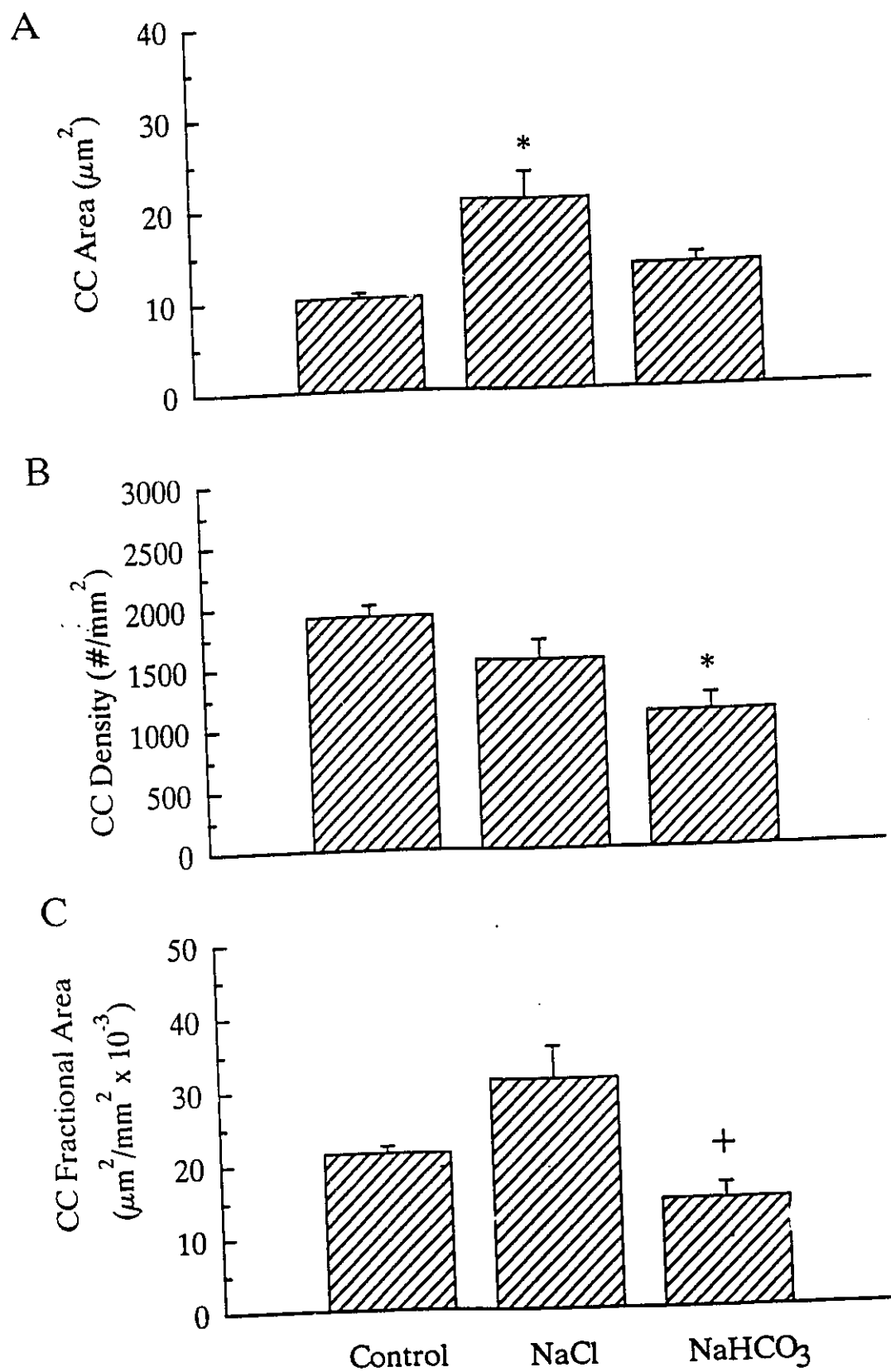
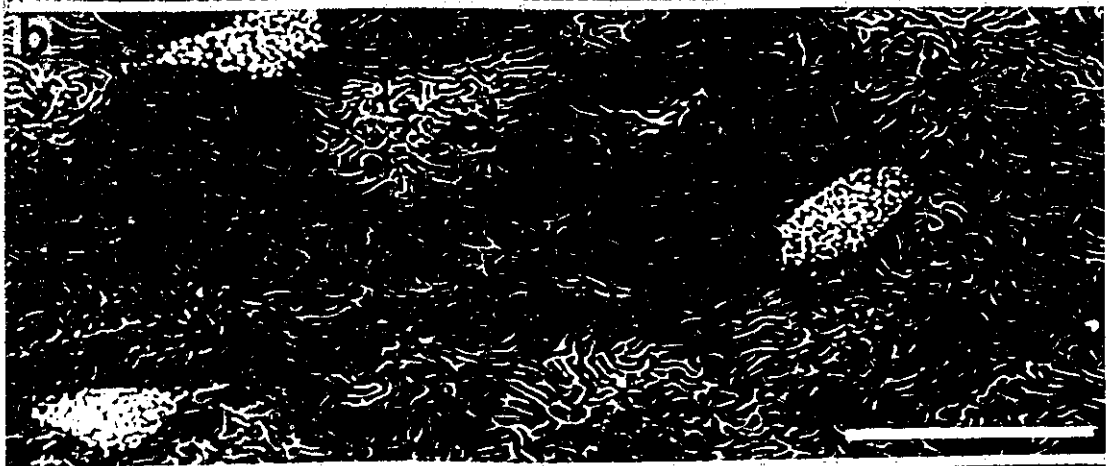
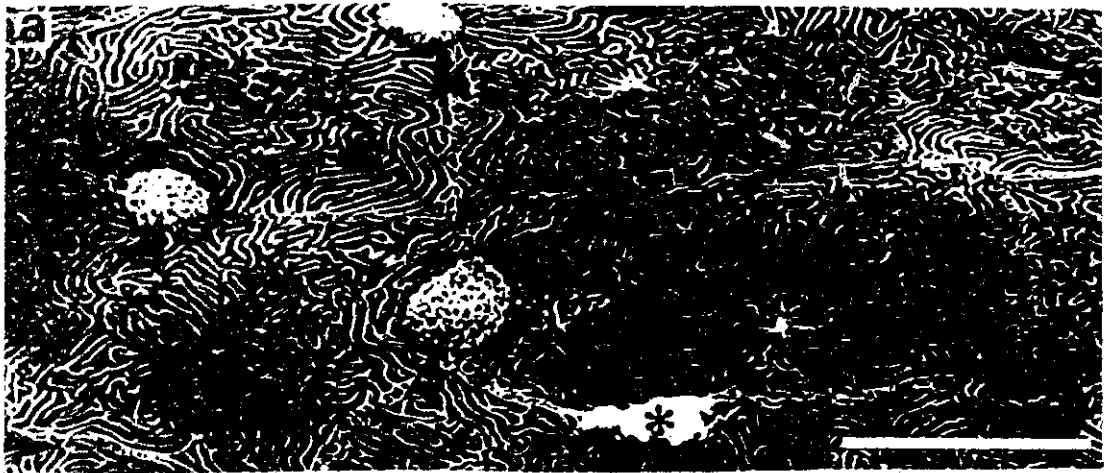


FIGURE 6.14

Representative low magnification scanning electron microscopy (SEM) photographs of the filament epithelium of American eel under control (non-infused) conditions (Fig 6.14a), after 24 h NaCl infusion (Fig 6.8b), and after 24 h infusion of NaHCO₃ (Fig 6.8c). Arrowheads indicate chloride cells. * indicate mucous cells. scale bar = 10 μ.



DISCUSSION

Inter-specific Differences in Gill Morphology between American Eel and Rainbow Trout

The present results confirm the findings of earlier studies showing considerable differences in gill epithelial CC morphology between eel and trout (Perry *et al*, 1992a, b). The CC fractional area (CCFA) of rainbow trout was considerably greater than that found in American eels. The reason for these considerable differences were due mainly to variations in the average CC apical exposure with only small differences in CC density. The external appearance of the CC was strikingly different in eel and trout. Eel CCs were characterized by an exceptionally microvilli rich apical membrane and small cells (apical membrane exposure) permitting easy identification of CCs from the adjacent PVCs and MCs (Fig 6.2c, d). In contrast, the CCs of rainbow trout displayed fewer and less pronounced apical membrane microvilli. The physiological significance of these differences in CCFA and CC appearance are unclear. However, in addition to its role in Cl⁻ uptake, the CC is thought to be the site of Ca²⁺ uptake (Perry and Wood, 1985; Ishihara and Mugiya, 1987; Perry and Flik, 1988; Perry *et al*, 1992a) and the differences noted may represent differences in the rate of Ca²⁺ uptake between the two species (Höbe *et al*, 1984a; Perry *et al*, 1992a).

An important point to note from this study is that characterization of the number of CCs (density) on the filament epithelium, a parameter often used to describe differences in freshwater fish, may not be a reliable indicator of the area of CCs exposed to the water, and hence the availability of the Cl⁻/HCO₃⁻ exchanger to function. In the present study, analysis of the CC surface density alone would reveal similar values in eel and trout and little difference in CC morphology. In addition, SEM observation of CCs only includes those cells which have direct contact with the water. CCs underlying the

surface of the gill are not considered to be functional and traditional means of counting CC density (e.g. light microscopy) cannot distinguish whether or not the cell is in contact with the water.

Blood Acid-Base Status During Infusion

There were profound alterations in the blood acid-base status of both species in response to NaHCO_3 infusion while NaCl infusion was without effect. In trout, plasma $[\text{HCO}_3^-]$ rose rapidly after the start of infusion but rapidly reached a level where further infusion resulted in no further increases. This elevation in $[\text{HCO}_3^-]$ caused a severe elevation in arterial pH. The $[\text{HCO}_3^-]$ that was attained ($\sim 22 \text{ mmol/l}$) was similar to the maximal values reached by rainbow trout during compensation from acidosis resulting from either hyperoxia (Wood and Jackson, 1980; Höbe *et al*, 1984c; Wood and LeMoigne, 1991) or hypercapnia (Perry, 1982; Perry *et al*, 1987a) and suggests that this value may represent a point above which $[\text{HCO}_3^-]$ cannot be elevated further.

In eels, however, plasma $[\text{HCO}_3^-]$ rose steadily throughout the infusion period. The $[\text{HCO}_3^-]$ at the 24 h period was astonishingly high; the value of $\sim 60 \text{ mmol/l}$ may be the highest value reported for a freshwater fish and is far higher than the bicarbonate threshold proposed by Claiborne and Heisler (1984). The continued rise in plasma $[\text{HCO}_3^-]$ did not cause further significant increases in arterial pH past the 8 h period owing to the exponential relationship between pH and HCO_3^- at elevated $[\text{HCO}_3^-]$ (Davenport, 1974). The large and continual increase in plasma $[\text{HCO}_3^-]$ in the eels cannot be explained by inadequate rates of base excretion because both trout and eel excreted equivalent amounts of base during the infusion although totally different mechanisms were used (see below).

There was no difference in the ability of trout and eels to clear the excess $[\text{HCO}_3^-]$

although return of arterial pH to control values after infusion had stopped was compromised in the eel. In trout, pH and $[\text{HCO}_3^-]$ were returned to control values by the 36 h period (12 h after infusion stopped) while in eel, there was only incomplete clearance of the accumulated $[\text{HCO}_3^-]$ over 24 h. In both species, however, plasma HCO_3^- decreased by approximately 20 mmol/l although totally different mechanisms were used (see below). Arterial pH remained high and unaltered over the entire 24 h post-infusion period owing to the exponential relationship between pH and HCO_3^- .

Ionic-Acidic/Basic Equivalent Fluxes During Infusion

To assess the effects of infusion (volume loading and/or Na^+ loading) on ionic-acidic/basic equivalent fluxes, infusion of NaCl was performed (sham). Differences in ion-acidic equivalent fluxes between NaCl-infused fish and NaHCO_3 -infused are considered to be the result of the acid-base disturbance (excess HCO_3^-) directly. Conversely, similar alterations in ion-acidic equivalent fluxes between NaCl-infused fish and NaHCO_3 -infused fish would presumably be the result of either the Na^+ or the volume load.

In both trout and eels NaHCO_3 infusion caused changes in branchial net ionic fluxes that resulted in *differential* losses of Na^+ over Cl^- , thus constraining a decrease in pH owing to changes in SID (Stewart, 1978, 1983). Clearly, however, the two species utilized very different strategies to manipulate net ionic and acidic/basic equivalent fluxes. In both eel and trout, $J_{\text{net}}^{\text{Na}^+}$ was not regulated during NaHCO_3 -infusion to compensate the alkalosis. Although there were significant losses of Na^+ during NaHCO_3 -infusion, the similar responses during NaCl-infusion indicate that this was caused by the Na^+ load associated with the infusion. There were significant reductions in $J_{\text{in}}^{\text{Na}^+}$ in NaHCO_3 -infused eels and not in NaCl-infused eels though the absolute values of these changes ($<50 \mu\text{mol/kg/h}$) were minimal compared to the increases in $J_{\text{out}}^{\text{Na}^+}$.

The predominant mechanism for the regulation of acid-base status in both eel and trout is manipulation of $J_{\text{net}}^{\text{Cl}^-}$. However, in the eel, owing to the absence of an appreciable influx component ($J_{\text{in}}^{\text{Cl}^-}$), efflux regulation ($J_{\text{out}}^{\text{Cl}^-}$) appears to be the only means available. In contrast, trout primarily utilize manipulation of the influx component ($J_{\text{in}}^{\text{Cl}^-}$) to manipulate $J_{\text{net}}^{\text{Cl}^-}$, although there is a role for manipulation of efflux ($J_{\text{out}}^{\text{Cl}^-}$) as well.

The results for rainbow trout support an earlier study by Goss and Wood (1990b) who demonstrated that during NaHCO_3 infusion, alterations in $J_{\text{net}}^{\text{Cl}^-}$ were quantitatively more important than variations in $J_{\text{net}}^{\text{Na}^+}$, and in turn, variations in $J_{\text{in}}^{\text{Cl}^-}$ were more important than variations in $J_{\text{out}}^{\text{Cl}^-}$. In addition, the results of the present study support the view that manipulation of the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism is the dominant mechanism for the regulation of acid-base disturbances in rainbow trout (Wood *et al*, 1984; Perry *et al*, 1987a; Goss and Wood, 1990a, b). On the other hand, the present results only partially confirm previous studies on American eel. In agreement with this study, Hyde and Perry (1989) reported regulation of hypercapnic acidosis in American eels was not accomplished with adjustments of $J_{\text{in}}^{\text{Cl}^-}$. In contrast, however, they reported large changes in $J_{\text{net}}^{\text{Na}^+}$. These differences may possibly be explained by differences in the mechanism of inducing the acid-base disturbance (hypercapnia induced respiratory acidosis *versus* - base infusion induced, metabolic alkalosis)

There are several possible mechanisms involved in the regulation of $\text{Cl}^-/\text{HCO}_3^-$ exchange in trout. Alterations in substrate availability (Wood and Goss, 1990; Goss and Wood, 1991) and elevated catecholamine levels (Girard and Payan, 1980; Perry *et al*, 1984a; Perry *et al*, 1987a; Vermette and Perry, 1988; McDonald *et al*, 1989) have been implicated as factors affecting Cl^- influx in trout while the contribution of variations in

morphology was demonstrated in Chapters 2-5. In both trout and catfish, the response to an acidosis is to cover the CCs and thereby limit $J_{in}^{Cl^-}$. Conversely, an alkalosis is regulated through an increase in CCFA and a corresponding increase in $J_{in}^{Cl^-}$. It is suggested that in eels, the low and inflexible rates of $J_{in}^{Cl^-}$ is due to the low CCFA present on the gill epithelium in eels and the lack of any significant alteration in CCFA during alkalosis. The mechanisms involved in active regulation of the efflux component have not been determined. Although active regulation of the paracellular permeabilities to ionic passage has been shown to occur in "leaky" epithelia (Madera, 1988), no comparable studies exist for "tight" epithelia at this point.

In both trout and eel, net acidic equivalent excretion ($J_{net}^{H^+}$) was approximately similar (150-200 $\mu\text{mol/kg/h}$) but not equal to the rate of infusion (900-1000 $\mu\text{mol/kg/h}$). Theoretically, this should result in an ever increasing $[\text{HCO}_3^-]$ in the fish. However, in trout, the plasma $[\text{HCO}_3^-]$ reached a maximal value of ~ 22 mmol/l while in eel, $[\text{HCO}_3^-]$ was ~ 60 mmol/l in the arterial blood and still rising. The expected rise in $[\text{HCO}_3^-]$ in eel and trout can be calculated using a 30% extracellular fluid volume (Milligan and Wood, 1982) and the difference between infusion rate and average excretion rate (200 $\mu\text{mol/kg/h}$) over a 24 h period. According to this analysis, $[\text{HCO}_3^-]$ should reach a value ~ 54 mmol/l higher than the initial value in eel and 46 mmol/l higher in trout. In the eel, plasma $[\text{HCO}_3^-]$ rose from a non-infused control value of 16 mmol/l to a maximal value of 60 mmol/l, a difference similar to that predicted from the measured infusion and excretion rates. In trout, however, maximal $[\text{HCO}_3^-]$ reached only 22 mmol/l and, thereafter, did not rise further. Since the measurement of $J_{net}^{H^+}$ encompasses whole body H^+ transfer (renal, branchial and integumental) into the water, and the extracellular acid-base status has been measured, the additional "missing" infused HCO_3^- must have been transferred

into the intracellular compartment and buffered. In the post-infusion period, trout were still clearing base (elevated $J_{\text{net}}^{\text{H}^+}$) at the 24 h period even though blood pH and $[\text{HCO}_3^-]$ had returned to normal. This suggests that clearance of the excess HCO_3^- transferred to the intracellular compartment continues once pH has returned to normal.

Although few studies have examined the intracellular responses to alkalosis, many studies focusing on acid-base responses to acidosis have demonstrated that intracellular pH (pH_i) is preferentially regulated in the essential and metabolically active tissues (Cameron, 1985; Heisler, 1989; Perry *et al*, 1988; Wood *et al*, 1990; Wood and LeMoigne, 1991). However, in each case, white muscle pH_i was less well regulated. Wood and LeMoigne (1991) found a clear role for "buffering" by the white muscle during the first 12 h of hypercapnic acidosis in the rainbow trout although they did not see any evidence of such a mechanism in the post-hypercapnic alkalotic period. Tang and Boutilier (1991) found that during a post-exercise acidosis, pH_e is preferentially regulated over white muscle pH_i and suggested that in an active pelagic species like the trout, maintenance of pH_e is advantageous to offset pH-induced interference with O_2 transport and support continued performance by the active glycolytic red muscle. Regardless, this short-term "buffering" role for white muscle is quantitatively important and deserves attention in further studies.

Gill Morphology and Base Infusion

In trout, CCFA was increased as a result of alkalosis. This is a similar result to that found in post-hypercapnic (Chapters 2 and 4), post-hyperoxic and NaHCO_3 -infused (Chapter 5) fish. Elevations in CCFA acting in conjunction with elevated levels of substrate (HCO_3^-) for the $\text{Cl}^-/\text{HCO}_3^-$ exchanger is the dominant mechanism used by trout to stimulate $\text{Cl}^-/\text{HCO}_3^-$ exchange and hence, aid in the clearance of the infused base load. In the eel, however, CCFA is small and is not significantly altered during metabolic

alkalosis. This corresponds with the extremely low rates of $J_{in}^{Cl^-}$ in eels and the inability to vary $J_{in}^{Cl^-}$ (Cl^-/HCO_3^- exchange) during an alkalosis (see also Hyde and Perry, 1987; 1989). This is despite the exceptionally high substrate availability that actually surpasses the K_m for HCO_3^- (33.3 mmol/l, Goss and Wood, 1991) suggesting that the Cl^-/HCO_3^- exchanger is either almost absent or non-functional in this species. Thus, morphological adjustments of Cl^-/HCO_3^- exchange, the predominant mechanisms utilized by trout, is unavailable to the eel. Consequently, the eel relies primarily on adjustments of Cl^- efflux and to a lesser extent, Na^+ uptake.

In summary, different strategies are utilized by eel and trout to regulate blood acid-base status during $NaHCO_3$ infusion. Trout utilize a combination of i) elevation of $J_{in}^{Cl^-}$ (Cl^-/HCO_3^- exchange) through elevation in both CCFA and substrate (HCO_3^-) availability, ii) reduction of Cl^- efflux (by unknown mechanisms) and iii) intracellular buffering. In contrast, eels rely predominantly on manipulations of $J_{net}^{Cl^-}$ through manipulation of the efflux component ($J_{out}^{Cl^-}$) only; a secondary mechanism appears to be a rapid reduction of $J_{in}^{Na^+}$, although the much larger and variable rate of Na^+ efflux negated any effect on $J_{net}^{Na^+}$. Manipulation of gill morphology and elevations in substrate availability to increase $J_{in}^{Cl^-}$ or the use of intracellular buffering are not utilized by eels to regulate the infused base load. It appears to be the absence of intracellular buffering that is responsible for the much larger levels of plasma $[HCO_3^-]$ that are attained in eels because both species excreted similar quantities of base during and after the infusion period, although by different mechanisms. If inter-specific differences in CCFA result in inter-specific differences in the mechanisms of acid-base regulation, then to what extent do intra-specific differences in the gill morphology account for differences in the ability of populations to acid-base regulate? This problem will be examined in Chapter 7.

CHAPTER 7

INTRA-SPECIFIC VARIATIONS IN THE RATES OF ACID-BASE COMPENSATION

INTRODUCTION

In freshwater fish, the gill is the site of Na^+ and Cl^- uptake from the environment. The Na^+ and Cl^- uptake mechanisms are essential to balance diffusional losses and urinary excretion. Further, the coupling of Na^+ uptake to acid equivalent (H^+/NH_4^+) excretion and Cl^- uptake to base equivalent ($\text{HCO}_3^-/\text{OH}^-$) excretion is the basis for an intimate relationship between ionic- and acid-base regulation in freshwater species (Krogh, 1938; Maetz, 1956, 1971, 1973; 1974; Maetz and Garia-Romeu, 1964; Cameron, 1976, 1978; Wood *et al.*, 1984; Perry *et al.*, 1987b; Avella *et al.*, 1990).

