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INTERRELATIONSHIP BETWEEN CHLORIDE CELL PROLIFERATION AND GAS TRANSFER IN THE RAINBOW TROUT

By

Shawn Donald Bindon, B.Sc (Hons)

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Author: Shawn Donald Bindon B.Sc. (Hons) (University of Ottawa)

Supervisors:
Dr. Steve F. Perry - Professor, Department of Biology, University of Ottawa
Dr. James C. Fenwick - Professor, Department of Biology, University of Ottawa

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INTERRELATIONSHIP BETWEEN CHLORIDE CELL PROLIFERATION
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GAS EXCHANGE IN THE RAINBOW TROUT

ABSTRACT

This thesis examines the morphological and physiological changes that occur in the gills of the rainbow trout, *Oncorhynchus mykiss*, as a result of chloride cell proliferation.

In the first component of this thesis (Chapter 2), the effects of chloride cell hyperplasia and hypertrophy on ion transport capability and the morphological diffusing capacity of the gill were evaluated. These experiments were performed to test the hypothesis that chloride cell proliferation benefits ionic regulation at the expense of efficient gas transfer. Following 10 days of treatment with cortisol and/or ovine growth hormone, the extent of hormonally induced chloride cell proliferation on the gill filament epithelium was assessed by determining the fractional surface area of exposed chloride cells using scanning electron microscopy. Both individual hormone treatments increased chloride cell fractional surface area (~2X) owing to the enlargement of individual chloride cells. The combined cortisol/oGH treatment increased chloride cell fractional area more (~6X) as both the size and numbers of chloride cells increased. Sham injections, consisting of a saline vehicle only, were without effect on chloride cell surface area or number.

Concomitant with the surface morphology results, significant increases in Na^+ ($J_{in}Na^+$) and Cl^- uptake ($J_{in}Cl^-$) were observed following treatment with the individual and combined hormone regimens. Conversely, the gas morphological diffusion capacity, as indicated by the morphological parameters measured, of hormone treated fish was reduced, and was inversely correlated to chloride cell fractional surface area. A pontential reduction in diffusion capacity resulted from an increase in the blood-to-water diffusion distance as quantified using transmission electron microscopy. Lamellar surface area, measured by light microscopy, was unaffected by the hormone treatments. The area of the inter-lamellar water channels was significantly reduced in hormone treated fish as a result of the thickening of the lamellar epithelia.

An extracorporeal circulatory shunt in combination with both environmental normoxia and hypoxia was used to assess the effects of chloride cell proliferation on respiratory function (Chapter 3). Arterial blood was routed from the coeliac artery through an external circuit in which pH (pH_a), partial pressure of oxygen (P_aO_2) and partial pressure of carbon dioxide (P_aCO_2) were monitored continuously.

Surface morphometric analysis showed that the hormone treatment increased the average chloride cell surface area by 2.7 x and chloride cell density by 2.2 x; the combined effect was a five-fold increase in chloride cell fractional area. While the P_aO_2 values of hormone-treated and control fish were similar at $P_wO_2 > 12.0$ kPa, the arterial O_2 tensions of treated fish were significantly lower than those of the control group for $P_wO_2 \leq 12.0$ kPa. In comparison, to control fish at all environmental O_2 tensions, the hormone-treated fish exhibited elevated P_aCO_2 values and a significant acidosis. The effects of chloride cell proliferation on blood gas parameters in hormone-treated fish were accompanied by significantly elevated ventilation amplitudes and lowered ventilation frequency.

The results in this study demonstrated i) that respiratory gas transfer is impaired by chloride cell proliferation, ii) that O_2 and CO_2 transfer are influenced differently, and iii) that partial compensation is achieved through physiological adjustments. The overall results of this thesis help give some basic insight into the compromises made when both ionoregulation and respiratory function are challenged.

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LIST OF ABBREVIATIONS

α	Greek letter alpha
β	Greek letter beta
A_{ls}	lamellar surface area
CC	chloride cell
CCFA	chloride cell fractional area
°C	degrees centigrade
CON	control
CORT	cortisol
DAP	dorsal aortic pressure
h	hours
I-LSpace	interlamellar space
$J_{in}Na^+$	rate of sodium ion influx
$J_{in}Cl^-$	rate of chloride ion influx
kg	kilogram
kPa	kilopascals
μm	microns
min	minutes
mg	milligram
mmol	millimoles
nm	nanometers
oGH	ovine growth hormone
PC	pavement cell
pH_a	arterial pH
P_aCO_2	arterial partial pressure of carbon dioxide
P_wO_2	partial pressure of oxygen in water
P_aO_2	arterial partial pressure of oxygen
P_IO_2	oxygen tension of inspired water
P_EO_2	oxygen tension of expired water
P_ICO_2	carbon dioxide tension of inspired water
P_ECO_2	carbon dioxide tension of expired water
SE	standard error of the mean
SEM	scanning electron microscopy
τ_h	harmonic mean distance
TEM	transmisssion electron microscopy
V_f	ventilatory frequency

CHAPTER 1

GENERAL INTRODUCTION

The teleost fish gill serves many roles, making it a preeminent organ in the maintenance of physiological homeostasis. It is involved in ionic regulation, acid-base regulation, osmotic regulation, and nitrogen excretion (see Laurent, 1989 for review). It is also, at least in most teleosts, the primary site of gas transfer. As such, it allows a fish to inhabit a wide range of different habitats that are frequently subjected to cyclic changes. To facilitate the heterogeneous physiological mechanisms required to adapt to new environmental situations and challenges, the gill has a complex anatomical design and a variety of cell types, each with unique physiological and morphological characteristics making them suitable for one or more physiological functions. The multifunctional nature of the gill and the various cell types found on it are not, however, always mutually exclusive and are, for the most part, directly or indirectly linked to each other. For example, the thin and abundant pavement cells are ideal sites for both osmotic movements and diffusive transfer of respiratory gases. The larger chloride cells are believed to be responsible for ion transport and are thought to link ion and pH regulatory functions via Cl^-/HCO_3^- and Na^+/H^+ exchange mechanisms. On the other hand, the larger and thicker chloride cells are poor sites for gas transfer (see below). Thus any adaptational strategy that employs an increase in the relative area of the gill occupied by chloride cells in order to enhance gill ion transport function, could jeopardize respiratory gas transfer, by reducing the total surface area occupied by the thin pavement cells and by increasing the average water-to-blood diffusion distance.

In light of the coupling of physiological functions, and the potential compromises that may exist between physiological functions, the focus of this thesis is an examination of the effects of chloride cell proliferation on gas transfer in the freshwater rainbow trout, *Oncorhynchus mykiss*. The parameters examined in this study were both morphological and physiological. The basic hypothesis tested in this thesis was that *the proliferation of chloride cells in the rainbow trout gill impairs gas transfer because such changes increase the average water-to-blood diffusion distance across*

the lamellar epithelium. The initial morphological studies utilized combinations of two different hormone treatments to induce chloride cell proliferation and hypertrophy. It was predicted that such chloride cell proliferation, while augmenting ionic uptake, would cause an increase in the water-to-blood diffusion distance and that the subsequent reduction in the morphological diffusing capacity would not be compensated for by an increase in lamellar surface area. Surface electron microscopic analysis was used to determine the degree to which the hormone treatments affected chloride cell morphometry. Following the morphometric analysis of the gill surface, a detailed sub-surface analysis was performed to determine the effect on the water-to-blood diffusion distance. Based on the results of the morphological study (Chapter 2), several predictions with respect to possible effects on respiratory physiology (i.e. blood gas levels, arterial pH, and ventilatory parameters) were made and tested (Chapter 3).