It is well established that fish adjust branchial ionic fluxes during acid-base disturbances to regulate blood pH (Claiborne and Heisler, 1984, 1986; Wood *et al.*, 1984; Perry *et al.*, 1987a; Goss and Wood, 1990a). However, the mechanisms underlying the compensatory adjustments of ionic fluxes have not been firmly established. A general theme that is emerging, however, from our recent studies in this area is that morphological alteration of the gill epithelium is a key mechanism of acid-base regulation (Chapters 2-5). During respiratory acidosis the surface area of CC's exposed to the water is markedly reduced thus resulting in fewer functional (exposed) $\text{Cl}^-/\text{HCO}_3^-$ exchangers; consequently the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange is decreased, an appropriate response to correct internal acidosis (Truchot, 1987; Wood, 1988, 1991a, b; Goss and Wood, 1990a). During metabolic alkalosis the exact opposite response occurs. The surface area of gill epithelial CCs is significantly increased, the number of exposed $\text{Cl}^-/\text{HCO}_3^-$ exchangers rises, and consequently $\text{Cl}^-/\text{HCO}_3^-$ exchange is increased, an appropriate response to correct internal alkalosis (Claiborne and Heisler, 1986; Goss and Wood, 1990b). This mode of regulation is only applicable to those species (e.g. trout, catfish) having sufficiently abundant gill chloride cells so as to allow the bimodal regulation of their surface area. Species such as

American eel that display extremely low branchial CC surface area (Perry *et al.*, 1992a, b) do not rely on morphological adjustment of $\text{Cl}^-/\text{HCO}_3^-$ exchange to regulate blood pH but instead adjust net Na^+ balance (Hyde and Perry, 1987) or passive Cl^- efflux (Chapter 6).

Given the presumed importance of morphological adjustment of $\text{Cl}^-/\text{HCO}_3^-$ exchange for acid-base regulation in trout, the present study was undertaken to examine further the relationship between gill morphology and the ability to regulate blood acid-base status. The experimental approach was to assess the impact of experimentally altered rates of $\text{Cl}^-/\text{HCO}_3^-$ exchange on the ability of fish to compensate metabolic alkalosis. The combined treatment of ovine growth hormone plus cortisol injections (Madsen, 1990b) was used as a tool to evoke an increase in CC surface area and thus $\text{Cl}^-/\text{HCO}_3^-$ exchange capacity. The predicted result is that an increased capacity for $\text{Cl}^-/\text{HCO}_3^-$ exchange would impart an increased ability to compensate an alkalosis. Owing to the lack of an adequate technique to consistently lower CC surface area, the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange was instead lowered by removal of external Cl^- . In this case, the predicted result is a reduced ability to compensate an alkalosis. Alternatively, the trout, when denied the use of the primary regulatory mechanism ($\text{Cl}^-/\text{HCO}_3^-$ exchange) might utilize strategies similar to those of the eel, a fish with limited $\text{Cl}^-/\text{HCO}_3^-$ exchange capacity.

METHODS

Experimental Animals

Rainbow trout *Oncorhynchus mykiss* [mean weight 300 ± 4 g (SE), $n = 108$], were obtained from Linwood Acres trout farm, Cambellcroft, Ontario and held as described in Chapter 4.

Animal Preparation

To allow infusion and blood sampling, fish were anaesthetized (MS222 1:10000; Sigma) and fitted with indwelling dorsal aortic cannulae according to the method of Soivio *et al* (1973). Fish were then placed in individual opaque acrylic boxes and allowed to recover for 48 h prior to experimentation.

For the cortisol/ovine growth hormone (Cort/oGH) treatment, fish ($n = 18$) were separated 10 days prior to the start of the experiment and injected intramuscularly with cortisol (4 mg/kg body weight; hydrocortisone 21-hemisuccinate sodium salt: Sigma; injection vehicle 0.9% NaCl, pH 7.8) daily for 10 days. On days 1, 3, 5, 7 and 9, fish were also injected intraperitoneally with ovine growth hormone (oGH: 2 μ g/g body weight: NIH; injection vehicle 0.9% NaCl, adjusted to pH 9.0 with NaOH). This procedure, adopted from the study of Madsen (1990b), is known to increase both gill Na^+/K^+ -ATPase activity and interlamellar CC density. Food was withheld for the final five days so as to minimize variations in acid output caused by feeding (Wood and Caldwell, 1978). In cort/oGH-treated fish, injections were performed during the post-surgery recovery period as required.

Infusion Regime

To induce a metabolic alkalosis, fish were infused for 24 h *via* the dorsal aortic cannulae with 140 mmol/l NaHCO_3 at a nominal infusion rate of 800 μ mol/k/h using a

syringe pump (Sage). The subsequent 24 h post-infusion period was also examined. Untreated trout infused with NaHCO_3 for 24 h served as a control for both cort/oGH and Cl^- free water exposed groups. Untreated trout actual infusion rate = 938 ± 22 $\mu\text{mol/kg/h}$, $n = 64$; cort/oGH-treated trout actual infusion rate = 989 ± 84 $\mu\text{mol/kg/h}$, $n = 18$; Cl^- free exposed trout actual infusion rate = 857 ± 43 $\mu\text{mol/kg/h}$.

Experimental Protocol

Series 1 - Effects of Cort/oGH Treatment on Blood Acid-Base Responses to NaHCO_3 Infusion

This series was designed to characterize alterations in blood acid-base status associated with infusion of 140 mmol/l NaHCO_3 in untreated trout ($n = 6$) and in cort/oGH-treated trout ($n = 6$). Blood samples (300 μl) were withdrawn at the following times: 0 (Pre), 1, 4, 8, 12, and 24 h after the start of infusion, and 25, 28, 32, 36, and 48 h (1, 4, 8, 12, 24 h) post-infusion. Blood samples were immediately analyzed for blood pH and then centrifuged at 10000g for 15 seconds, the plasma drawn off and analyzed for total CO_2 .

Series 2 - Effects of Cort/oGH treatment on Ion-Acidic/Basic Equivalent Fluxes and Gill Morphology during NaHCO_3 Infusion.

This series was designed to characterize the unidirectional and net ion and acidic/basic equivalent fluxes and the gill morphology during infusion. Based on the blood acid-base data, the following periods were chosen for analysis of the ionic-acidic/basic equivalent fluxes: 0 (non-infused control), 4, 12, 24, 28, 36 and 48 h. For untreated rainbow trout ($n = 56$), measurements of whole body ionic fluxes and morphometric measurements were accomplished as described in Chapter 6. For cort/oGH-treated fish ($n = 8$), the protocol was modified slightly owing to limitations in the amount of available

oGH. Instead of measuring the ionic and acidic/basic equivalent fluxes in separate fish for each time period as was performed in the untreated trout, the measurements were performed sequentially. Separate groups of fish were infused with either NaCl ($n = 6$) or NaHCO_3 ($n = 6$) at the appropriate rate for 24 h. These fish were then killed, the gills removed and fixed for examination in the SEM as described in Chapter 2.

Series 3 - Effects of Exposure to Cl⁻ free water on the Blood Acid-Base Responses to NaHCO_3 Infusion.

The third series was designed to characterize alterations in blood acid-base status associated with NaHCO_3 infusion in trout exposed to normal Ottawa Tapwater (termed OT; $n = 6$) and in trout exposed to Cl⁻ free water (termed CFW; $n = 6$). Series 3 and 4 were performed in Cl⁻ free water that was prepared to duplicate Ottawa tapwater in all other aspects as closely as possible. Ca^{2+} was maintained at the appropriate levels because of its well known role in regulating membrane permeability and trans-epithelial potential (Potts, 1984, McDonald and Rogano, 1986). This water was made by passing dechlorinated OT through a deionizing resin cartridge (Culligan Corp) and then adding back appropriate amounts of $\text{Ca}(\text{CO}_3)_2$ and Na^+ -Gluconate (BDH) to bring the levels of Ca^{2+} and Na^+ back to those normally found in OT (see Chapter 4). Gluconate is assumed to be an impermeant anion in this series of experiments. To dissolve the salts, the water was gassed with 100% CO_2 for at least 6 h. Following complete solution, the water was aerated vigorously to drive off the excess CO_2 and the temperature adjusted to the appropriate experimental temperature.

After the initial non-infused control period in OT, the water to the CFW group was replaced by flowing, temperature controlled CFW (0.2 l/min/box). The boxes were flushed with additional CFW (>2.0 l/min) to lower the external $[\text{Cl}^-]$ below 0.015 mmol/l

(c.f. Ottawa tapwater $[\text{Cl}^-] = 0.15 \text{ mmol/l}$), far below the measured K_m of the Cl^- transport mechanism (0.043 mmol/l - Chapter 4). At the same time, the fish were infused with NaHCO_3 and the blood sampled as outlined in Chapter 6.

Series 4 - Effects of Exposure to CFW on Ion-Acidic/Basic Equivalent Fluxes and Gill Morphology during NaHCO_3 Infusion.

The fourth series was designed to characterize the unidirectional and net ion and acidic/basic equivalent fluxes and the gill morphology during infusion. OT trout are the same fish as the untreated trout in the cort/oGH experiments above; the different series have been separated for clarity of presentation. To minimize the problems associated with increases in external $[\text{Cl}^-]$ during the flux measurements, the flux period was reduced to 0.75 h. In addition, owing to the low $[\text{Cl}^-]$ in the external water, $0.5 \mu\text{Ci}$ of $^{36}\text{Cl}^-$ was used instead of $1.0 \mu\text{Ci}$. Instead of measuring the ionic fluxes in separate fish for each time period, the measurements were performed sequentially. The net acidic/basic equivalent fluxes could not be measured accurately/precisely in this experiment owing to interference from the Na gluconate with the measurement of J^{TA} . Separate groups of fish were infused with a) NaCl ($n = 6$) or b) NaHCO_3 ($n = 6$) for 24 h. These fish were killed, the gills removed and fixed for examination in the SEM as described in Chapter 2.

Analytical techniques

The analytical techniques utilized in this Chapter are exactly as described in Chapter 6.

Statistical analysis

All data are presented as means ± 1 standard error (SE). Data for Figs 7.1 was analyzed by an unpaired Student's two-tailed t-test. Data for Figures 7.3 through 7.12 were analyzed by two-way ANOVA followed by Fishers LSD test for multiple comparisons

where the F-value of the ANOVA indicated significance. 5% was accepted as the fiducial limit of significance in all cases.

RESULTS

Effects of Cortisol/ovine Growth Hormone on Gill CC Morphology

Under control conditions, cortisol/ovine growth hormone (cort/oGH) treatment caused a large (250%) increase in CCFA [from $83951 \pm 9789 \mu\text{m}^2/\text{mm}^2$ in non-infused untreated fish to $296512 \pm 14287 \mu\text{m}^2/\text{mm}^2$ in non-infused cort/oGH-treated fish (Fig 7.1c)]. This increase was the result of an increase in both average CC area (from $34.6 \pm 2.0 \mu\text{m}^2$ to $59.2 \pm 3.0 \mu\text{m}^2$, Fig 7.1a) and CC density (from $2368 \pm 200 /\text{mm}^2$ to $5006 \pm 222 /\text{mm}^2$; Fig 7.1b). Representative low and high magnification SEM photographs illustrating the effects of cort/oGH treatment on gill CC morphology are shown in Fig 7.2. In untreated fish (Fig 7.2a, c, e), the CCs were located almost exclusively on the filament epithelium (Figs 7.2c). The CCs in untreated trout had characteristic surface morphologies as described earlier (Chapter 4-6). In cort/oGH-treated fish, CCs were abundant on both the lamellar (Fig 7.2b) and filament (Fig 7.2d) epithelia. In addition to the increased numbers of CCs in the cort/oGH-treated fish, the CCs presented a number of varying surface morphologies. Some CCs had large densely packed apical surface microvilli/microridges and have been arbitrarily termed CC_1 , other CCs were nearly totally devoid of apical microvilli and appeared smooth-surfaced, these have been arbitrarily termed CC_2 (Fig 7.2f). However, one must recognize that these two morphologies represent the extremes and that a variety of surface morphologies were observed.

Series 1 - Effect of Cort/oGH-treatment on the Blood Acid-Base Responses to NaHCO_3 Infusion.

NaCl-infusion and blood sampling did not result in any significant changes in blood acid-base status [pH, $[\text{HCO}_3^-]$ or P_{CO_2} ; Fig 7.3, filled circles (●)]. NaHCO_3 , on the other

FIGURE 7.1

Comparison of the gill filament chloride cell (CC) morphometry including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area for control untreated rainbow trout and after cortisol/ovine growth hormone treatment. * indicates significantly different from control untreated fish. Mean \pm SE.

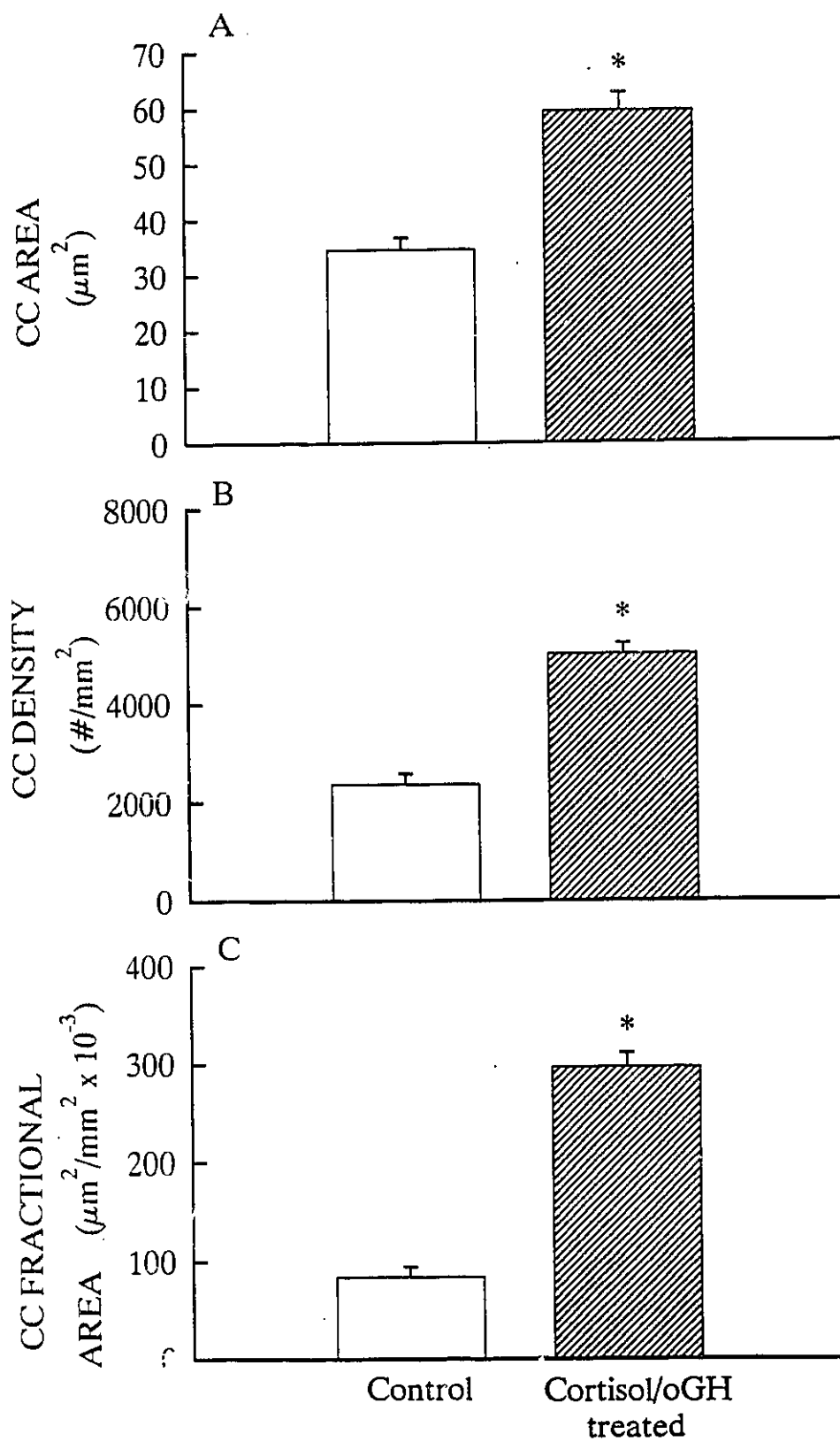


FIGURE 7.2

Representative low and high magnification scanning electron microscopy (SEM) photographs of the filament epithelium of rainbow trout under control conditions (Fig 6.8a, c, e) and after cortisol/ovine growth hormone treatment (Fig 7.1 b, d, f). Note the large increase in the chloride cell (CC) apical surface area in cortisol/oGH treated fish. In addition, in cortisol/oGH-treated fish, at least two distinct surface morphologies of CCs were noted. One type displayed a dense network of apical microvilli and microridges (CC₁) while others had only sparse microvilli (CC₂, Fig 7.1f). Arrowheads indicate chloride cells. MC = mucous cells Figs 6.8 a-d; scale bar = 10 μ . Figs 6.8 e,f, scale bar = 5 μ m.

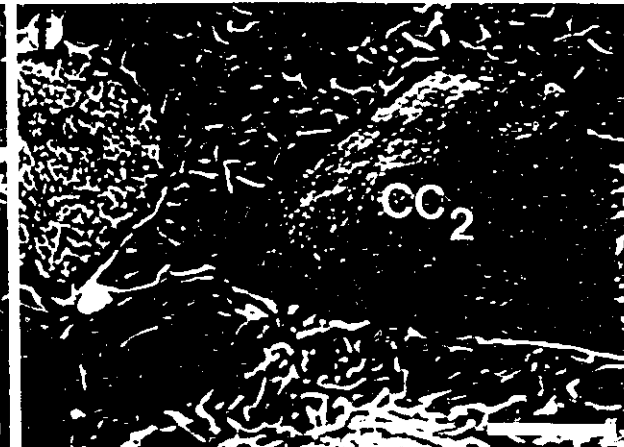
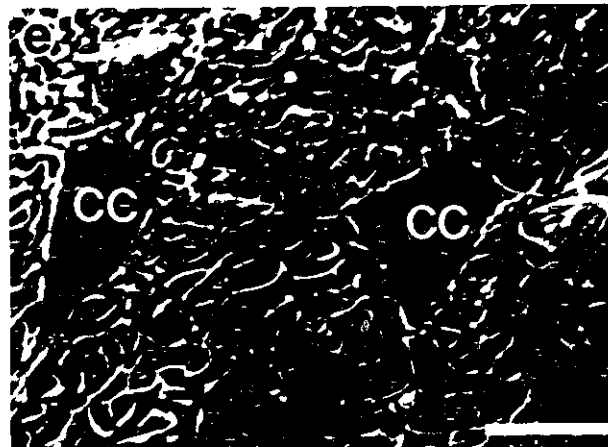
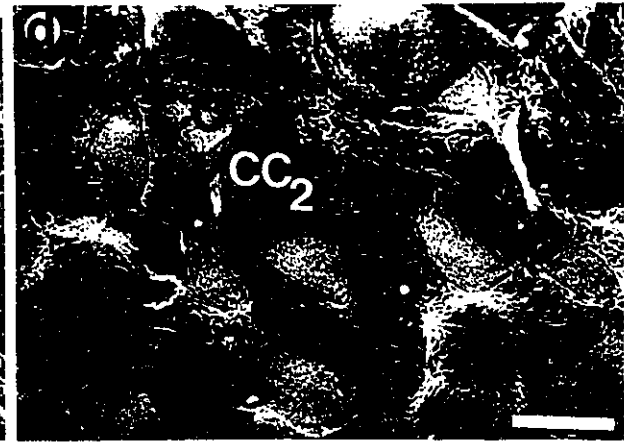
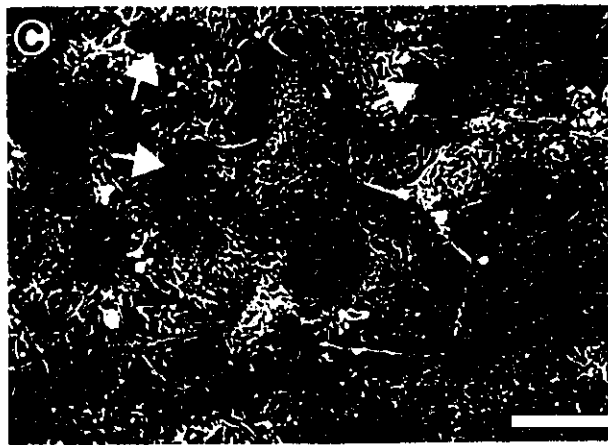
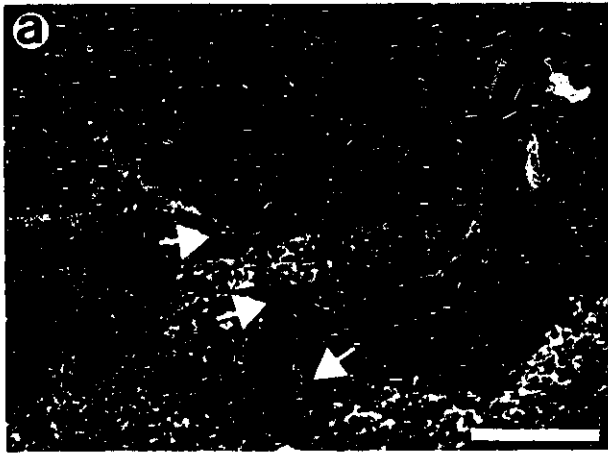
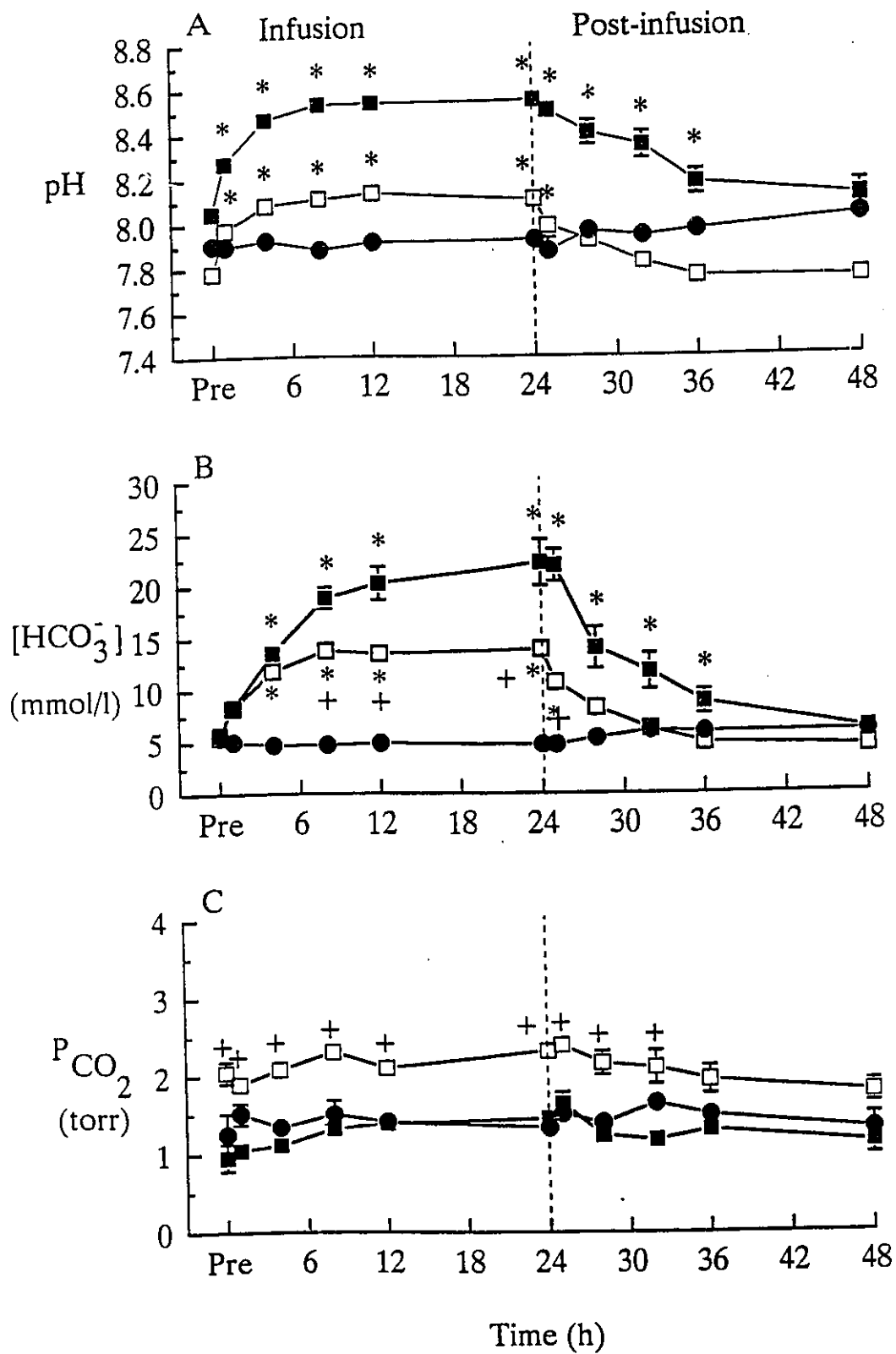


FIGURE 7.3

The temporal effects of 24 h of infusion of NaHCO_3 and the subsequent 24 h post-infusion period on the acid-base parameters (A) blood pH, (B) plasma $[\text{HCO}_3^-]$, and (C) blood P_{CO_2} in NaCl-infused [filled circles (●)] and NaHCO_3 -infused [filled squares (■)] control untreated fish and NaHCO_3 -infused cortisol/oGH-treated fish [open squares (□)]. * indicates significant difference from the initial Pre (control) value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO_3 -infused only). Mean \pm SE.

- Sham-NaCl infused
- Untreated NaHCO₃-infused
- Cort/oGH treated NaHCO₃-infused



hand caused significant increases in pH in both the untreated and cort/oGH-treated fish. In untreated fish, blood pH rose from 8.05 ± 0.01 to 8.53 ± 0.04 by 8 h (Fig 7.3A). Thereafter, pH did not rise appreciably throughout the infusion period. This elevation in blood pH was mainly caused by increases in plasma $[\text{HCO}_3^-]$ from 5.85 ± 0.44 mmol/l to 22.22 ± 2.18 mmol/l by 24 h (Fig 7.3B). In the post-infusion period, blood pH had returned to Pre values by 36 h (12 h post-infusion) owing to a reduction in plasma $[\text{HCO}_3^-]$ to Pre values (Fig 7.3b). Plasma P_{CO_2} remained elevated throughout the infusion and post-infusion period in the NaHCO_3 -infused untreated trout (Fig 7.3C).

Cort/oGH-treated trout also demonstrated an elevation in pH during NaHCO_3 infusion although the elevation was attenuated. Blood pH rose from 7.78 ± 0.02 to 8.11 ± 0.02 by 8 h (Fig 7.3A). After 8 h, blood pH did not rise despite the continued infusion. The attenuated elevation in blood pH was associated with a lower accumulation of plasma $[\text{HCO}_3^-]$. Plasma $[\text{HCO}_3^-]$ rose from 5.53 ± 0.47 mmol/l to 13.85 ± 0.50 mmol/l by the 8 h period (Fig 7.3B). Thereafter, plasma $[\text{HCO}_3^-]$ did not rise further despite continued NaHCO_3 infusion. In the post-infusion period, blood pH was returned to Pre values (by 4 h) owing to a rapid clearance of excess $[\text{HCO}_3^-]$.

Series 2 - Effects of Cort/oGH treatment on Ion-Acidic/Basic Equivalent Fluxes and Gill Morphology during NaHCO_3 Infusion.

Infusion resulted in significant net losses of Na^+ (negative $J_{\text{net}}^{\text{Na}^+}$) of approximately -600 to -900 $\mu\text{mol/kg/h}$ in both untreated- and cort/oGH-treated fish (Fig 7.4), although after 24 h, $J_{\text{net}}^{\text{Na}^+}$ was significantly greater in cort/oGH-treated fish. In the post-infusion period, $J_{\text{net}}^{\text{Na}^+}$ had returned to values near control by the 4 h period. In both untreated and cort/oGH-treated fish, $J_{\text{net}}^{\text{Cl}^-}$ was unchanged throughout the infusion period while in the post-infusion period, $J_{\text{net}}^{\text{Cl}^-}$ was significantly greater in the cort/oGH-

treated fish.

Both $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ were significantly higher under control conditions in cort/oGH-treated fish and similar trends were noted for both treatment groups in their response to $NaHCO_3$ infusion (Fig 7.5). $J_{in}^{Na^+}$ was reduced during infusion while $J_{in}^{Cl^-}$ was stimulated. $J_{in}^{Na^+}$ was significantly greater during infusion in the cort/oGH treated fish although the absolute value of change from control was greater. $J_{in}^{Cl^-}$ was significantly higher during infusion in the cort/oGH-treated fish. In the post-infusion period, $J_{in}^{Na^+}$ return to control values by the 12 h period in cort/oGH-treated fish while $J_{in}^{Na^+}$ remained low in the untreated fish. $J_{in}^{Cl^-}$ remained elevated throughout the post-infusion period in both treatment groups, with $J_{in}^{Cl^-}$ being higher in the cort/oGH treatment group at every period (Fig 7.5).

The trends in net acidic equivalent (H^+) transfer ($J_{net}^{H^+}$) in untreated and cort/oGH-treated fish were similar but there were pronounced differences in the magnitude of the fluxes (Fig 7.6B). In both groups, $J_{net}^{H^+}$ switched from a negative (H^+ excretion) value under control, non-infused conditions to a positive value indicating net H^+ uptake (or base excretion). However, cort/oGH-treated fish showed a dramatic increase in $J_{net}^{H^+}$ by the 12 h period and throughout infusion. In the post-infusion period, $J_{net}^{H^+}$ returned to control values by 36 h period in cort/oGH-treated fish while $J_{net}^{H^+}$ remained elevated throughout the post-infusion period in untreated trout. These alterations were mainly the result of manipulation of the titratable component (J^{TA}) to net acid transfer (Fig 7.6A). Throughout the post-infusion period, J^{Amm} was significantly greater in the cort/oGH-treated fish (Fig 7.6A).

Infusion of $NaHCO_3$ caused a significant 75% increase in CCFA in untreated trout from $83951 \pm 9789 \mu m^2/mm^2$ to $146597 \pm 10997 \mu m^2/mm^2$ by the 24 h period. The

FIGURE 7.4

The temporal effects of 24 h of infusion of NaHCO_3 in control untreated fish (open histograms) or cortisol/ovine growth hormone-treated fish (filled histograms) and the subsequent 24 h post-infusion period on the net fluxes of sodium ($J_{\text{net}}^{\text{Na}^+}$) and chloride ($J_{\text{net}}^{\text{Cl}^-}$). * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO_3 -infused only). Mean \pm SE.

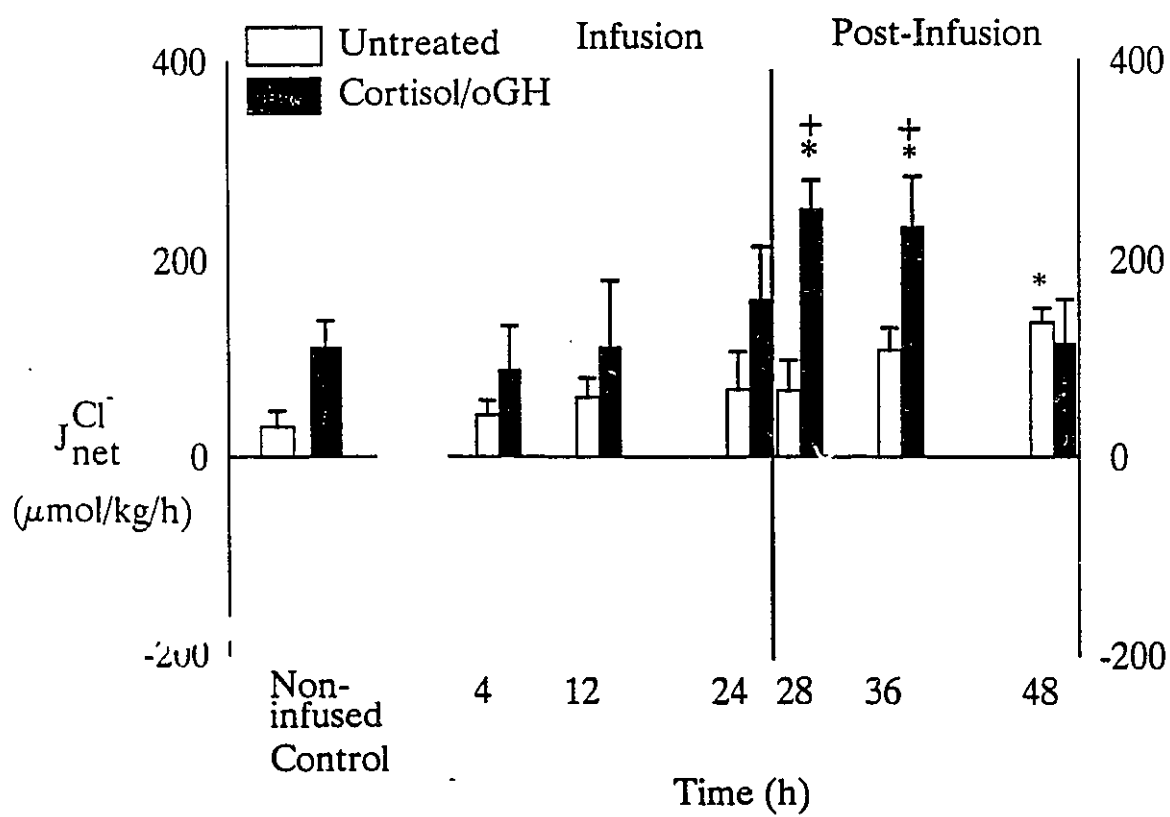
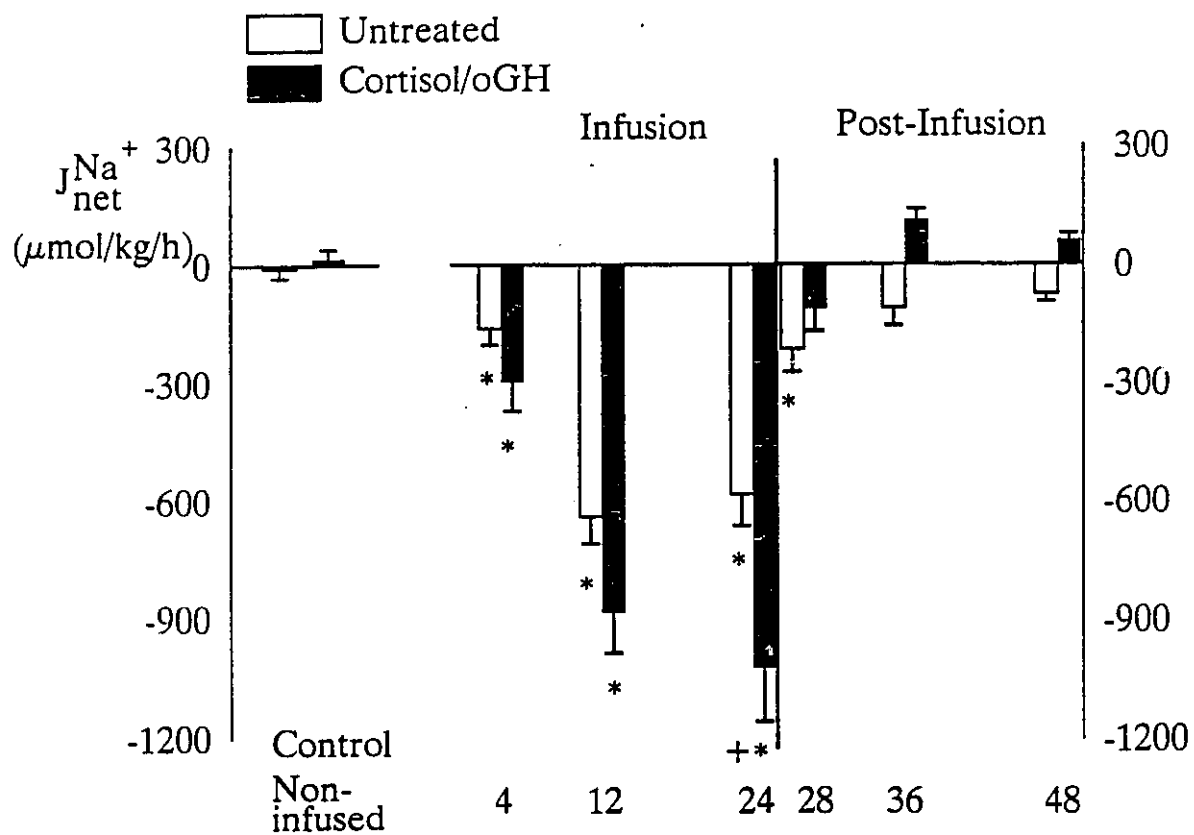


FIGURE 7.5

The temporal effects of 24 h of infusion of NaHCO_3 in control untreated fish (open histograms) or cortisol/ovine growth hormone-treated fish (filled histograms) and the subsequent 24 h post-infusion period on the influxes of sodium ($J_{\text{in}}^{\text{Na}^+}$) and chloride ($J_{\text{in}}^{\text{Cl}^-}$). * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl-infused value at that time period (NaHCO_3 -infused only). Mean \pm SE.

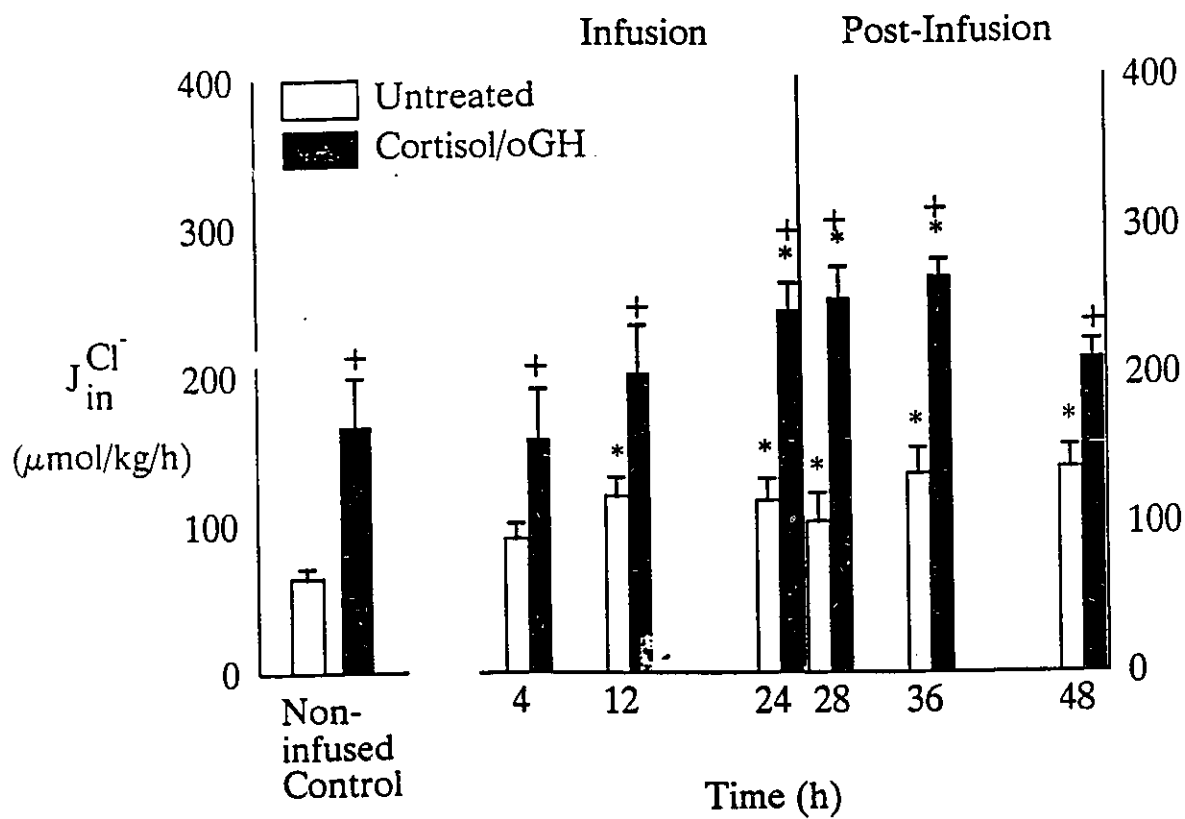
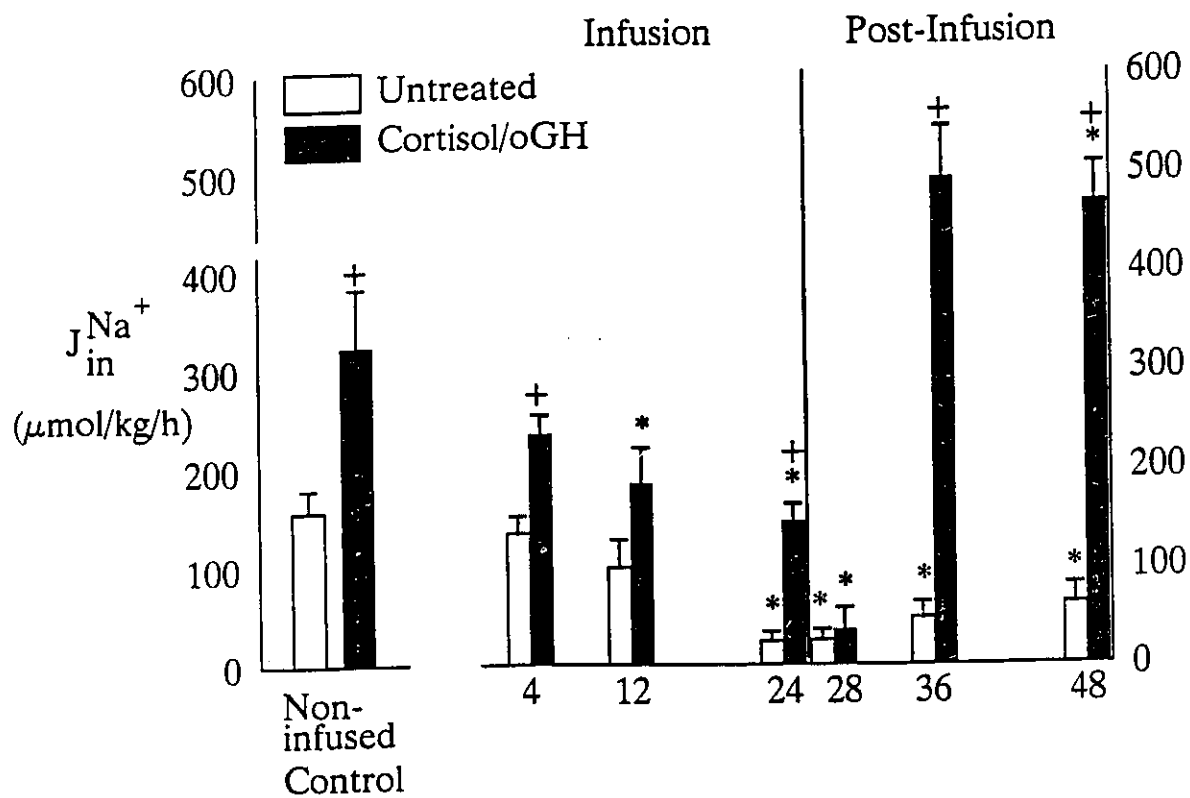


FIGURE 7.6

The temporal effects of 24 h of infusion of NaHCO_3 in control untreated fish (open histograms) or cortisol/ovine growth hormone-treated fish (filled histograms) and the subsequent 24 h post-infusion period on whole body flux rates of titratable alkalinity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents ($J_{\text{net}}^{\text{H}^+}$). Net acidic equivalent flux ($J_{\text{net}}^{\text{H}^+}$) is calculated as the sum of the two components, J^{TA} and J^{Amm} , signs considered. Positive values indicate acidic equivalent uptake (or base excretion) while negative values indicate acidic equivalent excretion. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the untreated group (Cortisol/oGH treatment group only). Mean \pm SE.