OSMOREGULATION AND THE CHLORIDE CELL

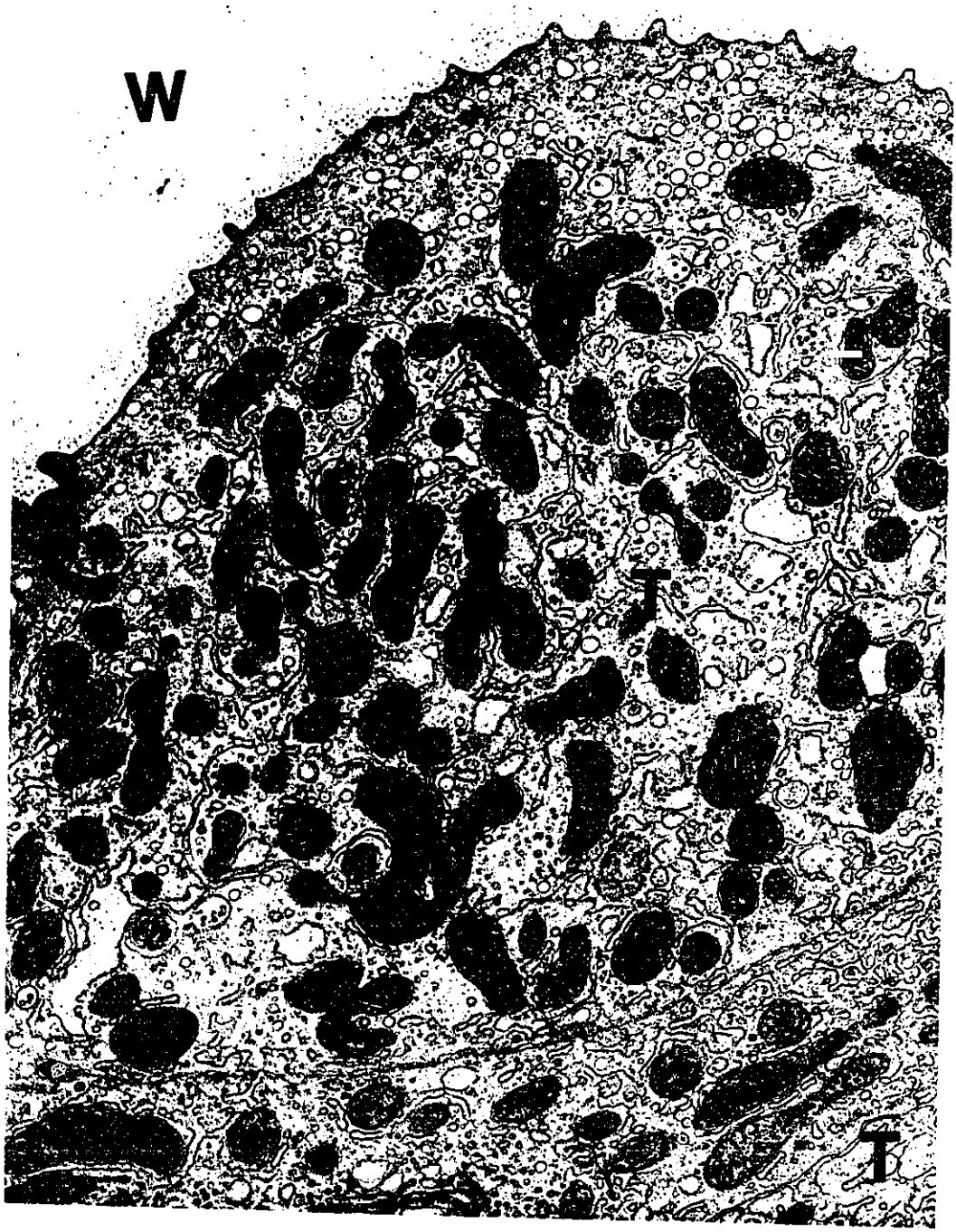
Both freshwater and seawater teleosts have heavy demands placed on their osmoregulatory systems in order to maintain a constant internal ionic environment. In order to meet these demands, they have evolved efficient mechanisms for adjustment. Indeed, these regulatory strategies allow some euryhaline teleosts to inhabit the complete range of aquatic environments from freshwater to seawater and even hypersaline water (with salinity as high as 2300 mosmol/kg) while still maintaining normal internal osmotic concentrations in the 250-500 mosmol/kg range (Maetz, 1971; McFarland, 1965; Parry, 1966). Their efficient regulatory mechanisms are, however, not without cost. For example, freshwater teleosts are hyperosmotic relative to their external environment and, as a result, face both an excessive influx of water and the loss of salt (endosmosis). To compensate, freshwater teleosts drink little, if any, water and excrete copious amounts of dilute urine. Ions which are lost in the urine or across

the body surfaces are replaced by the absorption of ions from the external environment across the gills. In contrast, seawater teleosts are hypo-osmotic to their external environment and this results in the osmotic loss of water and the constant uptake of ions (exosmosis). The osmoregulatory strategy for these fish is to drink large amounts of seawater and to excrete the excess Na^+ and Cl^- across the gills (Karnaky, 1980, 1986). In seawater, the chloride cell appears to be the primary cell responsible for the transport of Na^+ and Cl^-. In freshwater, however, the role of the chloride cell continues to be speculative and is based on indirect evidence which, in simplest terms, is based on studies that link increases in chloride cell morphometric parameters (i.e. number and size) to the enhanced uptake of Na^+, Cl^-, and Ca^{2+}. To date, however, there is no **direct** evidence that these cells are indeed the actual site of ionic uptake.