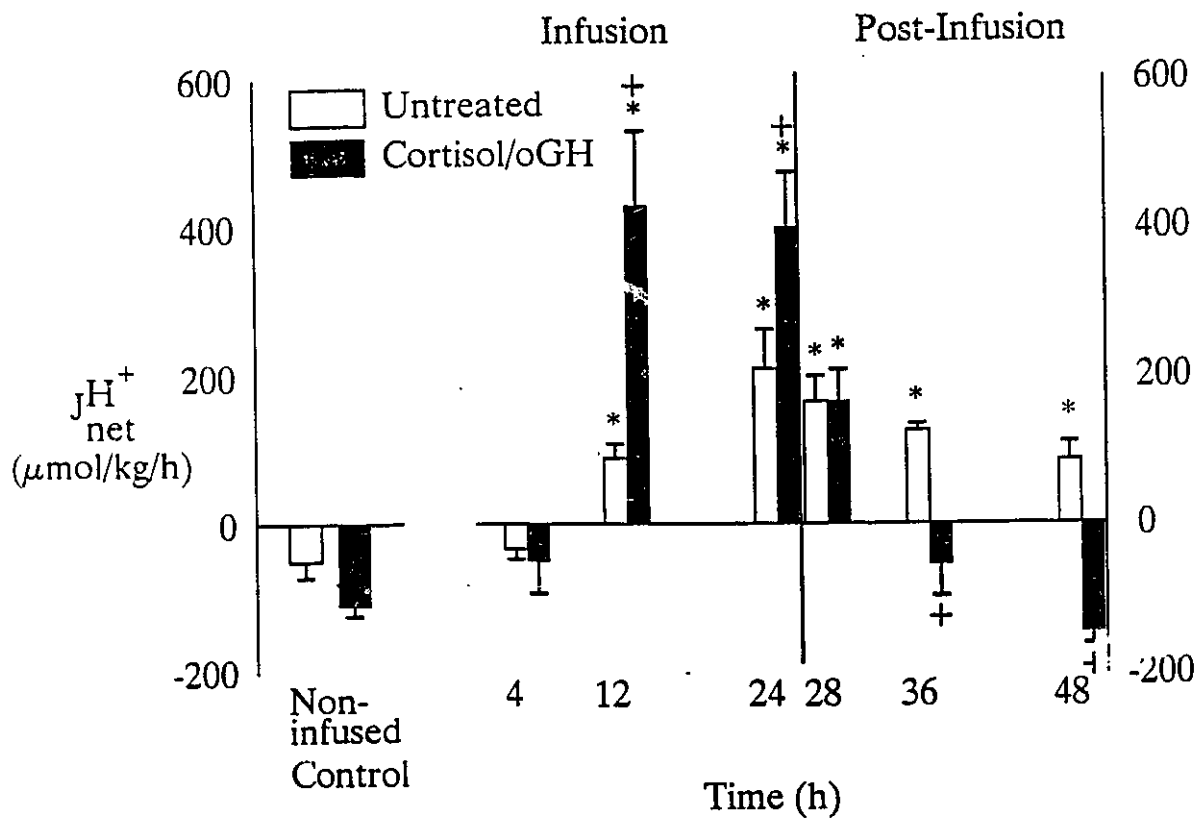
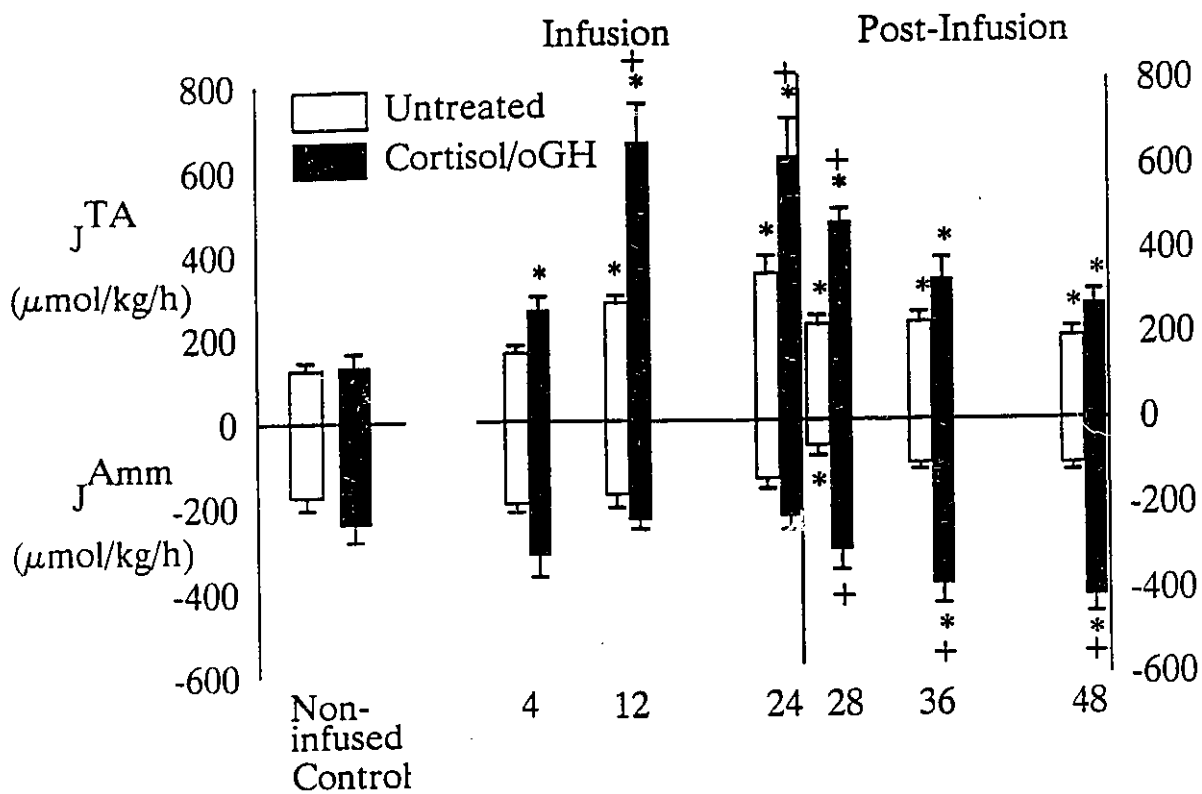
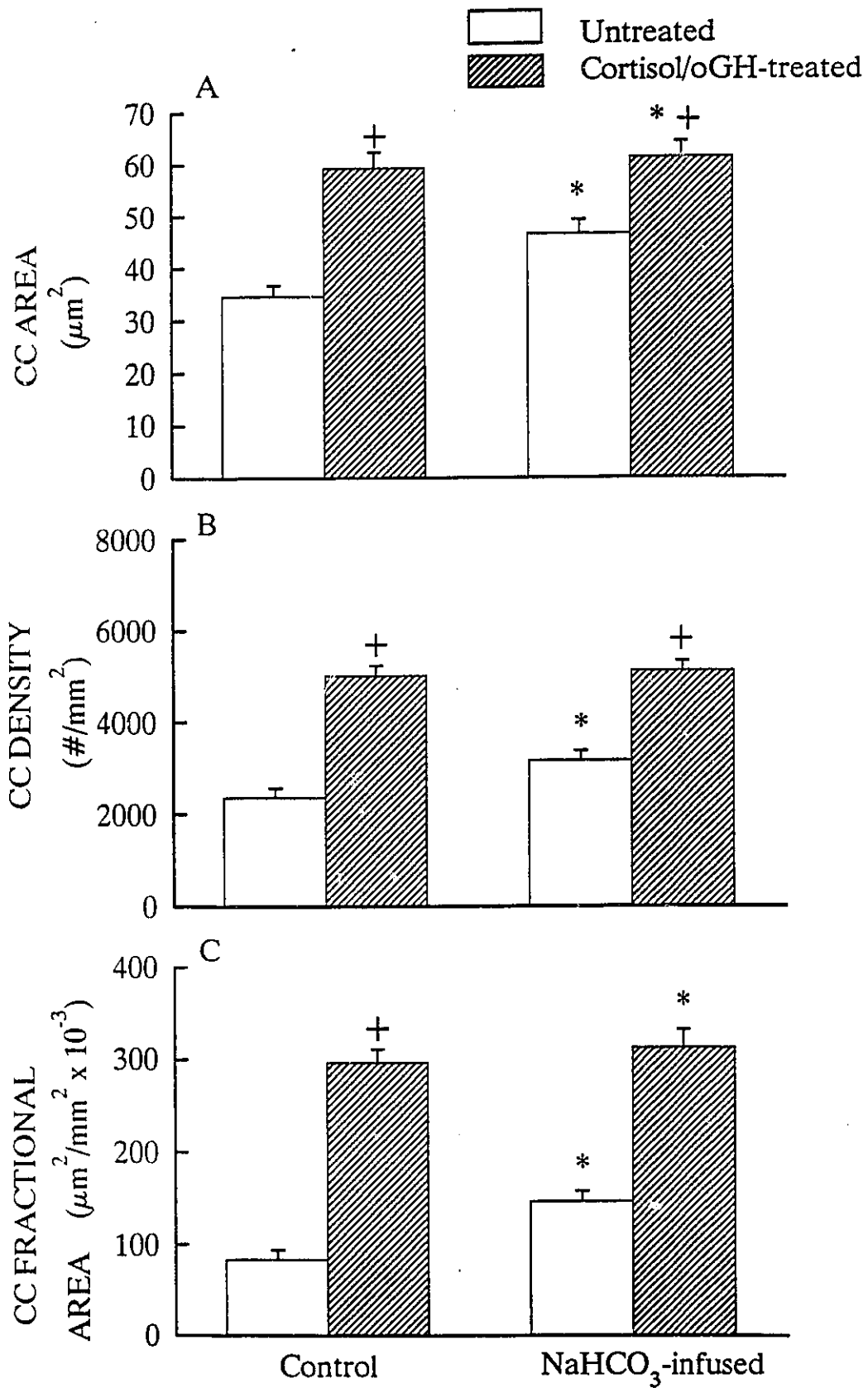


FIGURE 7.7

The effects of 24 h of chronic infusion of NaHCO_3 in control untreated fish (open histograms) or cortisol/ovine growth hormone-treated fish (filled histograms) 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 the initial non-infused control value. + indicates significant difference from the untreated group (Cortisol/oGH treatment group only). Mean \pm SE.



elevations in CCFA were due to significant elevations in both the average CC area (Fig 7.7A) and CC density (Fig 7.7B). In contrast, cort/oGH-treated trout, which already had significantly greater CCFA under control conditions ($296512 \pm 14287 \mu\text{m}^2/\text{mm}^2$ in non-infused fish) did not undergo any significant increase in CCFA (Fig 7.7C). Representative SEM photographs of the filament epithelium of untreated and cort/oGH-treated fish under control and after 24 h of NaHCO_3 infusion are shown in Fig 7.8. After 24 h of NaHCO_3 infusion, widely varying surface CC morphologies were evident on the gills with some CCs presenting few microvilli (CC_2) while others had large numbers of densely packed microvilli/microridges (CC_1). In control non-infused cort/oGH treated fish, CCFA was very large and in addition, the CCs already showed similar varying surface morphologies to those noted after 24 h infusion in untreated trout (Fig 7.8C; see Fig 7.2). As a result, infusion did not alter the surface CC morphologies in the cort/oGH-treated fish (Fig 7.8D).

Series 3 - Effects of Exposure to CFW on the Blood Acid-Base Responses to NaHCO_3 Infusion.

The blood acid-base status and ionic- acidic/basic equivalent fluxes of fish infused with NaHCO_3 while exposed to OT are shown for comparative purposes. These results were from the same fish which are termed "untreated" in Series 1 and 2.

To determine the effects of exposure to CFW, alone, on blood acid-base status, NaCl -infused (sham) experiments were performed. Exposure to CFW did not effect blood acid-base status during NaCl -infusion although there was a trend toward a gradual accumulation of HCO_3^- in the blood (Fig 7.9B). This trend continued throughout the post-infusion period and by 36 h, there was a significant accumulation of HCO_3^- in the plasma and a significant rise in blood pH by the 48 h period.

FIGURE 7.8

Representative low magnification scanning electron microscopy (SEM) photographs of the filament epithelium of rainbow trout in untreated (top panels) and cortisol/ovine growth hormone-treated fish (bottom panels) under control (non-infused) conditions (Fig 7.7A, C), and after 24 h NaHCO₃ infusion (Fig 7.7B, D). Note that NaHCO₃ infusion resulted in large increases in the average area of the CCs and a corresponding increase in CCFA in untreated fish while there were no further increases over control in the cort/oGH-treated fish. Arrowheads indicate chloride cells. Figs 6.8 a-c; scale bar = 10 μ . Figs 6.8 d-g, scale bar = 5 μ m.

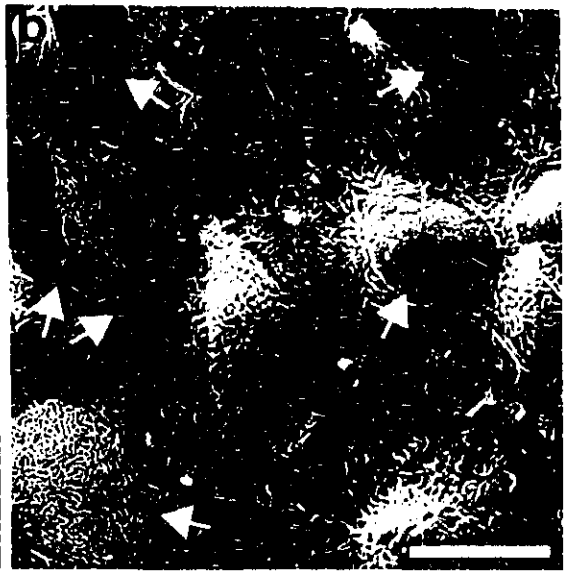
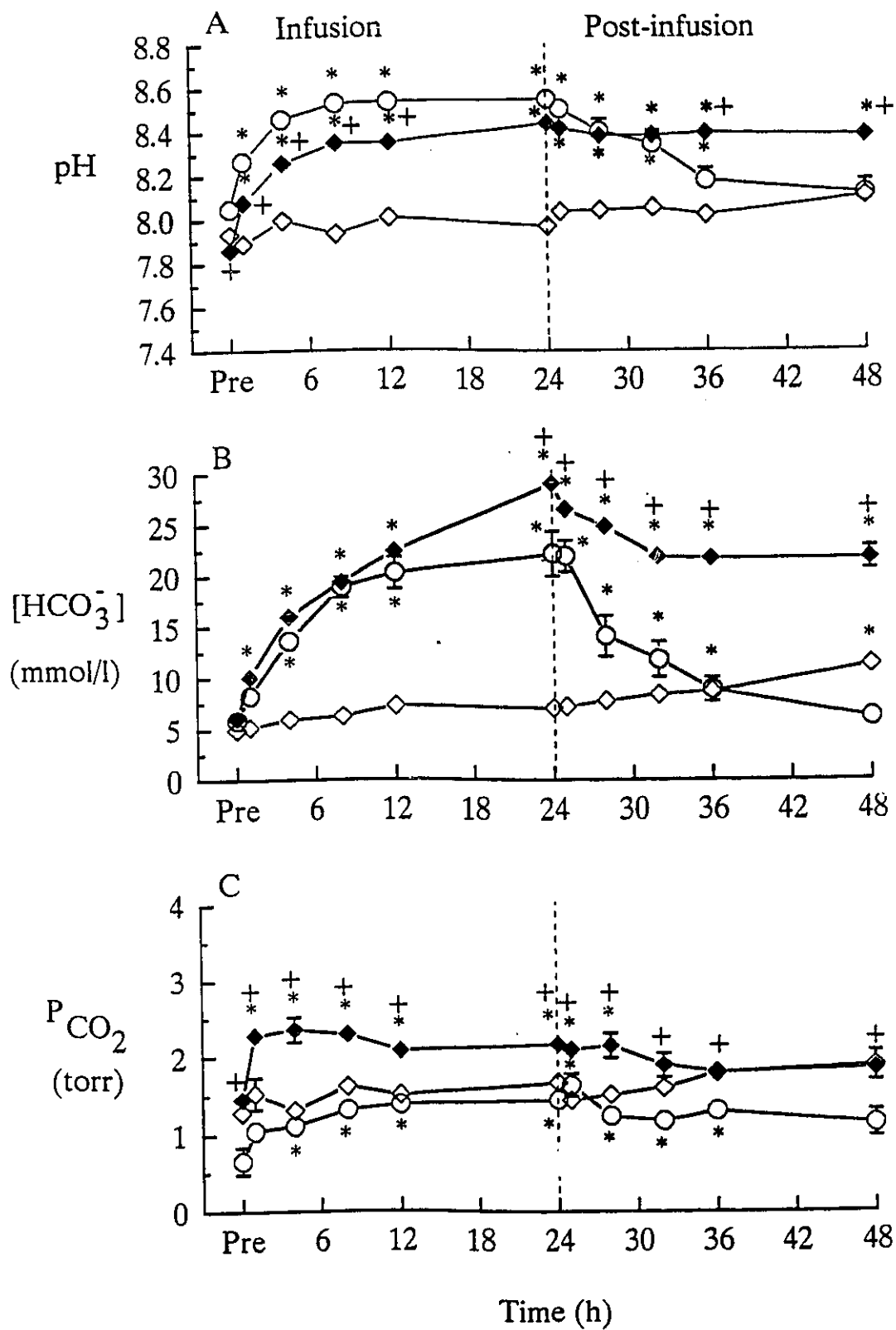


FIGURE 7.9

The temporal effects of 24 h of chronic infusion and the subsequent 24 h post-infusion period on the acid-base parameters (A) blood pH, (B) plasma $[\text{HCO}_3^-]$, and (C) blood P_{CO_2} in NaCl-infused [open diamonds (\diamond)] and NaHCO_3 -infused [filled diamonds (\blacklozenge)] fish exposed to Cl^- free water. NaHCO_3 -infused fish exposed to Ottawa tapwater are shown for comparison [open circles (\circ)]. * indicates significant difference from the initial Pre (control) value. + indicates significant difference from the NaHCO_3 -infused Ottawa tapwater value at that time period (NaHCO_3 -infused Cl^- free group only). Mean \pm SE.

- Normal Ottawa Tapwater
 ◇ NaCl infused } Cl⁻ free water
 ◆ NaHCO₃-infused



Infusion of NaHCO_3 during exposure to CFW resulted in a metabolic acidosis as in OT-treated trout. However, the accumulation of HCO_3^- in the plasma was significantly greater at the 24 h period (Fig 7.10B). Plasma $[\text{HCO}_3^-]$ rose from 6.05 ± 0.30 mmol/l in the Pre-infusion period to 29.1 ± 0.97 mmol/l by the 24 h period (compare with 22.2 maximum value in OT trout; Fig 7.9B). In contrast to OT exposed fish, CFW exposed fish were unable to regulate arterial pH and $[\text{HCO}_3^-]$ back to initial control values (Fig 7.9A, B) during the post-infusion period.

Series 4 - Effects of Exposure to CFW on Ion Equivalent Fluxes and Gill Morphology during NaHCO_3 Infusion.

Infusion resulted in a significant net Na^+ loss (negative $J_{\text{net}}^{\text{Na}^+}$) of approximately -600 to -800 $\mu\text{mol/kg/h}$ by the 12 h infusion period in both OT and CFW exposed fish. However, there were no significant differences in $J_{\text{net}}^{\text{Na}^+}$ between trout exposed to OT and those exposed to CFW (Fig 7.10). $J_{\text{net}}^{\text{Cl}^-}$ in OT trout remained positive and not significantly different from the non-infused control. In contrast, trout exposed to CFW had a significant, large net Cl^- loss ($-J_{\text{net}}^{\text{Cl}^-}$) of approximately -500 $\mu\text{mol/kg/h}$ by 12 h. The net Cl^- loss was sustained throughout the infusion and post-infusion periods (Fig 7.10) and was significantly greater than OT exposed fish at almost every period measured.

$J_{\text{in}}^{\text{Na}^+}$ was reduced in a similar fashion during the infusion and post-infusion in both the OT and CFW trout (Fig 7.11A). $J_{\text{in}}^{\text{Cl}^-}$ was reduced ($J_{\text{in}}^{\text{Cl}^-} = <20 \mu\text{mol/kg/h}$) in the trout exposed to CFW due to the unavailability of the external ion for the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism. Unfortunately, $J_{\text{in}}^{\text{Cl}^-}$ could not be completely abolished as $[\text{Cl}^-]$ in the water increased over the flux period due to the large negative $J_{\text{net}}^{\text{Cl}^-}$ (Fig 7.10), thereby providing a nominal amount of external substrate for the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. The reduction in $J_{\text{in}}^{\text{Cl}^-}$ was in marked contrast to the stimulation of $J_{\text{in}}^{\text{Cl}^-}$ noted in trout

FIGURE 7.10

The temporal effects of 24 h of infusion of NaHCO_3 in Ottawa tapwater (open histograms) or trout exposed to Cl^- free water (filled histograms) and the subsequent 24 h post-infusion period on the net fluxes of sodium ($J_{\text{net}}^{\text{Na}^+}$) and chloride ($J_{\text{net}}^{\text{Cl}^-}$). * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl -infused value at that time period (NaHCO_3 -infused only). Mean \pm SE.

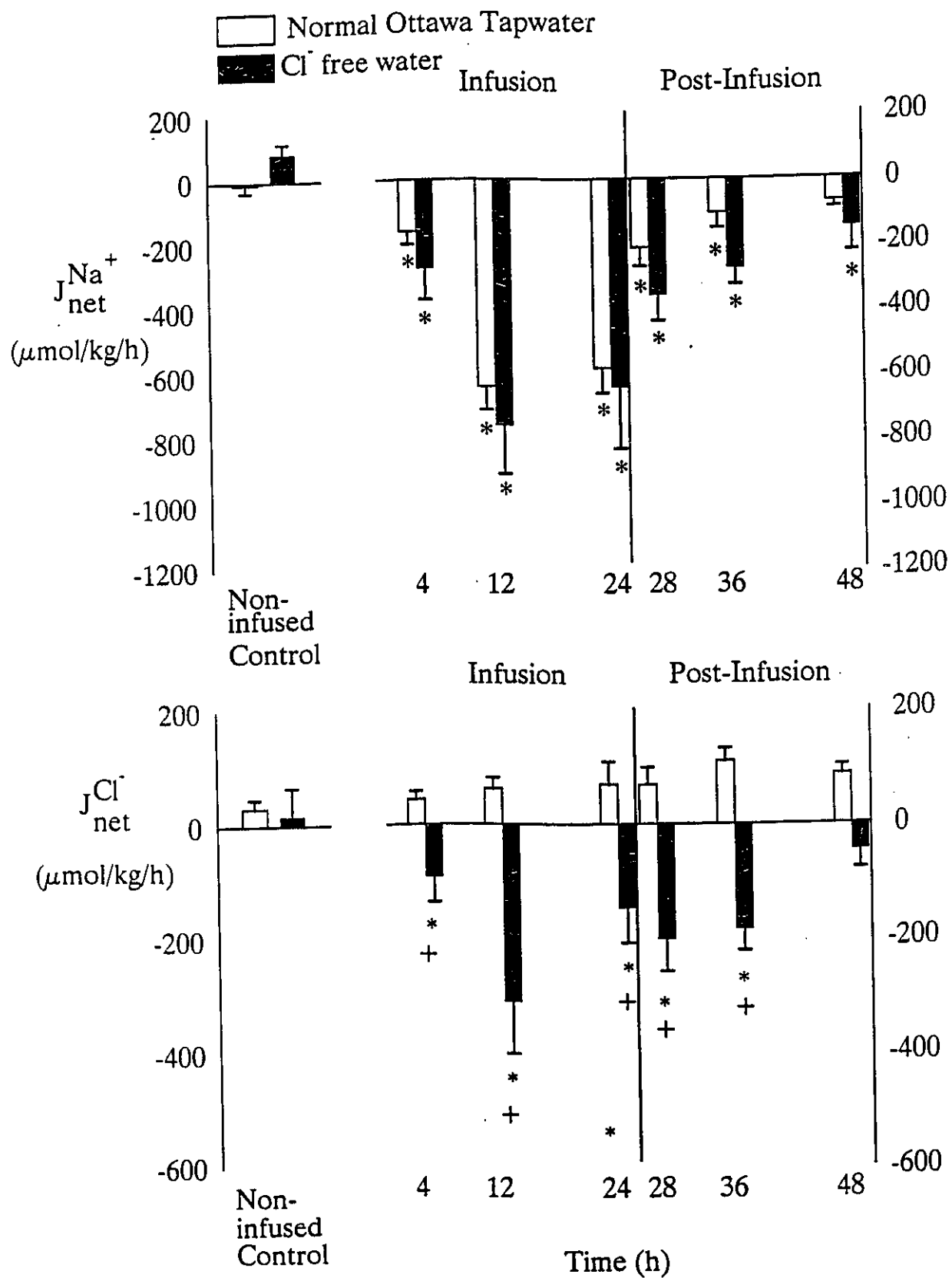
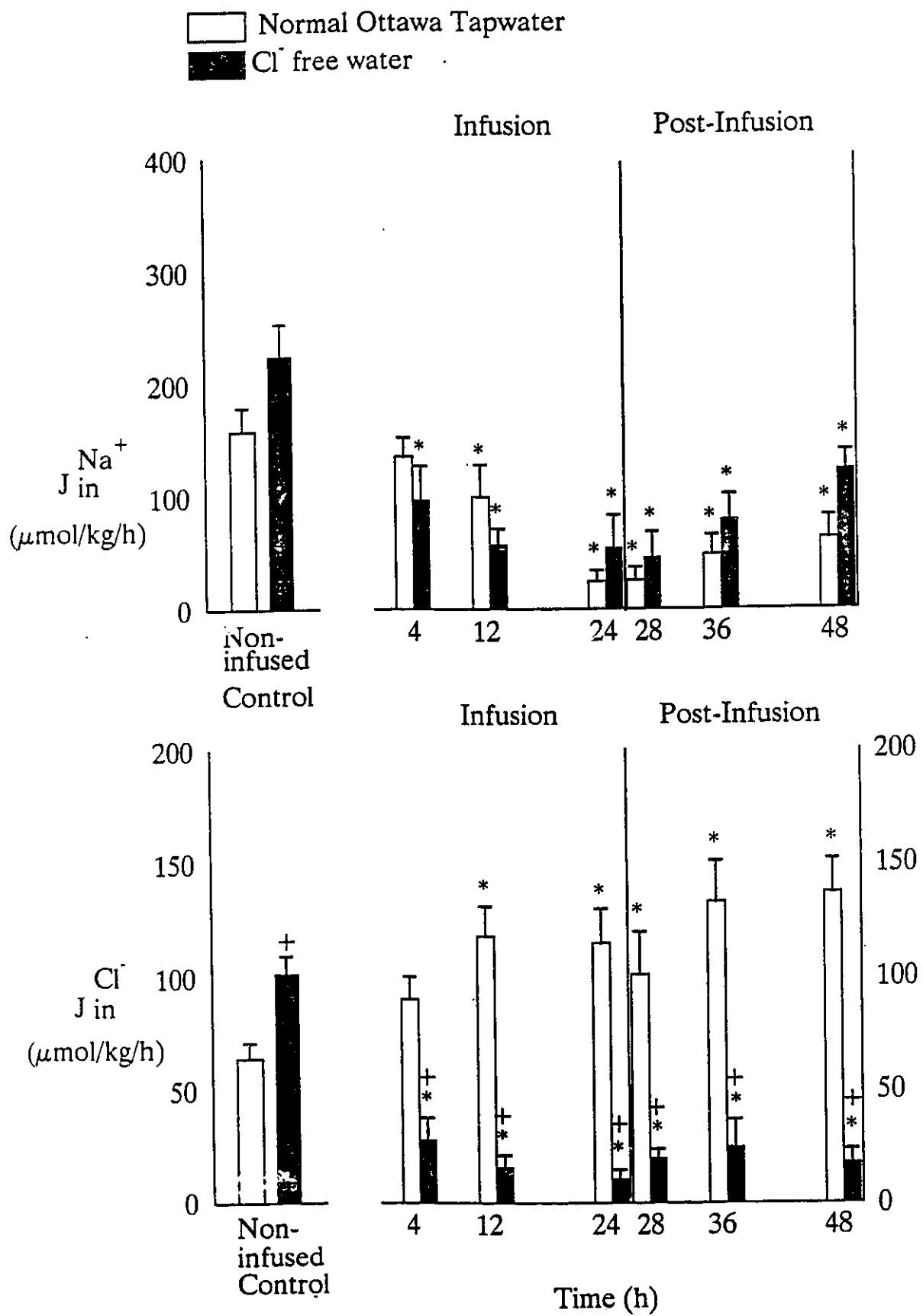


FIGURE 7.11

The temporal effects of 24 h of infusion of NaHCO_3 in Ottawa tapwater (open histograms) or trout exposed to Cl^- free water (filled histograms) and the subsequent 24 h post-infusion period on the influxes of sodium ($J_{\text{in}}^{\text{Na}^+}$) and chloride ($J_{\text{in}}^{\text{Cl}^-}$). * indicates significant difference from the initial non-infused control value. + indicates significant difference from the NaCl -infused value at that time period (NaHCO_3 -infused only). Mean \pm SE.



exposed to OT (Fig 7.11B). $J_{in}^{Cl^-}$ was significantly less at every time period in the CFW exposed trout compared to the OT exposed trout (Fig 7.11).

The effects of 24 h infusion of either NaCl or NaHCO₃ during exposure to CFW on CC morphometry are shown in Fig 7.12. For comparative purposes, the CC morphometry of control non-infused fish is shown as well. NaCl infusion and exposure to CFW resulted in a significant 40% reduction in CCFA similar to that seen in trout exposed to normal Ottawa tapwater (Chapter 6, Figure 6.8). CCFA was reduced from $83951 \pm 9789 \mu\text{m}^2/\text{mm}^2$ in control non-infused fish to $49931 \pm 10265 \mu\text{m}^2/\text{mm}^2$ in NaCl-infused, CFW exposed fish (Fig 7.12C). These reductions in CCFA were due to a reduction in average CC area only (Fig 7.12A). In contrast, 24 h of NaHCO₃ infusion in CFW exposed trout resulted in a large (150%) increase in CCFA to $211930 \pm 28676 \mu\text{m}^2/\text{mm}^2$. The increase in CCFA was the combined result of a 45% increase in average CC area (from $34.6 \pm 2.01 \mu\text{m}^2$ to $50.2 \pm 7.25 \mu\text{m}^2$; Fig 7.12A) and a large (100%) increase in CC density (from $2368 \pm 200 /\text{mm}^2$ to $4847 \pm 297 /\text{mm}^2$; Fig 7.12B). Fig 7.13 shows representative SEM photographs of the filamental epithelia of rainbow trout during a) control, b) 24 h NaCl-infused, CFW exposed and c) and 24 h NaHCO₃-infused, CFW exposed. Under control conditions, CCs were easily recognizable from adjacent PVCs and MCs by their numerous microvilli and sharp delimitation from the adjacent PVCs. After 24 h NaCl infusion in CFW, the CCs were much smaller in appearance. In addition, differentiation of CCs from MCs became more difficult. NaHCO₃ infusion for 24 h in CFW resulted in a sharply increased CCFA. These changes are clearly evident in Fig 7.13c showing large numerous CCs compared to control trout.

FIGURE 7.12

The effects of 24 h of infusion of NaHCO_3 in control Ottawa tapwater fish (open histograms) and NaCl or NaHCO_3 infusion in fish exposed to Cl^- free water (cross-hatched histograms) 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 the initial non-infused control value. + indicates significant difference from the NaCl -infused trout (CFW treatment group only). Mean \pm SE.

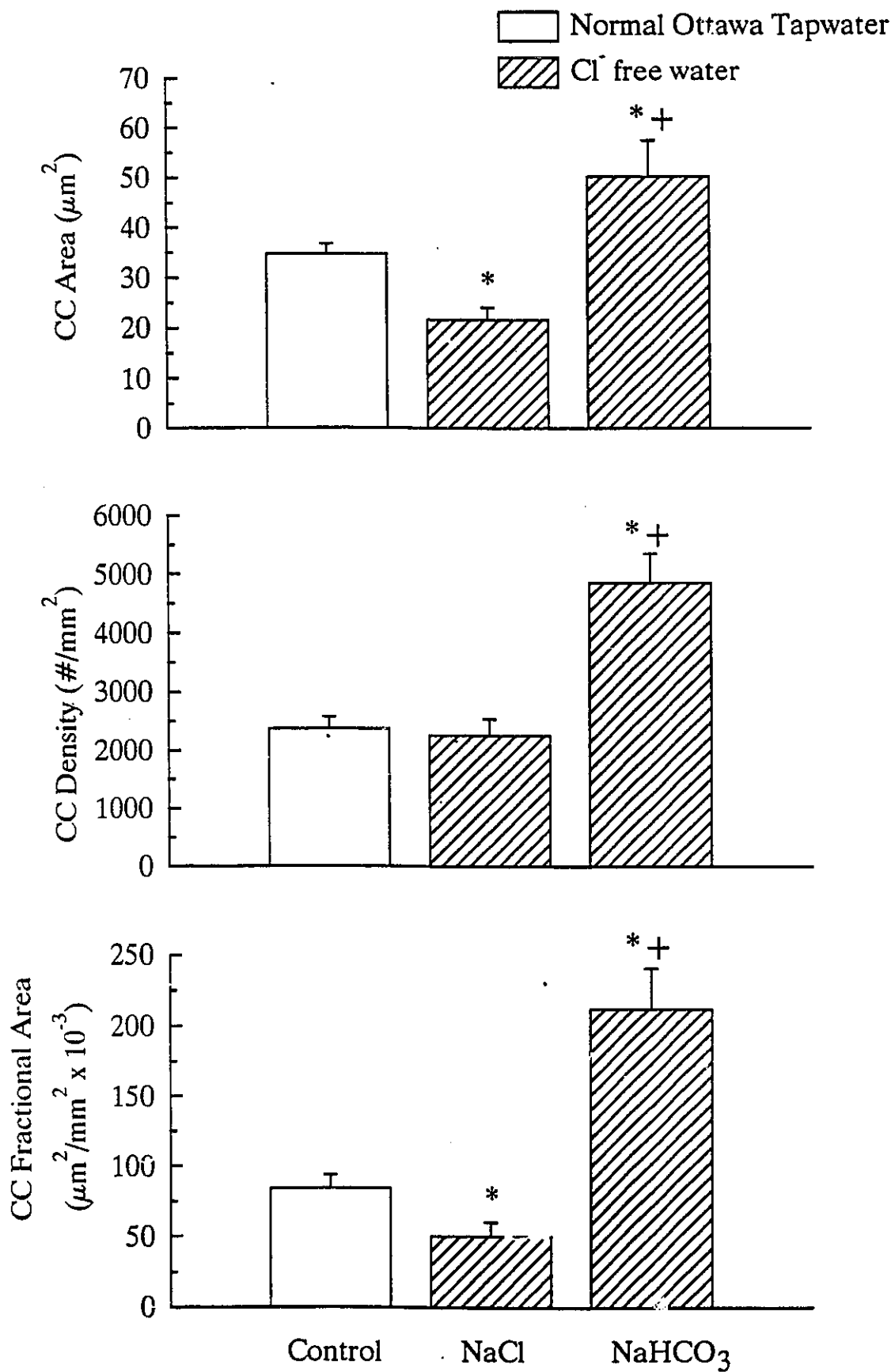
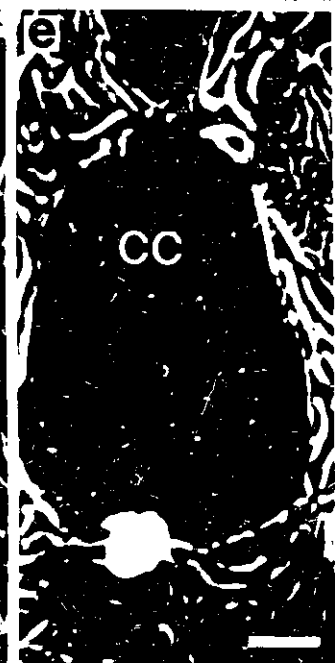
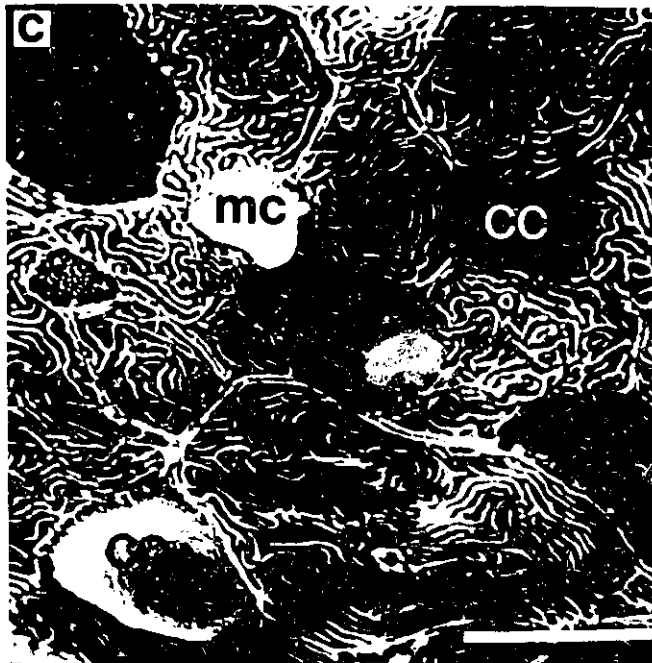
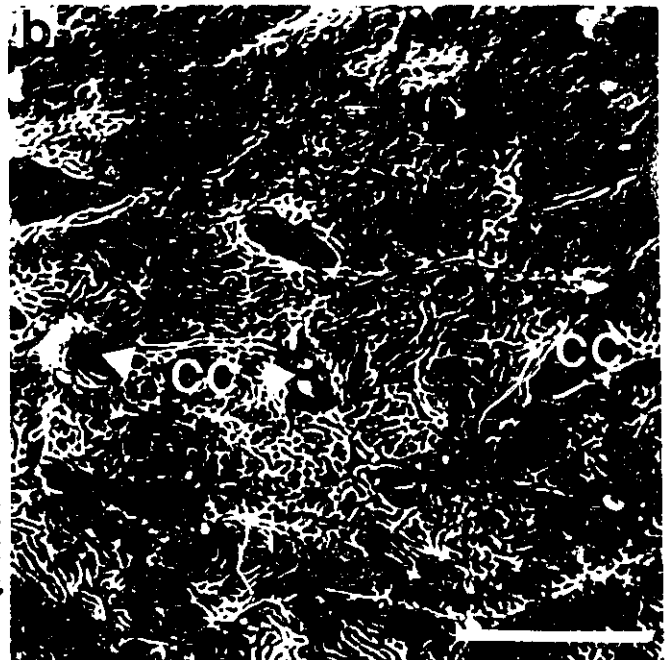


FIGURE 7.13

Representative low magnification scanning electron microscopy (SEM) photographs of the filament epithelium of rainbow trout in control Ottawa Tapwater fish (Fig 7.14A); 24 h NaCl-infused, Cl⁻ free exposed fish (Fig 7.14B, D); and NaHCO₃ infused, Cl⁻ free exposed fish (Fig 7.14C, E). Note that NaCl-infusion resulted in a reduction in CCs on the filamental epithelium while NaHCO₃ infusion in Cl⁻ free exposed fish resulted in large increases in the average area. CC indicate chloride cells. MC indicate mucous cells. Figs 6.8 a-c; scale bar = 10 μ. Figs 6.8 d-e, scale bar = 2 μm.



DISCUSSION

There were marked intra-specific differences in the ability of rainbow trout to regulate the metabolic alkalosis elicited by NaHCO_3 infusion. Populations of fish with increased rates of ionic uptake ($J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$) caused by cort/oGH treatment, had an increased ability to regulate whereas fish with reduced rates of $J_{\text{in}}^{\text{Cl}^-}$ (through removal of the external ion - Cl^-) had a decreased ability to regulate. These data demonstrate the crucial role of gill CCs and the associated $\text{Cl}^-/\text{HCO}_3^-$ exchange in the regulation of alkalosis in trout and are in agreement with previous studies which noted that manipulation of the branchial $\text{Cl}^-/\text{HCO}_3^-$ exchange is the dominant mechanism used to regulate metabolic alkalosis in freshwater teleost fish (Claiborne and Heisler, 1984, 1986; Wood *et al*, 1984; Perry *et al*, 1987a; Goss and Wood, 1990a, b). Although the effect of experimentally increasing CCFA on the ionic transport capacity has been well studied (e.g. Perry and Wood, 1985; Perry and Laurent, 1989; Laurent and Perry, 1990), this is only the second study to examine the effects of intra-specific changes in CCFA on compensation of an acid-base disturbance (Chapter 3 is the first) and the first to examine the effects increasing CCFA had on the compensation from an alkalosis.

Effects of Cortisol/ovine Growth Hormone Treatment on the Regulation of NaHCO_3 -infusion induced Alkalosis.

Increasing the CCFA and hence, the number of $\text{Cl}^-/\text{HCO}_3^-$ exchangers on the gill epithelium by chronic cortisol/oGH treatment imparted an increased ability to regulate blood acid-base status in the face of continued base (NaHCO_3) infusion. This occurred by increasing the flux of titratable alkalinity (J^{TA}). This corresponded with the increased $J_{\text{in}}^{\text{Cl}^-}$ noted and provides further evidence for the importance of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger in acid-base regulation (Wood *et al*, 1984; Perry *et al*, 1987b; Goss and Wood, 1990a, b).

In Chapter 6 it was demonstrated that NaCl-infusion caused an increase in net Na^+ loss ($-\text{J}_{\text{net}}^{\text{Na}^+}$) and that these were not significantly different from NaHCO_3 -infused fish. Therefore, manipulation of $\text{J}_{\text{net}}^{\text{Na}^+}$ was apparently not utilized as a means of acid-base regulation in those fish. There is evidence, however, for manipulation of $\text{J}_{\text{net}}^{\text{Na}^+}$ to aid in acid-base compensation in cort/oGH trout, although it is only significant at the 24 h period. The increase in Na^+ loss compared to untreated fish would act to decrease the SID and hence, reduce the alkalosis according to Stewart (1978, 1983). The mechanism responsible for this increase is unknown although changes in epithelial permeability likely occur as a result of cort/oGH treatment as demonstrated by the increases in $\text{J}_{\text{in}}^{\text{Cl}^-}$ and $\text{J}_{\text{in}}^{\text{Na}^+}$.

The effects of combined cortisol and growth hormone on gill morphology were similar to those previously reported (Madsen, 1990b) including an increase in both the size and density of lamellar and filament CCs. The increases in CCFA were correlated with increase rates of $\text{J}_{\text{in}}^{\text{Cl}^-}$ under control conditions in agreement with the results of Laurent and Perry (1990) and Chapter 3. There were no further increases in CCFA resulting from NaHCO_3 infusion in contrast to the large elevation noticed in untreated fish. It is conceivable that the CCFA displayed on the gill epithelium was already approaching the maximal value in those fish. The proliferation of CCs on the lamellar epithelium during cort/oGH treatment is thought to increase the blood-to-water diffusion distance (Shawn Bindon and Steve Perry, personal communication) and presumably must impair gas transfer. In the cort/oGH-treated fish, arterial P_{CO_2} was elevated under control conditions suggesting that gas transfer was indeed impaired. Thomas *et al* (1988) reported an impairment of gas transfer and proliferation of CCs in fish acclimated to low ionic strength water and suggested that the large numbers of CCs may have impaired gas

transfer

The appearance of differing external CC morphologies (CC₁ and CC₂) under control conditions and during infusion is intriguing. Whether all the CCs present in these groups have similar functions and the differences in morphology relate to the life cycle of the cell (Wendelaar-Bonga and van de Meij, 1989) or whether there exist sub-populations of CCs on the gill epithelium in analogy to other "tight" ion-transporting epithelia (e.g. frog skin: Rick *et al*, 1992; turtle bladder: Steinmetz, 1985; 1987; cortical collecting duct: Brown *et al*, 1988a, b) should be the focus of future investigation.

Effect of Cl⁻ Free Water on the Regulation of NaHCO₃-infusion induced Alkalosis.

Decreasing $J_{in}^{Cl^-}$ by removal of the external substrate (Cl⁻) for the Cl⁻/HCO₃⁻ exchanger from the external environment impaired the ability of rainbow trout to regulate blood acid-base status in the face of continued base (NaHCO₃) infusion. The impaired ability was the result of an inability to regulate $J_{net}^{Cl^-}$ through manipulation of $J_{in}^{Cl^-}$ even though there were increases in both CCFA and internal substrate (HCO₃⁻) availability which would otherwise act to increase $J_{in}^{Cl^-}$. Trout in CFW were able to modulate the efflux component of net Cl⁻ balance to contribute to acid-base regulation but this was not sufficient to attain the same degree of compensation as the OT trout. Net Cl⁻ loss was less in CFW trout than in OT trout. This response is similar to that seen in eels, although trout are unable to retain Cl⁻ as efficiently as eels. Eels, with no appreciable $J_{in}^{Cl^-}$ and no means of manipulation of $J_{in}^{Cl^-}$, rely primarily on regulation of Cl⁻ efflux to regulate acid-base status. The differential loss of Na⁺ over Cl⁻, resulting in a decrease in SID, will constrain a decrease in extracellular pH. Trout exposed to CFW had an increased net Cl⁻ loss but the increase was less than the increased Na⁺ loss. The mechanisms involved in the regulation of efflux are unclear although they likely represent alterations in tight

junctions, perhaps *via* alterations in selectivity (Madera, 1988).

Exposure to CFW during NaHCO_3 infusion resulted in large increases in CCFA but, owing to the absence of external Cl^- , $J_{\text{in}}^{\text{Cl}^-}$ remained low. This likely represents a "naive" attempt to increase $J_{\text{in}}^{\text{Cl}^-}$ by increasing CCFA. Continued lack of $J_{\text{in}}^{\text{Cl}^-}$ could potentially further stimulate increases in CCFA until the maximal value was reached. In general, the CCs in the CFW trout had few apical microvilli ornamenting the surface. The significance of these alteration are unknown to this point, although they may represent differences in the functioning or age of the cell as previously discussed.

The results of the CFW and cort/oGH experiments clearly demonstrate the importance of the branchial $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism in the regulation of blood acid-base status in freshwater fish. In addition, this Chapter demonstrated that selective regulation of Cl^- efflux was utilized in the compensation of metabolic alkalosis, although the mechanism(s) remain unresolved.

CHAPTER 8

**THE EFFECTS OF CHANGES IN WATER [NaCl] ON THE RATE OF ACID-BASE
COMPENSATION IN RAINBOW TROUT.**

INTRODUCTION

The rate at which fish compensate acid-base disturbances is highly variable both among species (inter-specific variability) and within a single species (intra-specific variability). The results of Chapter 7 demonstrated that variation in the surface area of CCs exposed on the gill epithelium is a key determinant of intra-specific differences in the ability of freshwater fish to regulate acid-base disturbances. Specifically, it was shown that a greater CC surface area results in an increased ability of rainbow trout to compensate metabolic alkalosis. Other studies have shown that compensation of blood acidosis is accelerated at higher water salinities and have attributed these effects to the elevated $[\text{NaCl}]$ directly (Kirsch, 1972; Perry, 1982; Boutilier *et al.*, 1986, Tang *et al.*, 1989a, b; Avella and Bornancin, 1990; Iwama and Heisler, 1991; Tang and Boutilier, 1991). However, these studies examined differences in acid-base compensation between freshwater- and seawater-adapted fish. Gill chloride cell function and morphology are totally different in seawater- and freshwater-adapted fish (Keys and Wilmer, 1932; Laurent and Dunel, 1980; Foskett and Scheffey, 1982; Foskett *et al.*, 1983; Laurent, 1984; Zadunaisky, 1984; Perry and Wood, 1985; Avella *et al.*, 1987; Leino *et al.*, 1987; Pisam *et al.*, 1987; Perry and Laurent, 1989; Franklin, 1990; King and Hossler, 1991). Therefore, interpretation of the variations in acid-base compensation are complicated by simultaneous alterations in 1) the basic function of the CC (*i.e.* freshwater- or seawater-adapted CC) 2) CC morphology (differences in CC surface area) and c) external substrate concentration (variations in $[\text{NaCl}]_e$).