The chloride cell is a large, granular, and acidophilic cell that was originally described by Keys and Wilmer (1932) in the gills of seawater eels. Since this initial discovery, much research has been done and, in the past ten years, the chloride cell and its potential role in teleost osmoregulation has been extensively reviewed (e.g. Evans, 1979; Degnan, 1984; Foskett *et al.*, 1983; Karnaky, 1980, 1986; Karnaky *et al.*, 1977). The chloride cell exhibits anatomical characteristics that distinguishes it from the branchial pavement and mucous cells. Using light microscopy, Karnaky (1980, 1986) described the chloride cell as a large, columnar, ovoid-shaped cell possessing a single nucleus which spans the entire epithelium from the apical surface on the external epithelium to the basal lamina. The initial electron microscopy studies were done by Doyle (1960), Kessel and Beams (1962), and Philpott (1962) and these have since been supplemented considerably with the focus on morphometry. Under the transmission electron microscope, the chloride cell shows a cytoplasm which is rich in mitochondria. The abundant mitochondria are thought to provide the large amount of ATP required for the chloride cell to pump ions in and out of the blood. There is also an

elaborate tubular system that is present in all regions of the cell with the exception of the Golgi region (Karnaky, 1986). This tubular system is an extension of the basolateral plasma membrane (Philpott, 1980; Laurent and Dunel, 1980; Sardet *et al.*, 1979) and is the site of numerous ion pumps, including the Na^+/K^+-ATPase (Sardet *et al.*, 1979; Philpott, 1980) and Ca^{2+}-ATPase (see review by Fenwick, 1989). This tubular system increases the total functional cell surface area in close contact with the mitochondria pool. Vesicles of various size, and believed to be of Golgi origin (Laurent and Dunel, 1980) can be found close to the apical surface which normally lies in contact with the external environment (Laurent, 1989). TEM studies, however, showed that the contact between the chloride cell and the environment could be reduced by a thin covering of pavement cell (Laurent, 1989). Figure 1.1 shows a representative transmission electron micrograph of a chloride cell. Scanning electron microscope studies of seawater adapted species (Sardet *et al.*, 1979; Thomson and Sargent, 1977; Laurent and Hebibi, 1989; Franklin, 1990; Maina, 1990 and others) showed that the apical surface is partially recessed into a crypt, a small invagination into the surface. A possible function of this crypt may be to accumulate mucous so as to facilitate ion transfer. Franklin and Davidson (1989) reported that the apical surface morphology of freshwater salmon, *Oncorhynchus nerka*, was variable presenting either a smooth surface or a surface covered to varying degrees with microvilli. These authors concluded that the differences in apical morphologies was likely correlated with functional differences between smooth and invaginated (microvilli) chloride cells. In addition, they proposed that the osmoregulatory status of the salmon could be determined by the distribution and the ratio of these two cell morphologies. Pisam *et al.* (1987) also reported two types of cell surfaces using TEM and labelled them as two distinct cell types (a and ß).

Figure 1.1. Representative transmission electron micrograph of the chloride cell showing the extensive basolateral tubular network (T) and numerous mitochondria (M). W = water, N = nucleus. Magnification = 6000X.


W
T
T

Function of the chloride cell in ion regulation and in acid-base balance

Although chloride cells are found in the skin, pseudobranch, and the opercular epithelium of fish (Laurent, 1984, 1989; Laurent and Dunel-Erb, 1984), their principal site is on the gill filaments (Laurent, 1984) where they are found irregularly spaced or in clusters (Laurent and Dunel, 1980). The chloride cells are found on both the filament and the lamellar epithelia (Laurent *et al.*, 1985), with the greatest density at the filamental end (base) of the lamellae (Boyd *et al.*, 1980; Laurent and Dunel, 1980; Matthiej and Stroband, 1971; Laurent, 1989). No differences appear to exist between the morphologies of lamellar and filamental chloride cells.

Although Na^+ and Cl^- uptake by the gills was described by Krogh as early as 1938, the exact location of the Na^+ and Cl^- transporters remains controversial. But, many studies, including those of Laurent and Dunel (1980), Laurent *et al.* (1985), and Laurent and Perry (1990), postulate that the chloride cell is the site of Na^+ and Cl^- uptake. This hypothesis is supported by the morphological features of the chloride cell described earlier. Further, the role of chloride cells in ion transport is strongly suggested by the morphological and physiological changes in the chloride cell which occur during acclimation to changing ionic levels. Perry and Laurent (1989) reported that both the total number and size of the chloride cells increased when fish were exposed to water with artificially low concentrations of Na^+ and Cl^-. Further, the resulting increase in total chloride cell apical surface area was positively correlated with the increase in both Na^+ and Cl^- influx rates (Perry and Laurent, 1989).

Morphological changes associated with environmental and internal acidosis/ alkalosis suggest a functional role of the chloride cell in the maintenance of acid-base balance. Leino and McCormick (1984) showed that acidification of the ambient medium was followed by increases in the absolute number of chloride cells, the percentage of chloride cells on the lamellae, and the number of chloride cells with an apical pit. Goss *et al.* (1992) investigated the role of the chloride cell during respiratory

acidosis. During periods of alkalosis, the number of chloride cells on the gill was increased. In contrast, during acidosis, the total chloride cell surface area was reduced with the reduction appearing to result from the covering of the apical chloride cell surfaces by pavement cells. It was not clear if this was an adaptation to reduce chloride cell exposure or to increase that of the pavement cell, a cell that is also suspected of being involved in acid-base balance (Goss *et al.*, 1992).