In a recent study, Iwama and Heisler (1991) demonstrated that chronic elevation of external salinity from 3 mmol/l to 100 mmol/l $[\text{NaCl}]$ resulted in a significantly greater rate of acid-base compensation during hypercapnia in rainbow trout while a further

increase to 300 mmol/l, a level closer to seawater $[\text{NaCl}]_e$, caused only slightly greater rates of compensation. The absence of gill morphological data, however, does not allow the relative contributions of variations in morphology to be discerned from the direct effects of elevated $[\text{NaCl}]_e$.

$J_{in}^{\text{Cl}^-}$ and $J_{in}^{\text{Na}^+}$ are known to be directly altered by acute adjustments of external ion concentrations over a relatively small (<2 mmol/l) range of salinities (Shaw, 1959; de Renzis and Maetz, 1973; Wood and Randall, 1973; Avella *et al.*, 1987; Frain, 1987; Avella and Bornancin; 1990; Goss and Wood, 1990a, b) and alterations in freshwater $[\text{NaCl}]_e$ within a similar range may cause significant variations in gill CC surface area (Laurent and Dunel, 1980; Höbe *et al.*, 1984a, b; Spry and Wood, 1988; Perry and Laurent, 1989; Laurent and Perry, 1991). Furthermore, the rate of acid-base compensation during metabolic alkalosis is related to the gill CC surface areas (Chapters 6, 7). Thus, the goal of the present study was to determine the relative roles of altered $[\text{NaCl}]_e$ and altered CC morphology on blood acid-base balance during experimentally induced metabolic alkalosis. This involved examination of the responses to base (NaHCO_3) infusion of rainbow trout (*Oncorhynchus mykiss*) acclimated to two different salinities known to cause differing rates of $J_{in}^{\text{Cl}^-}$, $J_{in}^{\text{Na}^+}$, (Wood and Randall, 1973; Frain, 1987; Avella and Bornancin, 1990; Goss and Wood, 1990a, b) and CC morphology (Laurent *et al.*, 1985; Perry and Laurent, 1989).

METHODS

Experimental Animals and Acclimation Conditions

Rainbow trout (*Oncorhynchus mykiss*) [mean weight = 301 ± 12 g (SE) $n = 109$] were obtained from Linwood Acres Trout Farm (Cambellcroft, Ontario) and held in large (500 l) fibreglass tanks (Living Stream, Toledo, Ohio) supplied with flowing dechlorinated Ottawa tap water ($\text{Na}^+ = 0.15$ mmol/l; $\text{Cl}^- = 0.18$ mmol/l; $\text{Ca}^{2+} = 0.42$ mmol/l, $\text{pH} = 7.70$; $\text{temp} = 5\text{-}14^\circ\text{C}$). All fish were held for at least one month prior to experimentation. Fish were fed daily to satiation with commercial trout pellets (Martin Feed Mills). Food was withheld 48 h prior to experimentation to minimize variation in acid output caused by feeding (Wood and Caldwell, 1978). Photoperiod was held constant at 12L:12D. At least six weeks prior to experimentation, fish were divided into two groups. One group was maintained in a 500 l tank containing normal Ottawa tapwater while the other was transferred to an adjacent tank containing elevated NaCl concentration ($[\text{NaCl}]_e$). This was achieved by titration of the inflowing water (4 l/min) with a stock solution of NaCl (1.35 mol/l NaCl) using a peristaltic pump to yield nominal NaCl concentrations of 1.5 mmol/l, 10X higher than normal Ottawa tapwater. This $[\text{NaCl}]_e$ was chosen as it is known to elevate both $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ (see Chapter 4). Actual $[\text{NaCl}]_e$ in the high NaCl-acclimated group was found to be 1.36 ± 0.08 mmol/l ($n = 108$).

To allow for infusion and repetitive blood sampling, trout were anaesthetized using their acclimation medium as a source of water (MS222 1:10000; Sigma, adjusted to pH 7.0 with NaHCO_3) and fitted with an indwelling dorsal aortic cannulae filled with heparinized Cortland saline (Wolf, 1963) according to the method of Soivio *et al* (1973). The fish were allowed to recover in opaque acrylic boxes supplied with acclimation water for at least 48 h prior to experimentation.

Experimental Protocol

Series 1 - Gill Morphology

To determine the effects of acclimation to various salinities on gill morphology, gill filaments of cannulated fish from each acclimation group (tapwater-acclimated, $n = 22$ high NaCl-acclimated, $n = 6$) were fixed and the CC morphometry examined in the SEM according to the methods outlined in Chapter 2.

Series 2 - Effects of High $[\text{NaCl}]_e$ Acclimation and Acute Exposure to Elevated $[\text{NaCl}]_e$ on the Blood Acid-Base Response to NaHCO_3 Infusion.

The second series of experiments was designed to characterize the alteration in blood acid-base status during 24 h infusion of NaHCO_3 and the subsequent 24 h post-infusion period under three different experimental conditions. Ottawa tapwater-acclimated fish exposed to Ottawa tapwater served as a control group (infusion rate = $893 \pm 63 \mu\text{mol/kg/h}$; $n = 6$). To determine the combined effects of differences in CC morphology and elevations in external substrate on the blood acid-base responses to NaHCO_3 infusion, chronically high NaCl-acclimated trout were exposed to their acclimation water throughout the experimental period (infusion rate = $917 \pm 102 \mu\text{mol/kg/h}$; $n = 7$). To determine the effect of elevation in external substrate alone on the blood acid-base response to NaHCO_3 infusion, Ottawa tapwater-acclimated trout were acutely exposed to an elevated external NaCl concentration. After an initial control (Pre) sample in Ottawa tapwater, infusion of NaHCO_3 ($826 \pm 71 \mu\text{mol/kg/h}$; $n = 6$) was started. Simultaneously, the inflowing water flow to the box was stopped and replaced by rapidly flowing (5 l/min) water from the high NaCl acclimation tank thereby rapidly elevating the $[\text{NaCl}]_e$ to $\sim 1.5 \text{ mmol/l}$. After an initial period of about 10 min, the flow rate was reduced to 0.5 l/min and maintained at that rate for the entire 24 h infusion and 24 h

post-infusion period.

In each of the above treatment groups, repetitive blood samples (300 μ l) were withdrawn from the arterial cannulae at the following times: 0 (Pre), 1, 4, 8, 12, and 24 h after the start of infusion. In the post-infusion period, blood samples were withdrawn at 25, 28, 32, 36, and 48 h (1, 4, 8, 12, 24 h post-infusion). Blood samples were analyzed immediately for pH. The blood was then centrifuged at 10000g for 15 sec, the plasma drawn off and analyzed for total CO₂.

Series 3 - Effects of Chronic Acclimation to Elevated [NaCl]_e on the Ion-Acidic/Basic Equivalent Fluxes during Infusion.

The third series of experiments was designed to characterize the unidirectional and net ion and acidic/basic equivalent fluxes during and after NaHCO₃ infusion in fish acclimated to Ottawa tapwater or high [NaCl]_e. Unfortunately, measurement of the ion and acidic/basic equivalent fluxes during acute exposure to high [NaCl]_e was not performed. For Ottawa tapwater rainbow trout (n = 56), measurements of whole body ionic fluxes were accomplished as described in Chapter 6. At the end of the flux period, the trout were killed by spinal section, the left gill arch removed and fixed for examination in the scanning electron microscope (SEM) as described in Chapter 2.

For high [NaCl]_e-acclimated fish, the protocol was modified slightly. Instead of measuring the ionic and acidic/basic equivalent fluxes in separate fish for each time period as was done for Ottawa tapwater trout, the measurements were performed sequentially. Separate groups of fish were infused with either NaCl (sham: infusion rate = 786 ± 82 μ mol/kg/h; n = 5) or NaHCO₃ (infusion rate = 908 ± 48 μ mol/kg/h; n = 6) for 24 h to determine the effects of infusion on net and unidirectional ion and acidic/basic equivalent fluxes. The following periods were chosen for analysis of the ionic-acidic/basic equivalent

fluxes. Following an initial non-infused control (Pre), the 4, 12, and 24 h infusion, and the 28, 36 and 48 h periods (4, 12, 24 h post-infusion) were examined.

Analytical techniques

The analytical techniques utilized in this Chapter are exactly as described in Chapter 6.

Statistical analyses

All data are presented as means \pm 1 standard error (SE). Data for Fig 8.1 was analyzed by an unpaired Student's two-tailed t-test. Data for figures 8.2 to 8.4 were analyzed by two-way ANOVA followed by Fishers LSD test for multiple comparisons where the F-value of the ANOVA indicated significance. 5% was accepted as the fiducial limit of significance in all cases.

RESULTS

Series 1 - Gill Morphology

Acclimation to 1.5 mmol/l $[\text{NaCl}]_e$ caused a 65% increase in CCFA from $83951 \pm 9789 \mu\text{m}^2/\text{mm}^2$ in Ottawa tapwater-acclimated fish to $138357 \pm 13460 \mu\text{m}^2/\text{mm}^2$ in the high NaCl-acclimated fish. The increases in CCFA were caused entirely by increases in average CC area from $34.6 \pm 2.01 \mu\text{m}^2$ in tapwater-acclimated trout to $53.9 \pm 3.04 \mu\text{m}^2$ in high NaCl-acclimated trout; CC density was unchanged during high NaCl acclimation (Fig 8.1).

Series 2 - Effects of High $[\text{NaCl}]_e$ Acclimation and Acute Exposure to Elevated $[\text{NaCl}]_e$ on the Blood Acid-Base Responses to NaHCO_3 Infusion.

To clarify presentation of data, the effects of NaCl infusion (140 mmol/l; $884 \pm 44 \mu\text{mol}/\text{kg}/\text{h}$) on blood acid-base status are not shown. NaCl-infusion did not cause any significant changes from the control (Pre) values for any of the blood acid-base variables measured, pH, $[\text{HCO}_3^-]$ and P_{CO_2} .

In all three treatment groups, infusion of NaHCO_3 resulted in a severe metabolic alkalosis (elevation in pH owing to increased $[\text{HCO}_3^-]$ coupled with a significant rise in P_{CO_2} (Fig 8.2). However, there were significant differences in the degree of alkalosis and the increases in plasma $[\text{HCO}_3^-]$ observed. In fish acclimated to, and experimented on, in Ottawa tapwater, infusion of NaHCO_3 caused an elevation of pH from a control value of 8.05 ± 0.01 to 8.54 ± 0.04 by the 8 h period; there were no further increases in blood pH throughout the remaining 16 h of infusion (Fig 8.2a). Plasma $[\text{HCO}_3^-]$ rose from 5.85 ± 0.44 to $22.2 \pm 2.2 \text{ mmol}/\text{l}$ after 24 h of infusion and fell rapidly to control levels during the post-infusion period (Fig 5.2b). There was a slight rise in arterial P_{CO_2} (respiratory acidosis) during NaHCO_3 infusion; this elevation in P_{CO_2} persisted to the 36 h (12 h post-

FIGURE 8.1

Comparison of the gill filament chloride cell (CC) morphometry including (A) surface area of individual CCs, (B) surface CC density, and (C) CC fractional area for rainbow trout (*Oncorhynchus mykiss*) acclimated to either Ottawa tapwater or high (1.5 mmol/l) NaCl (Series 1). Mean \pm SE. * = significantly different from tapwater acclimated value, $P < 0.05$.

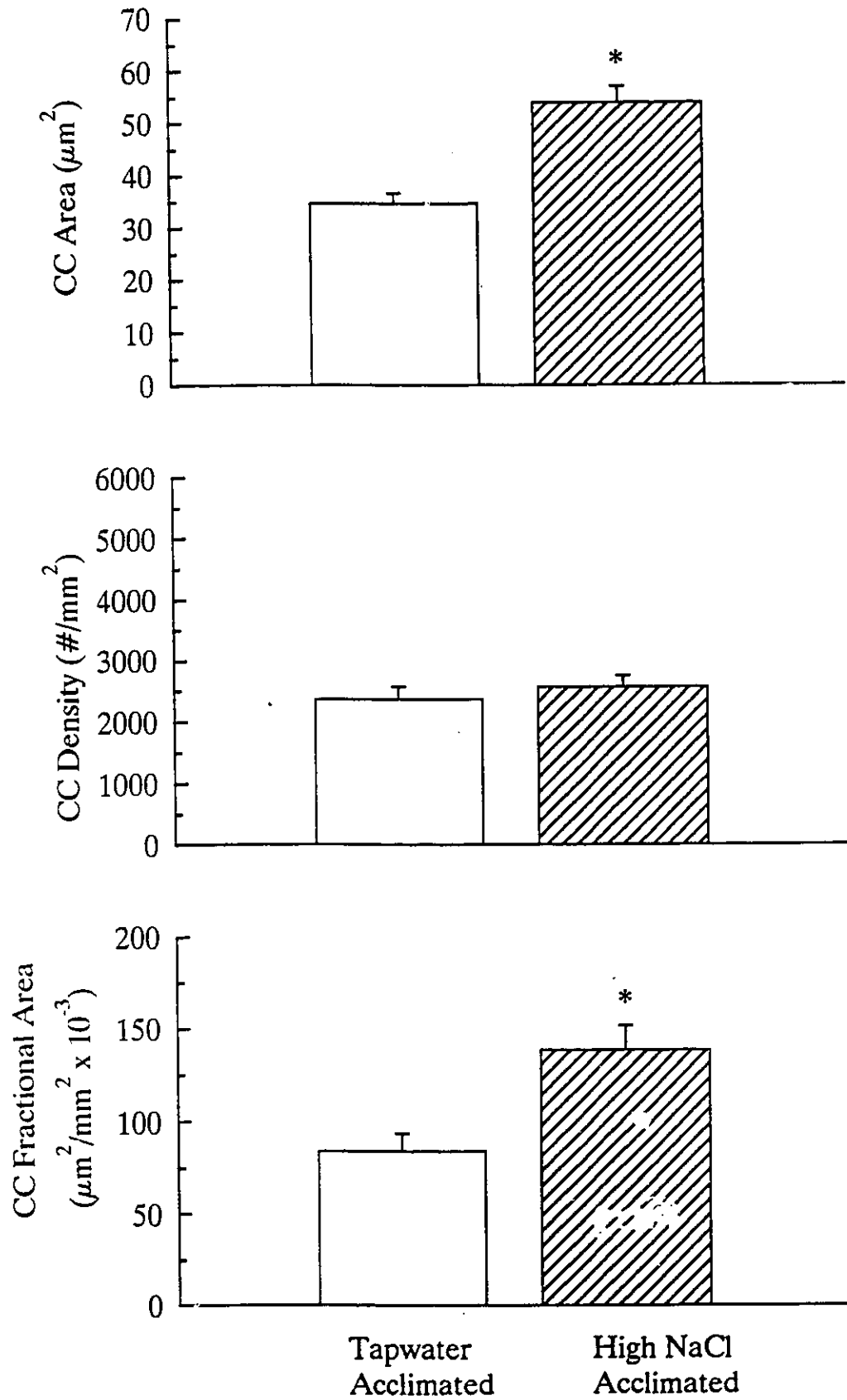
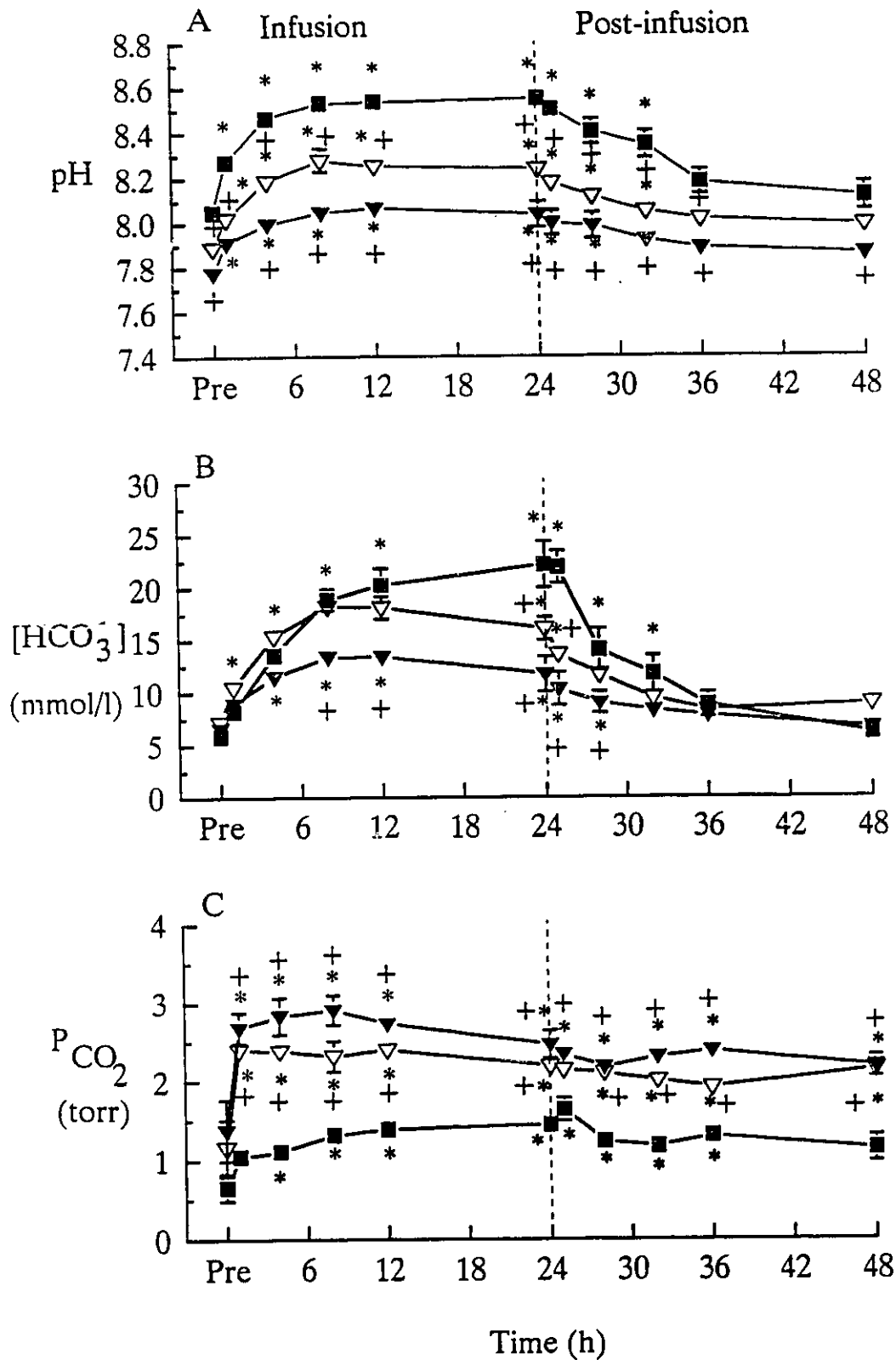


FIGURE 8.2

The temporal effects of 24 h of chronic infusion of NaHCO_3 and the subsequent 24 h post-infusion period on the acid-base variables in rainbow trout A) blood pH, (B) plasma $[\text{HCO}_3^-]$, and (C) plasma P_{CO_2} in three experimental groups, 1) Ottawa tapwater-acclimated (■), 2) Ottawa tapwater-acclimated; acute high NaCl-exposed (▼), 3) high NaCl-acclimated (▽). * indicates significant difference from the initial non-infused control value. + indicates significant difference from the Ottawa tapwater value at that time period only. Mean \pm SE.

- Ottawa Tapwater
- ▽ Acute High NaCl exposed
- ▼ High NaCl acclimated



infusion) period (Fig 8.2c).

The increase in pH during NaHCO_3 infusion was attenuated by acute exposure to elevated $[\text{NaCl}]_e$ (Fig 8.2a). The reason for the differences are two-fold. First, there was a greater elevation in P_{CO_2} in the acutely high NaCl-exposed trout that probably contributed to a lowering of pH (Fig 8.2C). Secondly, there was significantly less HCO_3^- accumulation in the plasma by the 24 h period (22.2 ± 2.2 mmol/l *versus* 16.3 ± 1.1 mmol/l; Fig 8.2b). In the post-infusion period, blood pH and plasma $[\text{HCO}_3^-]$ were returned to control values more rapidly (by the 8 h period) in acutely high NaCl-exposed trout.

High NaCl-acclimated fish showed the greatest ability to regulate the infused base load. Infusion of NaHCO_3 in the high NaCl-acclimated trout caused a smaller rise in blood pH for similar reasons as described for the acutely high NaCl-exposed fish. i.e a reduced rise in plasma $[\text{HCO}_3^-]$ and elevation of P_{CO_2} . In the post-infusion period, blood pH and plasma $[\text{HCO}_3^-]$ were returned rapidly to control values by the 8 h period although arterial P_{CO_2} remained elevated throughout the post-infusion period. Interestingly, arterial blood pH was significantly lower in the high NaCl-acclimated fish under control conditions owing to an elevated P_{CO_2} (1.38 ± 0.39 torr *versus* 0.66 ± 0.17 torr in Ottawa tapwater fish).

Series 3 - Effects of Chronic Acclimation to Elevated $[\text{NaCl}]_e$ on the Ion-Acidic/Basic Equivalent Fluxes during Infusion.

In non-infused controls, elevation of the $[\text{NaCl}]_e$ resulted in significant stimulations of both $J_{\text{in}}^{\text{Na}^+}$ and $J_{\text{in}}^{\text{Cl}^-}$ compared to Ottawa tapwater-acclimated trout (Fig 8.3). NaHCO_3 infusion in Ottawa tapwater-acclimated (open histograms) trout resulted in a significant drop in $J_{\text{in}}^{\text{Na}^+}$ to <50 $\mu\text{mol/kg/h}$ by the 24 h period (Fig 8.3a) while $J_{\text{in}}^{\text{Cl}^-}$ was

significantly stimulated to $115 \pm 15 \mu\text{mol/kg/h}$ by the 24 h infusion period (Fig 8.3b). The reduced rates of $J_{\text{in}}^{\text{Na}^+}$ and elevated rates of $J_{\text{in}}^{\text{Cl}^-}$ were maintained throughout the post-infusion period (Fig 8.3).

NaHCO_3 infusion in high NaCl-acclimated trout caused a marked decrease in $J_{\text{in}}^{\text{Na}^+}$ similar to that seen in Ottawa tapwater-acclimated trout (Fig 8.3b). $J_{\text{in}}^{\text{Na}^+}$ was almost completely abolished by the 24 h period. $J_{\text{in}}^{\text{Na}^+}$ returned to control values by the 36 h (12 h post-infusion) period in contrast to Ottawa tapwater-acclimated trout which remained depressed throughout the post-infusion period. $J_{\text{in}}^{\text{Cl}^-}$ was significantly elevated during NaHCO_3 infusion in the high NaCl-acclimated trout. This elevation was greater than that seen in Ottawa tapwater-acclimated trout. $J_{\text{in}}^{\text{Cl}^-}$ was elevated significantly by the 12 h period and remained elevated throughout the infusion and post-infusion periods. NaCl infusion in high NaCl-acclimated trout did not effect $J_{\text{in}}^{\text{Na}^+}$ at any time while $J_{\text{in}}^{\text{Cl}^-}$ was reduced in the infusion period only.

Under control conditions, net acidic equivalent excretion (negative $J_{\text{net}}^{\text{H}^+}$) was significantly greater in high NaCl-acclimated trout compared to Ottawa tapwater-acclimated trout (Fig 8.4b). On the other hand, the two components of $J_{\text{net}}^{\text{H}^+}$, J^{Amm} and J^{TA} were not significantly altered by NaCl acclimation (Fig 8.4a). $J_{\text{net}}^{\text{H}^+}$ was not significantly altered by NaCl infusion except for a slight increase after 24 h of infusion.

NaHCO_3 infusion in both Ottawa tapwater-acclimated trout (open histograms) and high NaCl-acclimated trout (solid histograms) caused significant increases in J^{TA} resulting in a positive $J_{\text{net}}^{\text{H}^+}$ (base excretion). However, the high NaCl-acclimation resulted in a more rapid (by 4 h) and greater elevation in J^{TA} ($716 \pm 113 \mu\text{mol/kg/h}$ at 24 h; high NaCl acclimated *versus* $371 \pm 43 \mu\text{mol/kg/h}$ at 24 h; Ottawa tapwater acclimated). J^{Amm} did not change as a result of NaHCO_3 infusion in either group. The alterations in J^{TA} resulted in

a positive $J_{\text{net}}^{\text{H}^+}$ throughout the infusion and post-infusion periods in both groups. The greater J^{TA} noted in the high NaCl-acclimated trout contributed to a greater $J_{\text{net}}^{\text{H}^+}$ at each period in this group (Fig 8.4).

FIGURE 8.3

The temporal effects of 24 h of chronic infusion of NaHCO_3 or NaCl and the subsequent 24 h post-infusion period on the influxes of sodium ($J_{\text{in}}^{\text{Na}^+}$) and chloride ($J_{\text{in}}^{\text{Cl}^-}$) in Ottawa tapwater-acclimated trout and high NaCl -acclimated trout (Series 3) * indicates significant difference from the initial non-infused control value. + indicates significant difference from the Ottawa tapwater value at that time period only. Mean \pm SE.

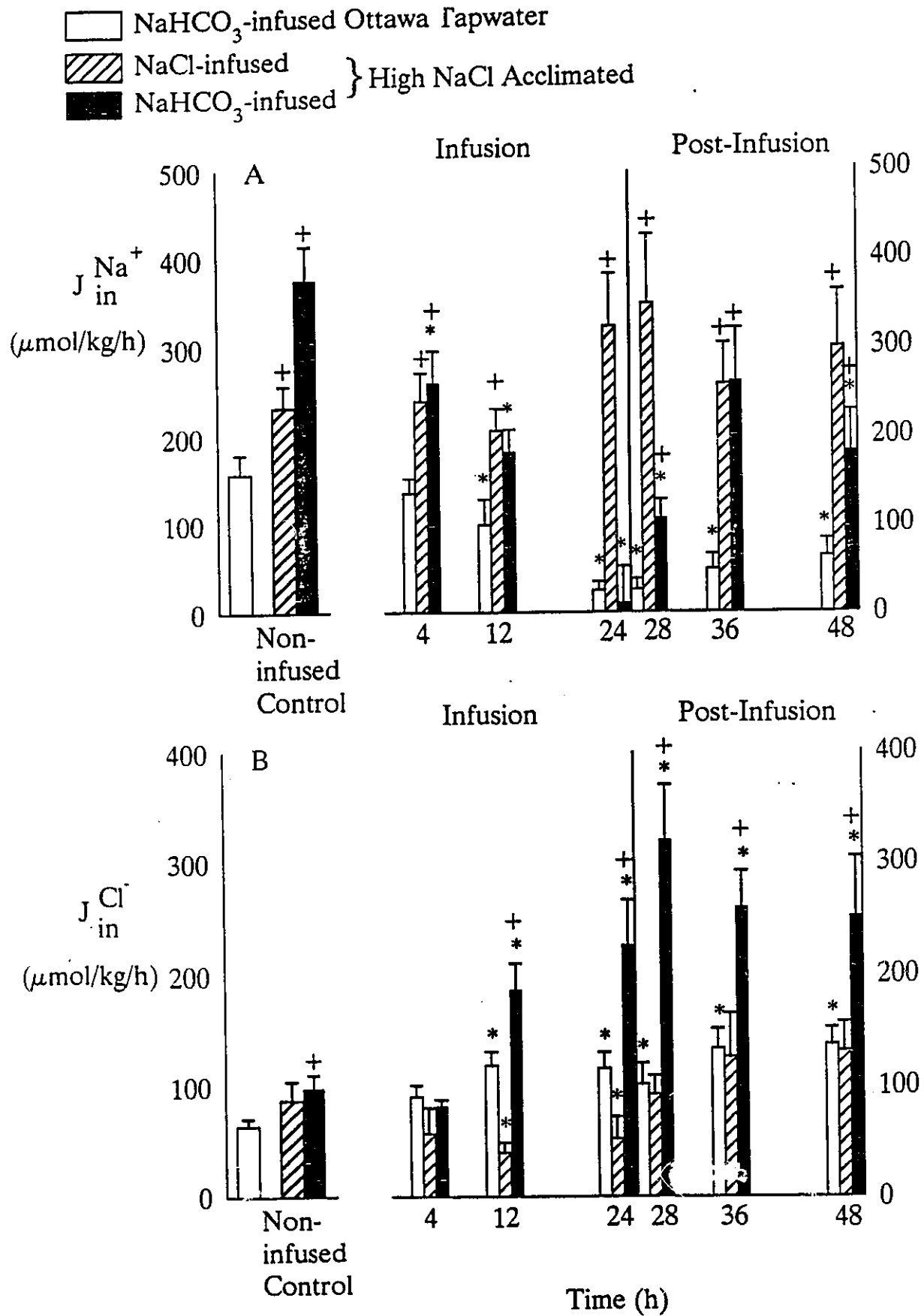
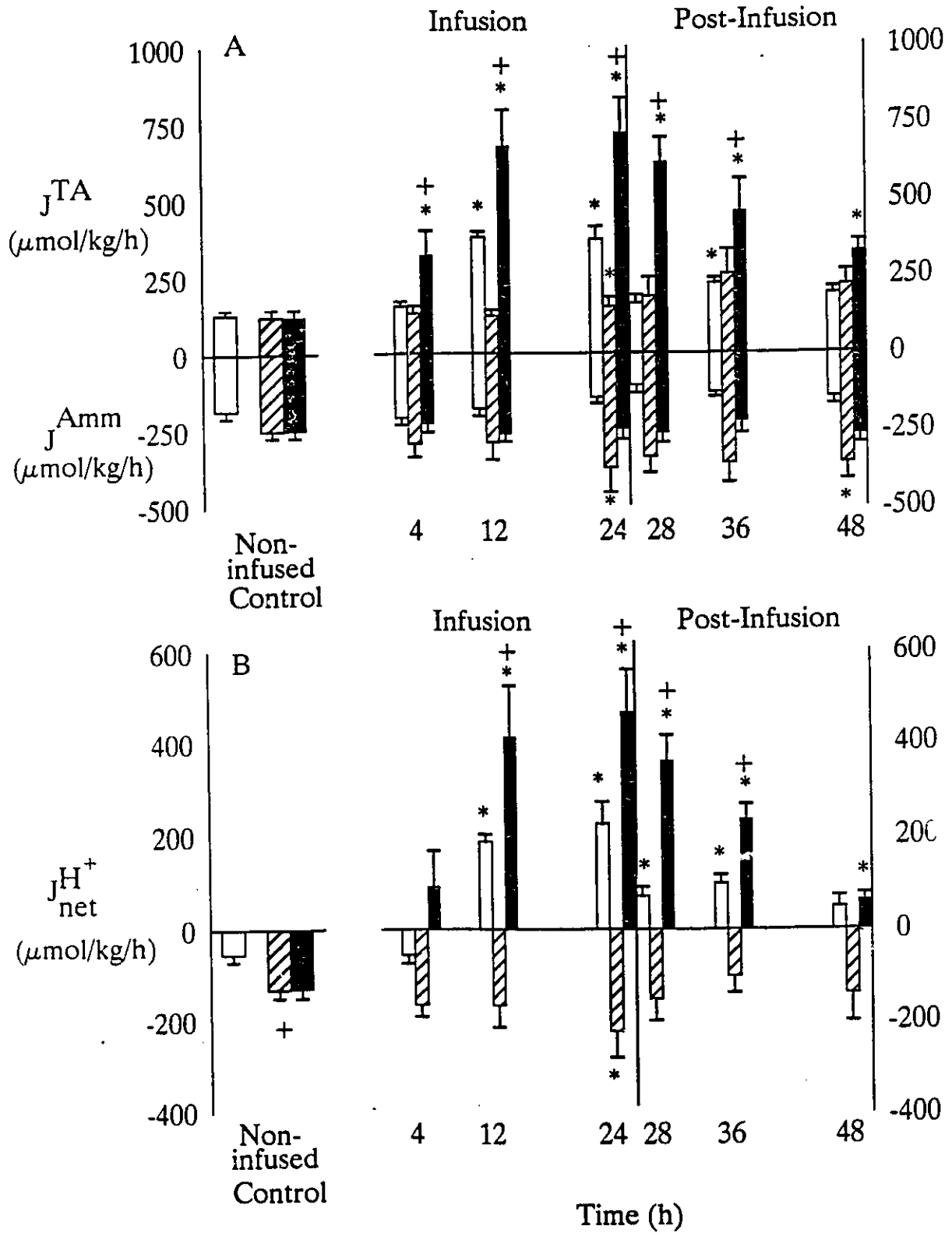


FIGURE 8.4

The temporal effects in rainbow trout of 24 h of chronic infusion of NaHCO_3 or NaCl and the subsequent 24 h post-infusion period on whole body flux rates of titratable alkalinity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents ($J_{\text{net}}^{\text{H}^+}$) in Ottawa tapwater-acclimated trout and high NaCl -acclimated trout (Series 3). Net acidic equivalent flux ($J_{\text{net}}^{\text{H}^+}$) is calculated as the sum of the two components, J^{TA} and J^{Amm} , signs considered. Positive values indicate acidic equivalent uptake (or base excretion) while negative values indicate acidic equivalent excretion. * indicates significant difference from the initial non-infused control value. + indicates significant difference from the Ottawa tapwater value at that time period only. Mean \pm SE.

NaHCO₃-infused-Normal Ottawa Tapwater
 NaCl-infused
 NaHCO₃-infused
 } High NaCl Acclimated



DISCUSSION

An elevation in environmental $[\text{NaCl}]_e$ clearly enhanced compensation of metabolic alkalosis induced by NaHCO_3 infusion. Both the magnitude of the alkalosis and the rate of return to control values after cessation of infusion were reduced at higher $[\text{NaCl}]_e$ (acute exposure) while increased CCFA associated with the high NaCl acclimation resulted in a further attenuation of the alkalosis. The chronically high NaCl-acclimated (1.36 mmol/l) fish showed the greatest ability to regulate with the infused base load. These findings confirm the results of earlier studies which showed a positive relationship between water salinity and the rate of compensation/degree of acid-base disturbance (Perry, 1982; Heisler, 1984; Tang *et al.*, 1989; Iwama and Heisler, 1991; Tang and Boutilier, 1991). The effect of placing freshwater animals in increasing external $[\text{NaCl}]_e$ has been investigated thoroughly since the classic experiments of Shaw (1959). In general, these experiments demonstrate that elevations in external substrate concentrations result in increased rates for both $J_{\text{in}}^{\text{Na}^+}$ and $J_{\text{in}}^{\text{Cl}^-}$ (Maetz, 1972; de Renzis and Maetz, 1973; de Renzis, 1975). Assuming that the increases in $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ are not accounted for wholly through increases in self exchange (Na^+/Na^+ and Cl^-/Cl^- exchange), then the increased rates of $J_{\text{in}}^{\text{Na}^+}$ should allow for a greater reduction in the rates of acidic equivalent excretion *via* the Na^+ uptake mechanism (Na^+/H^+ exchange - Na^+ channel/ H^+ -ATPase) and the increased rates of $J_{\text{in}}^{\text{Cl}^-}$ should allow for a greater rate of basic equivalent excretion *via* the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism. The increased capacity for manipulation of these mechanisms should allow for a more rapid compensation from an alkalosis. This study demonstrates conclusively that even small elevations in external substrate (to 1.5 mmol/l) alone, results in an increased capacity to regulate metabolic alkalosis (acute exposure) while increases in CCFA further increases the capacity (high

NaCl-acclimated).

The elevation in external $[\text{NaCl}]_e$ from 0.15 mmol/l to 1.36 mmol/l caused significant elevation of CCFA. The reasons for the larger CCFA in the high NaCl-acclimated fish are unknown and indeed, the response was unexpected. Owing to the 10-fold reduction in the chemical gradient for passive branchial ion loss in the high NaCl-acclimated fish, it was initially expected that CCFA would be reduced because there would be less need for ionic uptake to balance ion losses. Regardless of the mechanism or the physiological significance, the increase in CCFA in the high $[\text{NaCl}]_e$ acclimated fish was used as tool to determine the relative contributions of alteration in morphology and external substrate availability to acid-base regulation. Thus, the values of this particular group represent the combined effects of increased CCFA and increased external substrate availability. The effect of increased $[\text{NaCl}]_e$, alone, on acid-base compensation was measured in the acute exposed trout acclimated to Ottawa tapwater. The possibility that morphological changes occurred during the infusion period was not examined in this study. However, it was demonstrated in Chapter 6 that NaHCO_3 infusion caused an increase in CCFA.

As noted in Chapter 6, arterial P_{CO_2} was significantly elevated in NaHCO_3 infused fish above control values, however, the elevations were greater in fish exposed to high NaCl water (either acute or chronically acclimated). The reasons for this elevation are not clear at this point. Iwama and Heisler (1991) noted that during hypercapnic acidosis, arterial P_{CO_2} was significantly higher in fish exposed to higher salinities. They suggested that at low external salinities, improved efficiency of CO_2 transfer caused by morphological alterations of the gill could account for the variation in P_{CO_2} . As mentioned in Chapter 7, increases in gill CCs may cause increases in the blood-water

diffusion distance and this may explain the elevated P_{CO_2} in the high NaCl-acclimated fish. Additionally, the increased P_{CO_2} in all fish may represent an increased rate of HCO_3^- dehydration at the uncatalyzed rate in the plasma. Hypo-ventilation/hypo-perfusion of the gill are also possible mechanisms for elevating P_{CO_2} .

Exposure to increased $[\text{NaCl}]_e$ resulted in elevated rates for both $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ under control conditions while during alkalosis, $J_{\text{in}}^{\text{Cl}^-}$ was stimulated and $J_{\text{in}}^{\text{Na}^+}$ was reduced. The pattern of alterations in unidirectional ion fluxes are in general agreement with those of previous workers (Cameron, 1976; Wood *et al*, 1984; Perry *et al*, 1987a; Goss and Wood, 1990b). These are appropriate responses if Cl^- is exchanged for a basic equivalent (HCO_3^-) and Na^+ is exchanged for an acidic equivalent (H^+ , NH_4^+). The differences between Ottawa tapwater-acclimated trout and high NaCl-acclimated trout were only in the magnitude of the alterations. The absolute change from control values for both $J_{\text{in}}^{\text{Cl}^-}$ and $J_{\text{in}}^{\text{Na}^+}$ was much larger in high NaCl-acclimated trout and hence, the rates of net acidic equivalent uptake (base excretion = positive $J_{\text{net}}^{\text{H}^+}$) were higher in the high NaCl-acclimated fish. Unfortunately, net and unidirectional ionic fluxes were not determined for acutely high NaCl-exposed trout. According to the blood acid-base data, however, the fluxes would presumably be intermediate between those of Ottawa tapwater trout and high NaCl-acclimated fish.

In the present study, changes in $J_{\text{net}}^{\text{H}^+}$ were solely the result of alteration in J^{TA} as has been repeatedly shown throughout the thesis. Furthermore, there were no obvious relationships between $J_{\text{in}}^{\text{Na}^+}$ and either J^{Amm} or $J_{\text{net}}^{\text{H}^+}$. The data certainly did not yield the simple 1:1 stoichiometry between $J_{\text{in}}^{\text{Na}^+}$ and J^{Amm} that has been reported under resting conditions in other studies (e.g. McDonald and Milligan, 1988; McDonald and Prior, 1988). It seems likely, therefore, that branchial total ammonia (NH_3 , NH_4^+)

excretion occurred as both NH_3 and NH_4^+ in the present experiments. During alkalosis, NH_3 diffusion would likely increase due to the increased blood pH_a and the subsequent increase in the plasma $\text{NH}_3/\text{NH}_4^+$ ratio and increase in the P_{NH_3} gradient. This may explain the maintenance of J^{Amm} at times of severely inhibited $J_{\text{in}}^{\text{Na}^+}$. These data, therefore, favour the flexible model of Wright and Wood (1985) whereby ammonia excretion may be either by non-ionic diffusion or by apical extrusion in exchange for Na^+ .

Elevation of J^{TA} during alkalosis is the most direct mechanism for the excretion of the excess HCO_3^- from the blood (Wood *et al*, 1984; Wheatly *et al*, 1984; Perry *et al*, 1987a, b; Goss and Wood, 1990a, b). The greater elevation in J^{TA} in the high NaCl-acclimated fish corresponds directly with the elevation in $J_{\text{in}}^{\text{Cl}^-}$ and suggests that manipulation of the $\text{Cl}^-/\text{HCO}_3^-$ exchange is the dominant mechanism underlying the regulation of acid-base status during acid-base disturbances in general agreement with previous studies (de Renzis and Maetz, 1973; Toews *et al*, 1983; Claiborne and Heisler, 1984, 1986; Wood *et al*, 1984; Perry *et al*, 1987a; Goss and Wood, 1990a, b). Acute increases in $[\text{NaCl}]_e$ allow for augmentation of $J_{\text{in}}^{\text{Cl}^-}$ and hence an increased capacity to excrete HCO_3^- while chronic acclimation to high $[\text{NaCl}]_e$ further increases the capacity for acid-base regulation owing to morphological alteration of $J_{\text{in}}^{\text{Cl}^-}$.

In summary, elevations of the external NaCl concentration, alone, result in an increased capacity to regulate an alkalosis induced by NaHCO_3 infusion. The increase in substrate availability resulted in an increased $J_{\text{in}}^{\text{Cl}^-}$, and hence an increased capacity to excrete basic equivalents *via* the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism. The increase in $J_{\text{in}}^{\text{Na}^+}$ under control conditions allows for an increased capacity to reduce $J_{\text{in}}^{\text{Na}^+}$ and therefore reduce the absolute net acidic equivalent excretion during the alkalosis. Additionally, morphological alteration which occurs as a result of acclimation to the varying

environmental salinities affects the degree of acid-base disturbance and the rate of compensation. A greater CCFA will attenuate the increase in $[\text{HCO}_3^-]$ and increase the rate of compensation from an alkalosis owing to the presence of an increased number of Cl/HCO_3^- exchangers. The above mechanisms combined to result in an increased capacity to regulate a NaHCO_3 -infusion induced alkalosis under increasing environmental salinities.

CHAPTER 9
GENERAL DISCUSSION

This thesis provides new insight into the mechanisms of acid-base regulation in freshwater fish. Branchial mechanisms utilized by freshwater fish to regulate internal acid-base status were examined employing physiological and morphological tools and employing a wide variety of experimental treatments which induced both respiratory (hyperoxia, hypercapnia) and metabolic (post-hyperoxia, post-hypercapnia, HCl infusion, NaHCO₃ infusion) acid-base disturbances. Acid-base regulation was achieved by appropriate adjustments of Na⁺ and Cl⁻ net fluxes across the gills which, in turn, were accomplished by variable contributions of three different branchial mechanisms; i) morphological adjustments to the gill epithelium. ii) changes in internal (H⁺, HCO₃⁻) and external (Na⁺, HCO₃⁻) substrate availability, and iii) *differential* changes in Na⁺ versus Cl⁻ net flux through regulation of Cl⁻ efflux. These data helped to elucidate the reasons for both intra- and inter-specific differences in the ability of fish to regulate acid-base disturbances.

Morphological Regulation

An important contribution of the thesis is the demonstration that freshwater fish dynamically adjust their gill morphological characteristics to correct acid-base disturbances. Ionic transport capacity, and hence the capacity to excrete acidic or basic equivalents is regulated by adjustments of the number of functional transporters present on the gill epithelial surface. Cameron and Iwama (1987), were the first to suggest that morphological alteration of the fish gill epithelia might be involved in the regulation of acid-base status. Furthermore, Cameron (1989) suggested that future studies on the mechanisms of acid-base status should focus on the relationships between morphological alteration of the gill and the physiological adjustments during acid-base disturbances.

This thesis also gives insight into the location of the Na⁺ and Cl⁻ transporters with respect to cell type. An obvious and important feature of these data is the

correspondence between the changes in $J_{in}^{Cl^-}$ and CCFA and the lack of correspondence between $J_{in}^{Na^+}$ and CCFA. When CCFA is reduced, $J_{in}^{Cl^-}$ is reduced while when CCFA is increased, $J_{in}^{Cl^-}$ is increased. The degree of change in $J_{in}^{Cl^-}$ roughly corresponds to the degree of change in CCFA suggesting that the Cl^-/HCO_3^- exchange mechanism is located on the CC. Long lasting reductions of $J_{in}^{Cl^-}$ to compensate acidosis are accomplished by physical covering of the CC's (and hence Cl^-/HCO_3^- exchange sites) by adjacent PVC's. This method of lowering $J_{max}^{Cl^-}$ can be viewed as essential to counteract the opposing influence of elevated internal $[HCO_3^-]$ during compensation of a respiratory acidosis which would then tend to stimulate $J_{in}^{Cl^-}$ and impede compensation. The lack of correspondence between $J_{in}^{Na^+}$ and CCFA suggests that the Na^+ uptake mechanism (either Na^+/H^+ exchange or a H^+ ATPase/ Na^+ channel) is not associated with the CC. In addition, evidence of morphological changes in PVCs both externally and ultrastructurally during hypercapnic acidosis in bullheads (Chapter 2 and 3) suggest an increase in the metabolism of the PVC, perhaps associated with H^+ excretion and Na^+ uptake.

The localization of the Cl^-/HCO_3^- exchanger to the CC provided insight into the reasons for inter- and intra-specific variability in the rates of acid-base compensation. Due to the relationship between the CC and the Cl^-/HCO_3^- exchanger, variation in the fractional area of CCs and hence the number of available exchangers is one factor responsible for the inter- and intra-specific variability noted in the rates of compensation from an alkalosis. An increased CCFA imparts an increased ability to compensate a metabolic alkalosis (Chapters 6 and 7) but does not alter the ability of fish to compensate a respiratory acidosis (Chapter 3). Morphological alteration of the gill CC and PVC are important mechanisms for acid-base balance in freshwater fish.