In summary, the morphology of the gill, including the chloride cell, appears to be strongly affected by periods of ionic challenge. These morphological changes, however, cannot be ignored, especially given the involvement of the gill in gas transfer which is extremely dependent on morphological parameters.

THE GILL AND GAS TRANSFER

Although small in mass, the gill possesses the general characteristics of an effective gas exchanger. It has a large surface area over which gas exchange can take place, has a thin water-to-blood epithelial barrier (especially at the lamellae) and enjoys a high degree of perfusion by the blood and ventilation by the water (Dejours, 1981). Morphological changes of the gill associated with increased ionoregulation can effect these characteristics and, in general, could alter normal respiratory gas transfer.

Perry and McDonald (1993) described gas transfer as a series of stages, including ventilation, branchial diffusion, blood gas transport, and tissue diffusion in which each stage is dependent on the preceeding to maintain the efficient transfer of gases. For efficient, diffusive gas transfer to occur, the two respiratory media, in this case water and blood, must be in constant motion in order to maintain sufficient partial pressure differences across the gill diffusion barrier. During this process, deoxygenated blood continually enters the gill vasculature, and in particular the lamellae, and departs as oxygenated blood after "stripping" oxygen from the well-

oxygenated water. To maintain the partial pressure of O_2 in the water, the water at the surface of the lamellae is constantly renewed by a process of ventilation called gill irrigation (Hughes and Morgan, 1973). For a review of this mechanism, see Milsom (1989) and Perry and McDonald (1993). This gill ventilation presents a large volume of water to the gas transfer surface (Randall *et al.*, 1991). Ventilation is achieved through two pumping systems (Hughes, 1963), one pre- (buccal) and the other post- (opercular) branchial. This system drives an almost continuous flow of water into the mouth, across the gills and then out the opercular opening. During particularly active periods, some fish may "ram ventilate" (Roberts, 1975) in which active breathing is suspended and water flow occurs through an open mouth.

Resistance to water flow through the oral cavity occurs at three locations: the mouth, the operculum and at the gills. Changes in gill morphology can alter the degree by which gill resistance contributes to the overall resistance. Within the gill region, the two major paths of water flow are axial or between the filaments and interlamellar or passing through adjacent lamellae and both these pathways are primary sources of resistance to water flow. Previous studies that have examined gill morphometry (see Hughes and Morgan, 1973 for review) showed that the interlamellar space is very small, a characteristic consistent with efficient transfer of O_2 and CO_2. But this arrangement results in a significant resistance to the flow of water. This interlamellar resistance is encountered along the entire length of all lamellae over the entire length of the filament. According to Hughes (1972), the major proportion of the water flows through this resistance so it can be predicted that any morphological changes in this area could significantly alter water flow. This thesis examines the effect of alterations to the interlamellar space resulting from changes in lamellar morphometry and, as a consequence, gill interlamellar resistance to water flow.

As with water flow, a high degree of blood flow is required for a fish to maintain appropriate partial pressure differences of gases across the lamellae. As a result,

each filament receives an abundant supply of deoxygenated blood via an afferent filament artery. This route is termed the systemic branchial circulation (Laurent, 1989) as it not only allows for the oxygenation of blood but also provides this blood to the rest of the systemic circulation. Morphologically, each lamella is composed of blood spaces, or lacuna which are separated by the flanges of pillar cells (Laurent, 1984). Once the blood has traversed the lamellae it passes into the efferent arterioles and arteries and then into the dorsal aorta.

Mechanism and quantification of oxygen and carbon dioxide diffusion

The quantitative description of the diffusion of a respiratory gas across an epithelial surface is given by Fick's equation:

$$M_X = \frac{(K_X)(A)(\Delta P_X)}{D}$$

where M_X is the rate of gas transfer for a particular gas (X). K_X is defined as Krogh's constant of diffusion (also called the permeation coefficient), A is the surface area over which gas exchange is able to take place, ΔP_X is the difference in partial pressure across the gas exchange barrier, and D is the mean diffusion distance across the barrier. In the case of the fish gill D is the water-to-blood barrier thickness. From this equation it is obvious that gill morphology is an important factor in controlling gas transfer as changes in gill morphometry can effect the various parameters that effect gas transfer.

Generally, the barrier thickness and lamellar surface area are the variables that have the greatest effect on gas transfer and therefore will occupy a central role in this thesis. The diffusion distance across the lamellae varies along the length of the entire gas exchange surface (Hughes and Morgan, 1973) and therefore a mean diffusion distance must be used to represent this parameter. The measurement of this water-to-blood diffusion distance can be performed utilizing the light microscope but, in

general, is more accurately performed using TEM. The mathematical expression of this parameter utilizes the "mean harmonic distance" method developed by Weibel and Knight (1964) for assessing the mammalian lung. Unlike earlier methodology (Hughes, 1972), this method accounts for the fact that the movement of gases does not necessarily occur over the shortest distance.

Morphologically, the branchial epithelium is composed of a basement membrane and pillar cell flanges together with several epithelial cells, including the pavement, mucous, and chloride cells. Of these, the large chloride cells could present the greatest barrier to diffusion such that increased chloride cell numbers on the lamellae would reduce M_X by increasing D and possibly by reducing K_X. Assuming that the water-to-blood barrier will thicken concomitantly with chloride cell proliferation, it is also postulated that the diffusion distance (D) component of Fick's Law will increase and lead to a decrease in M_X.

The surface area of the gill can be divided into two domains: *anatomical* surface area and the *functional* surface area. The anatomical area describes the total area that is potentially available for gas exchange, whereas the functional area is that which is perfused and ventilated. Depending on environmental and physiological conditions (e.g. environmental hypoxia or exercise, respectively), the functional area can change. For example, during periods of quiet ventilation, only the proximal lamellae are functional. During periods of exercise, however, blood pressure increases and this may lead to the recruitment of distal lamellae for gas exchange (Perry and McDonald, 1993). . At the level of the individual lamellae, morphological changes may result in the expansion of the lamellae producing an overall increase in surface area. It is the premise of this thesis that an increase in gas exchange surface area might accompany the predicted increase in the water-to-blood barrier distance and this is examined in Chapter 2.

To date there are only two studies that have directly evaluated the

consequences of gill chloride cell proliferation on respiratory gas transfer. The first, a study by Thomas *et al.* (1988), examined the effects of high and low external ion concentrations on the ability of rainbow trout to transfer gases. Their morphological analysis showed that trout acclimated to low ionic strength water resulted in an increased gill chloride cell fractional area, with the increase being particularly evident on the lamellae. The authors speculated that this resulted in a considerable reduction in the gas exchange surface and may have possibly increased the mean barrier distance. Unfortunately, a detailed morphological analysis of the lamellae was not performed. These authors also reported that trout displaying chloride cell proliferation were more sensitive to acute hypoxia. A second study (Laurent and Hebibi, 1989), however, which examined only the morphological responses of the trout gill to varying ionic levels, reported a thinning of the mean water-to-blood diffusion barrier in response to ion-poor water. Clearly, an examination involving both morphological and physiological responses is required. This is the main focus of this thesis.

PROTOCOLS AND ANALYSES

In this thesis, chloride cell proliferation was induced by using three hormone injection treatments; namely cortisol, ovine growth hormone, and both hormones together. These treatments were used simply as tools to induce a range of chloride cell proliferation. The study was not intended to investigate the role or action of these hormones *per se*.

Light and both scanning and transmission electron microscopy were used to examine gill morphometry. Scanning electron microscopy was used to examine surface parameters such as size and numbers of chloride cells in order to test the effectiveness of the hormonal treatments. Ion uptake rates were measured to confirm that functional chloride cell proliferation occurred. Transmission electron microscopy was used to measure the water-to-blood diffusion distance. Finally, light microscopy

was used to assess lamellar surface area.

On the basis of the morphological results presented in Chapter 2, certian predictions were made regarding the effects of the morphological changes on respiratory function. For this part of the study an experimental set-up, termed the extracorporeal circulation (Thomas and LeRuz, 1982; described in Chapter 3), was used to measure water and blood gas parameters in order to test the prediction that gas diffusion was impaired. Specifically, on the basis of the increased water-to-blood diffusion distance, it was predicted that arterial PO_2 would be lowered and that PCO_2 would be higher. Chapter 3 also reports on experiments designed to test the ability of chloride cell-rich trout to compensate for hypoxia. Results on the examination of ventilation parameters and their possible adjustments are presented in the same chapter.