Substrate Availability

The relative contributions of alteration in internal (HCO_3^- , H^+) and external (Cl^- , Na^+) substrate availability to acid-base regulation were examined. Change in internal counter-ion availability play an integral role in the clearance of basic/acidic equivalents as required to regulate to "normal" acid-base status. Each of the transport mechanisms involved in acid-base regulation (Na^+/H^+ exchange, H^+ pump/ Na^+ channel, $\text{Cl}^-/\text{HCO}_3^-$ exchanges) also have an internal active site for the counter-ion. Wood and Goss (1990) and Goss and Wood (1991) demonstrated that the internal counter-ion concentration is limiting on the rate of functioning of these transporters and that increases in $[\text{HCO}_3^-]$ result in increases in $J_{\max}^{\text{Cl}^-}$ while increases in H^+ resulted in increases in $J_{\max}^{\text{Na}^+}$. Therefore, in any situation where acid-base status is being altered, the effect of alteration of the internal counter-ion concentration must be taken into account.

According to this model, however, there are several instances where changes in extracellular acid-base status would inappropriately affect branchial $\text{Cl}^-/\text{HCO}_3^-$ exchange. For example, extracellular HCO_3^- levels are elevated during uncompensated respiratory acidosis, and further elevated during, and after, metabolic compensation. At these times, the model predicts a stimulation of $\text{Cl}^-/\text{HCO}_3^-$ exchange which would exacerbate rather than correct the acidosis yet clearly, $J_{\text{in}}^{\text{Cl}^-}$ remains the same or is reduced in almost all cases (Cameron, 1976; Wood *et al* 1984; Cameron and Iwama, 1987; Perry *et al* 1987a; Goss and Wood, 1990a, b; Goss and Wood, 1991; Goss *et al* 1992). This apparent paradox (a lower $J_{\text{in}}^{\text{Cl}^-}$ at a time when $[\text{HCO}_3^-]_a$ is elevated) is solved by the morphological modification of the gill reducing the absolute number of functional exchange sites and, hence, $J_{\text{in}}^{\text{Cl}^-}$ at this time.

Efflux Regulation.

The contribution of regulation of Cl^- efflux ($J_{\text{out}}^{\text{Cl}^-}$) as a means of acid-base

compensation during periods of alkalosis was demonstrated. *Differential* net loss or gain of strong ions by mechanisms other than the branchial exchangers have only recently been suggested as possible means of regulating an acid-base disturbance (McDonald *et al*, 1989a; Goss and Wood, 1990a, b; Wood and Goss, 1991; Goss and Wood, 1991; Gonzales and McDonald, 1992). In both trout and eel, manipulation of $J_{out}^{Cl^-}$ is a mechanism for the regulation of $J_{net}^{Cl^-}$ although in eels, it is the sole mechanism available due to the absence of an appreciable influx ($J_{in}^{Cl^-}$). *Differential* loss of Na^+ over Cl^- during metabolic alkalosis is an important mechanism of acid-base regulation in trout when use of the Cl^-/HCO_3^- exchanger is denied.

An Updated Model for the Fish Gill

This thesis presents evidence for a number of mechanisms which are variably utilized to regulate acid-base status. A model incorporating the proposed mechanisms and their proposed locations is shown in Fig 9.1 (compare with Figure 1.1). For simplicity, the concentration of the external ion(s) are assumed to be constant in this model. As was demonstrated in Chapter 8, variation in the external ion concentration will variably affect the ability of freshwater fish to regulate an acid-base disturbance. Increases in external ion concentration will aid acid-base compensation through increases in the capacity of the transporters to function, conversely, decreases in external ion availability will reduce the capacity to acid-base compensate (e.g. exposure to Cl^- free water). In the model, the relative size of the lettering for the counter-ion (H^+ , HCO_3^-) represents the relative changes in the concentration of that particular counter-ion during the various acid-base disturbances. In addition, the relative size of the arrows represent the relative "rate" for that transporter as determined by the substrate availability. The mechanism(s) involved in Na^+ uptake (Na^+/H^+ exchange, H^+ pump/ Na^+ channel) remain controversial (Avella and

Bornancin, 1989; Lin and Randall, 1991) and therefore, both have been indicated in each panel accompanied by a question mark (?). Cell junctions between CCs and PVCs are indicated by double arrowheads.

Control: Under control conditions, the $\text{Cl}^-/\text{HCO}_3^-$ exchange system located on the CC and the Na^+/H^+ exchange system (H^+ pump/ Na^+ channel) located on the PVC, function at a "normal" rate according to the amount of internal substrate available (blood acid-base status) under those acclimation conditions (Fig 9.1A).

Metabolic acidosis: During a metabolic acidosis (e.g. HCl infusion, exercise) there is a reduction in $[\text{HCO}_3^-]$ and an increase in $[\text{H}^+]$. These changes in counter-ion availability would result in an decreased rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange and an increased rate of Na^+/H^+ exchange (H^+ pump/ Na^+ channel) activity according to the two-substrate model (Fig 9.1B). Morphologically, there was an increase in CCFA during HCl infusion, an unexpected and counter-productive response. This increase may be due to hormonal alterations as a result of the stressful treatment of HCl infusion (Tang and Boutilier, 1989c).

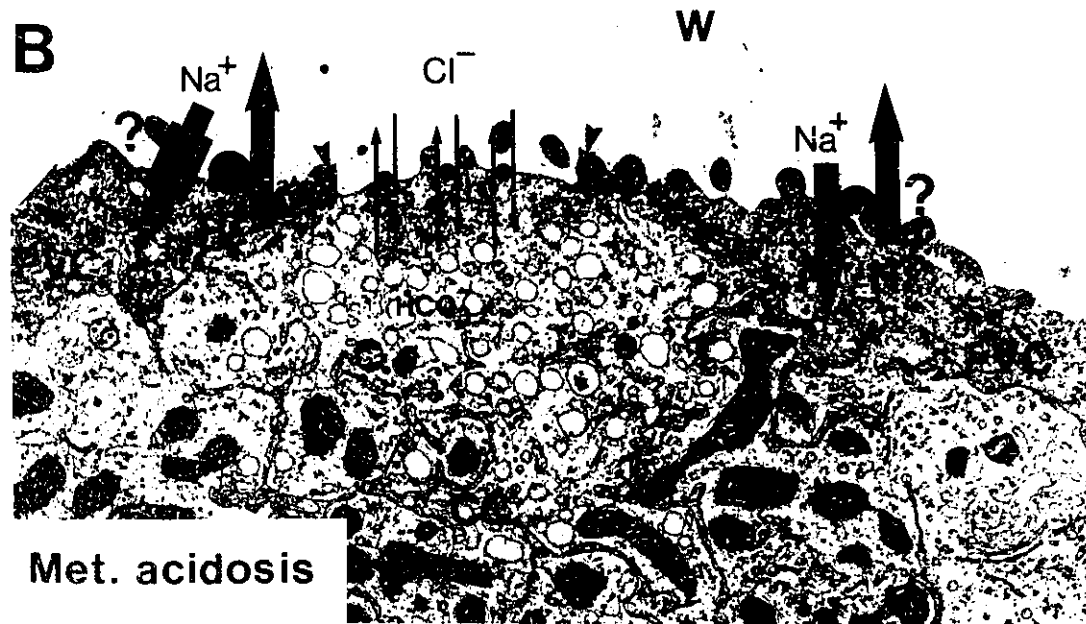
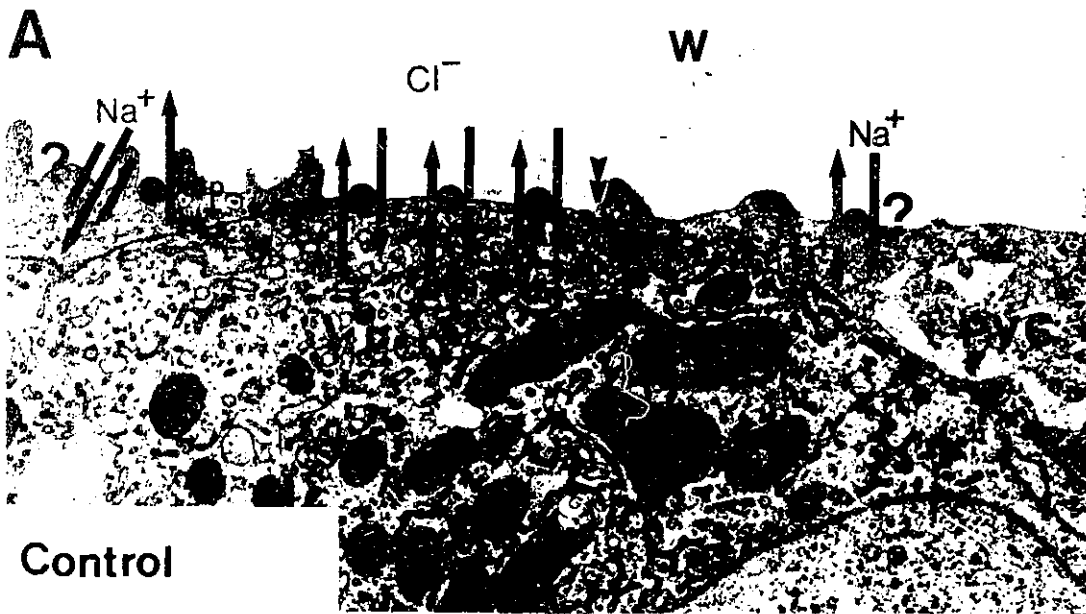
Respiratory acidosis: During, and after, compensation of a respiratory acidosis (e.g. hyperoxia, hypercapnia), plasma $[\text{HCO}_3^-]$ (internal substrate) is greatly elevated, yet $J_{\text{in}}^{\text{Cl}^-}$ is reduced. This is accomplished by a reduction in the apical exposure of the CC, thereby reducing the number of $\text{Cl}^-/\text{HCO}_3^-$ exchangers which are exposed to the water (Fig 9.1C). This may be accomplished either by covering the CC by adjacent PVCs or actual removal of the $\text{Cl}^-/\text{HCO}_3^-$ exchanges from the plasma membrane. This would aid retention of HCO_3^- in the face of a greatly increased "rate" of $\text{Cl}^-/\text{HCO}_3^-$ exchange. In addition to the alterations in CCs noted, many PVCs undergo changes in their ultrastructure including a more apical polarization and an increase in the number of

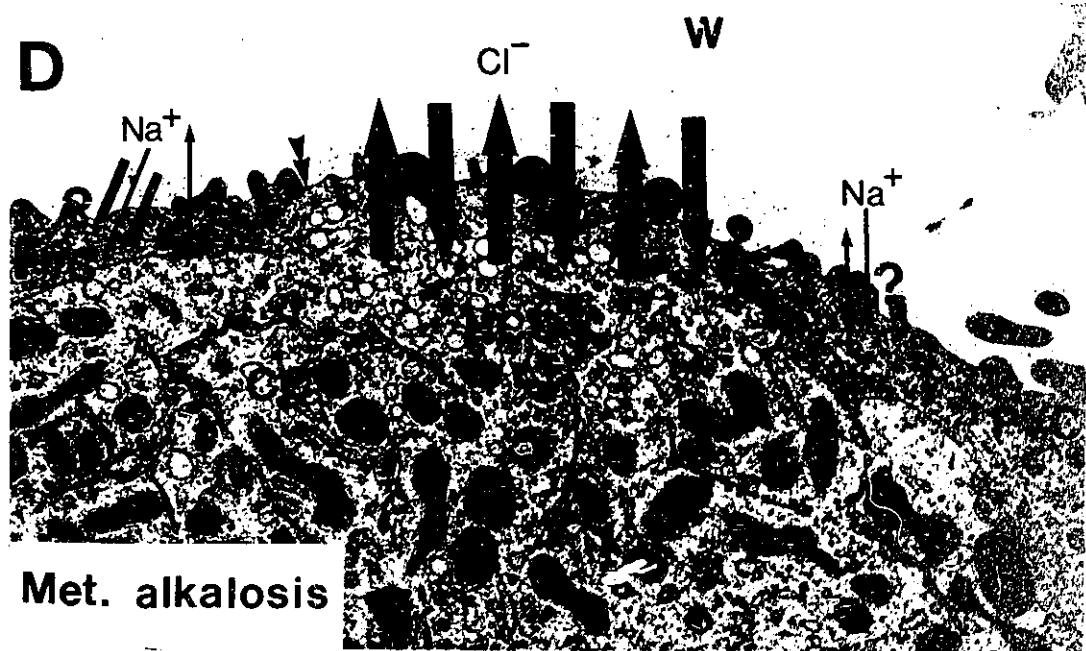
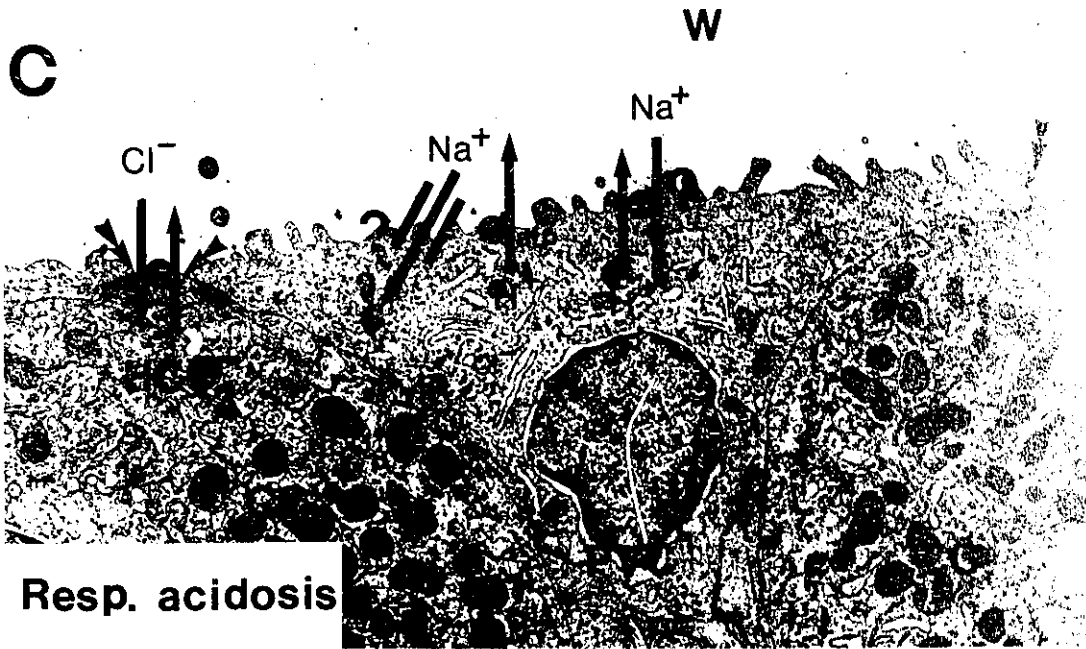
mitochondria present. We suggest that this accounts for increased H^+ excretion through the Na^+/H^+ exchange (H^+ pump/ Na^+ channel) mechanism during an acidosis.

Metabolic Alkalosis: During a metabolic alkalosis (eg. post-hyperoxia, post-hypercapnia, $NaHCO_3$ infusion), there were a number of strategies employed to correct the acid-base disturbance. A major contributor to acid-base regulation is through manipulation of the influx component. Morphological regulation of the fractional area of exposed chloride cells is a primary mechanism for the manipulation of the Cl^-/HCO_3^- exchanger. Increasing the number of Cl^-/HCO_3^- exchangers results in an increase in the capacity to excrete base, and aids in the regulation of the alkalosis. At the same time, since $[HCO_3^-]$ is greatly elevated and $[H^+]$ is reduced, the "rate" of Cl^-/HCO_3^- exchange is increased and "rate" of Na^+/H^+ exchange (H^+ pump/ Na^+ channel) is reduced. In addition to these branchial mechanisms, "intracellular buffering", most likely by the white muscle (Wood and LeMoigne, 1991), was noted in the $NaHCO_3$ -infused trout. The combination of these mechanisms probably accounts for the relatively rapid (24 h) compensation from metabolic alkalosis as opposed to the slower (48-72 h) compensation from either respiratory or metabolic acidosis.

Figure 9.1

Diagrammatic model illustrating the various mechanisms employed for acid-base regulation under control conditions (6A), during a metabolic acidosis (6B), respiratory acidosis (6C), and metabolic alkalosis (6D). The size of the arrows connected to the ion transport mechanisms indicates the relative "rates" according to the two-substrate model while the size of the lettering relative to control indicates the relative concentrations during each acid-base disturbance. Note that during a metabolic acidosis, $[\text{HCO}_3^-]$ is reduced and so is the "rate" for $\text{Cl}^-/\text{HCO}_3^-$ exchange while $[\text{H}^+]$ is increased resulting in an increase in the "rate" of Na^+/H^+ exchange (H^+ pump/ Na^+ channel) activity. During a respiratory acidosis, note the reduction in CC apical surface area and reduction in the number of $\text{Cl}^-/\text{HCO}_3^-$ exchangers present. During a metabolic alkalosis, there is a combination of an increase in CC area (hence increased number of $\text{Cl}^-/\text{HCO}_3^-$ exchangers) and an increased "rate" for the $\text{Cl}^-/\text{HCO}_3^-$ exchangers and decreased "rate" for the Na^+/H^+ exchanger (H^+ pump/ Na^+ channel). CC = chloride cell, PVC = pavement cell, W = water. See text for further details.





Future Directions

The freshwater fish gill continues to serve as a useful model for the examination of acid-base regulating epithelia yet clearly, there are several aspects of the model that remain controversial or unresolved. In particular, future studies should focus on the following areas:

1). Definitive localization and quantification of the $\text{Cl}^-/\text{HCO}_3^-$ and Na^+/H^+ (H^+ pump/ Na^+ channel) mechanisms.

Although I have presented evidence for the placement of these transport mechanisms on the CC and PVC respectively, the evidence is only circumstantial and correlational. Precise localization of the transporters through the use of advanced cellular localization techniques such as immunocytochemistry, lectin binding and autoradiography are required. Furthermore, the effects of alterations in acid-base status on the numbers of transporters, the cellular localization and the cellular functioning should also be examined to confirm that changes in transport numbers correlate with changes in J_{max} .

2). The mechanism(s) linking Na^+ uptake and H^+ excretion.

Elucidation of the mechanism(s) involved in Na^+ uptake and H^+ excretion is essential. The two current models (Na^+/H^+ and H^+ pump/ Na^+ channel mechanisms) should be tested using morphological, immunocytochemical, pharmacological and/or electrophysiological techniques. A multidisciplinary approach employing these powerful techniques for the precise determination of the physiological characteristics of Na^+ transport in the freshwater fish gill and the methods of regulation.

3). The mechanism(s) responsible for morphological adjustments in gill epithelia.

The mechanisms controlling these alterations in gill morphology remain unclear. The possibilities for regulation of the gill morphology are numerous. Experimentally

elevated cortisol titres are known to cause increases in CCFA (Perry and Wood, 1985; Laurent and Perry, 1990) and growth hormone is another humoral agent which has demonstrated the ability to cause dramatic alteration in the gill CC morphology (Madsen, 1990b). However, the role these hormones play in the regulation of the morphological responses to acid-base disturbances remains to be investigated. In addition, the possible involvement of other humoral agents (eg. prolactin, atriopeptin) should be investigated.

In addition, the reasons for the varied external appearance of the gill CCs of salmonids from highly ornamented with microvilli (CC₁) to possessing few microvilli or "bald" CCs (CC₂) should be examined to determine whether there are more than one cell type present as suggested by some researchers (Pisam *et al* 1987; Franklin and Davison, 1989; Franklin, 1990) or whether these varying morphologies simply represent varying stages in the cell cycle as suggested by others (Wendelaar-Bonga and van der Meij, 1989).

4). Intracellular Buffering During Acid-base Disturbances.

Many studies including the present thesis have suggested a role for intracellular buffering, with particular mention of the white muscle compartment, in functioning to maintain extracellular acid-base balance (Heisler and Neumann, 1980; Wood and LeMoigne, 1990; Tang and Boutilier, 1991). Further examination of the mechanisms involved in this buffering and its relative importance to overall acid-base regulation are required.

5). The contribution of neural control in the regulation of acid-base status.

It is known that both adrenergic and cholinergic neurons are present in the gill (Bailly *et al* 1989; Dunel-Erb *et al* 1989) and the former have been implicated in the control of branchial Ca²⁺ uptake (Donald, 1989). However, to my knowledge, no studies have been conducted to determine if there is a neuronal component in the regulation of

acid-base status. Direct neuronal control of the activities of the $\text{Cl}^-/\text{HCO}_3^-$ and Na^+/H^+ exchange (H^+ pump/ Na^+ channel) systems remains a possibility which should be investigated.

6). The importance of the gill boundary layer in ionic and acid-base regulation.

The importance of the gill boundary layer in ionoregulation, CO_2 excretion and NH_3 excretion in fish living in freshwater has recently received a great deal of attention (Wright *et al* 1986; Wright *et al* 1989; Playle and Wood, 1989; Randall *et al* 1991). Water chemistry in the boundary layer may be very different than in the bulk medium (Handy, 1989). Differences in the pH, ion concentrations and/or acidic/basic equivalent concentrations in the gill microenvironment will affect substrate availability and competition for the external substrate site and, hence, acidic/basic equivalent transfers to/from the fish. The importance of changes in this microenvironment and its effect on acid-base regulation should be investigated.

7). Importance of catecholamines in control of acid-base transfers.

Pharmacological doses of catecholamines have been shown to variably alter the rates of influx for both Na^+ and Cl^- in FW fish. Initially, β -adrenergic stimulation was found to stimulate $J_{\text{in}}^{\text{Na}^+}$ (Girard and Payan, 1977; Payan and Girard, 1978; McDonald *et al* 1989b) and inhibit $J_{\text{in}}^{\text{Cl}^-}$ (Perry *et al* 1984). Catecholamines are released during some acid-base disturbances (eg. acid infusion: Boutilier *et al* 1986; Tang *et al* 1988a; exercise: Tang and Boutilier, 1988b; hypercapnia: Perry *et al* 1987a). However, these surges in catecholamines are transient in nature and do not necessarily correspond directly to the alterations in ion fluxes. Furthermore, it has recently been shown that during hyperoxic acidosis, catecholamines are not released (Perry *et al* 1989) despite the alteration of ionic fluxes which occur (Wood *et al* 1984; Goss and Wood, 1990a). Therefore, the role of

catecholamines in the compensation of acid-base remains unclear. Future research should focus on the role of catecholamines in controlling ionic and acidic/basic equivalent fluxes at the gill.

8). Mechanisms and control of efflux of ions.

Although it has been shown that there is a role for *differential* efflux of strong ions in the regulation of acid-base status during metabolic alkalosis *via* manipulation of Cl⁻ efflux. However, the route of transport (presumably paracellular) and the control of this mechanism has not been investigated although it is known that Ca²⁺ is important in regulating tight junction formation (Gonzalez-Mariscal *et al* 1990). Future studies should focus on mechanisms regulating ion loss and examination of the controlling mechanism(s).

Answers to these and other questions that arise require the adoption of new interdisciplinary experimental approaches and methods. With vigilance to the problems of the whole animal, cellular and molecular approaches to the study of acid-base regulation in freshwater fish should form the basis of exciting research for decades to come.

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