CHAPTER 2

THE EFFECTS OF BRANCHIAL CHLORIDE CELL PROLIFERATION ON MORPHOLOGICAL DIFFUSING CAPACITY IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

INTRODUCTION

As discussed in Chapter 1, the epithelial surface of the fish gill is comprised of three cell types; pavement cells, chloride cells and mucous cells. The pavement cell is the most common cell type and is located on both the filament and lamellar surfaces. Although no precise physiological function has been assigned to the pavement cell, it is clear that these cells form an expansive and thin surface separating the internal and external environments across which the respiratory gases, O_2 and CO_2, diffuse. The chloride cells are found predominantly at the bases of lamellae on the trailing edge of the filament or in the inter-lamellar regions. In trout, the chloride cells occur only sparsely on the lamellae (Laurent, 1984) except under conditions requiring an enhancement of ion transport capacity (see below). Although direct evidence is lacking, abundant correlative data support the view that the chloride cell is the site of Na^+ and Cl^- uptake in freshwater teleosts (Laurent *et al.*, 1985; Perry and Laurent, 1989,1993; Laurent and Perry,1990; Perry *et al.*, 1992b). Indeed, the proliferation of branchial chloride cells in trout exposed to ion-poor water (Perry and Laurent, 1989; Yoshikawa *et al.*, 1993) is thought to be an important adaptive response tc optimise the ion transport capacity of the gill.

The proliferation of chloride cells in response to ion-poor environments occurs on both the filament and lamellar epithelia. The lamellar chloride cells, unlike the flattened pavement cells, are large diameter spherical cells that often protrude above the general surface of the epithelium. Thus, the proliferation of lamellar chloride cells, although presumably beneficial for increasing the capacity of the gill to transport ions, may compromise respiratory gas transfer (Perry and Laurent, 1993). Thomas *et al.* (1988) reported that trout naturally adapted to ion-poor water were characterized by unusually low arterial blood O_2 partial pressures and were less resistant to hypoxia. These effects were attributed to a preponderance of lamellar chloride cells. On the othert hand, Laurent and Hebibi (1989) demonstrated an increase in gill

diffusive conductance in trout acclimated to ion-poor water owing to a reduction of the blood-to-water diffusion distance and an increase in lamellar surface area.

The purpose of the present study was to evaluate the simultaneous effects of gill chloride cell proliferation on ionic uptake and respiratory gas transfer to test the hypothesis that whereas an increased lamellar chloride cell surface area enhances ion transport capacity, it concomitantly impairs gas transfer. This was accomplished by using a variety of established hormone treatments as tools to induce a wide range of chloride cell surface areas. Branchial ion transport capacity was determined by measuring the unidirectional influxes of Na^+ and Cl^- whereas the potential consequences of chloride cell proliferation on gas transfer were evaluated by measuring the lamellar surface area, the area of the inter-lamellar water channel, and the blood-to-water barrier thickness.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex (mean mass 59 ± 2 g for morphometric study, n = 6 per group; 196 ± 10 g for ion uptake study, n = 6 per group) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario), and transferred to 300 l tanks at the University of Ottawa fish facility. Fish were supplied with continuously flowing, aerated, and dechlorinated City of Ottawa tap water ($[Na^+]$ = 0.15 mM; $[Cl^-]$ = 0.15 mM; $[Ca^{2+}]$ = 0.45 mM; pH = 7.50; temperature range 12-15°C). The photoperiod was kept constant at 12 h light: 12 h dark. After at least one month in the facility, fish were divided into six groups and placed into smaller (200 l) tanks. Fish were fed three times per week with a commercial trout diet (Martin). Fish were not fed 48 h prior to morphometric or ion uptake studies.

Injection Protocol

Fish were divided into the following six groups: control, cortisol treatment, cortisol sham, ovine growth hormone (oGH) treatment, combined oGH/cortisol treatment, and oGH/cortisol sham. The control group was left untreated for 10 days, the cortisol treatment group received 10 daily injections (8 $mg \cdot kg^{-1}$ intramuscularly; Madsen, 1990a,b) of hydrocortisone-21-hemisuccinate sodium salt (Sigma) and the oGH treatment group received intraperitoneal injections every second day over the ten day treatment period with 2 $mg \cdot kg^{-1}$ oGH (NIH - NIADDK, Bethesda, MD; Madsen, 1990a,b). The combined treatment fish were given both injection protocols. In all cases, the injection vehicle was a 0.1 ml solution of saline (0.9% w/v NaCl, pH 7.0). Prior to injections, the fish were anaesthetised in a 0.1% (w/v) solution of aminobenzoic acid ethyl ester (MS-222; Sigma) until the point where they began to

lose equilibrium. Sham injected fish were treated identically to the treated fish except that they were injected with vehicle alone.

Tissue Fixation

The morphometric analyses were performed using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy. In each case, gill tissue was excised and fixed in 5% gluteraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) at 4°C for 1 h. The fixed tissue was rinsed in buffer and post-fixed in 1% osmium tetroxide at 24°C for 1 h. Mazzone *et al.* (1980) showed that this fixation technique causes less shrinkage of respiratory tissue than others commonly used (e.g. formaldehyde). After fixation, the tissues were stored in a sodium cacodylate buffer until used.

Measurement of chloride cell surface parameters

Cell surface parameters were analysed using SEM. Fish were killed by a blow to the head. The central portion of the second gill arch (left side) was immediately removed from the fish; the isolated area contained several pairs of filaments which were separated from the gill arch tissue. Each pair (anterior and posterior) remained attached at the septum. All excision work was performed in buffer to prevent tissue dehydration prior to fixation. Following fixation, each filament pair was dehydrated in an ethanol series (70, 85, 95, and 2 x 100%) then bathed twice in 1,1,1,3,3,3-hexylmethyldisilizan (Aldrich) and air-dried. Pairs of filaments were attached with silver paint (Electron Microscopy Sciences, Fort Washington, VA) to SEM specimen stubs suitable for a Phillips 500 scanning electron microscope. The tissue was carefully oriented so that the lateral side of the filaments were parallel to the face plane of the stub plate. The microscope was focused on the trailing edge of the filament epithelium near the septum (approximately 5 - 10 lamellae distal from the

septum) and close to where the lamellae meet the filament. Random areas on both anterior and posterior filaments were photographed at a magnification of ~1000 x. Five pictures per fish were randomly selected for use in subsequent measurements. Apical chloride cell area was determined by tracing the cell perimeter on a calibrated digitizer tablet that was linked to a microcomputer utilising specialised software (Sigmascan, Jandel Scientific). In addition, the chloride cell fractional area (CCFA) and cell density were calculated using the following equations:

$$CCFA = \frac{\text{Area of whole and partial cells}}{\text{Picture area} \times 10^{-6}}$$

$$\text{Cell Density} = CCFA/\text{Average whole cell area}$$

Ion uptake studies

The unidirectional influx rates of Na^+ ($J_{in}Na^+$) or Cl^- ($J_{in}Cl^-$) were determined in each treatment group by monitoring the disappearance of ^{22}Na (as NaCl; New England Nuclear) or ^{36}Cl (as HCl; New England Nuclear) from the ambient water as follows. Before each experiment, fish were placed into opaque acrylic flux boxes (approximate volume = 5 l) that were supplied with running and aerated dechlorinated water. After 24 h, water flow to the boxes was stopped and the water volume reduced to 3.5 l. ^{22}Na (1.0 μCi) or ^{36}Cl (1.0 μCi) was added to each box and allowed to mix for 15 min. The mixing was accomplished by vigorous aeration of the static water. Water temperature was stabilised by partially immersing the flux boxes in cooling water. After the initial mixing time, water samples (20 ml) were withdrawn from the boxes at the beginning of the flux period and 4 h later to determine ^{22}Na or ^{36}Cl specific activity (DPM μmol^{-1}).

Water ^{22}Na and ^{36}Cl activities were measured on 5 ml samples using liquid scintillation counting (Canberra Packard 2500TR). Total Na^+ and Cl^- concentrations in water were assessed by flame emission spectrophotometry (Varian Spectra AA10)

and colorimetry, respectively. From these measurements, whole body $J_{in}Na^+$ and $J_{in}Cl^-$ were determined as described by Maetz (1956). Backflux correction was not required.

Determination of blood-to-water barrier distance

The mean blood-to-water barrier thickness was calculated from the lamellar harmonic mean thickness (τ_h) using TEM micrographs as previously described by Weibel and Knight (1964). This method involves measuring the harmonic mean intercept length (l_h) of several lines randomly crossing the barrier. The harmonic mean thickness is then calculated using the equation:

$$\tau_h = 2/3\ (l_h)$$

Following the treatment regime, twelve sections of gills were removed and dorsal, middle, and ventral sections from the first and second gill arches were isolated from each side. A single filament pair was isolated from each section and fixed, and then filament pairs were separated at the septum and dehydrated in an alcohol series as described above. A portion of the filament containing lamellae was isolated by first separating the anterior and posterior filaments at the septum. The filament was next cut parallel to the lamellae near the septum and again approximately 20-25 lamellae further towards the distal end. Tissue, oriented so that lamellae would be cross-sectioned when cut, was embedded in Araldite. Twenty-four sample blocks were made per fish .

A random number generator was used to select five blocks which were then trimmed under a stereo microscope. Ultrathin (50-70 nm) cross-sections were cut across the entire length of the lamellae. Of these, 10 randomly picked sections (per block; 50 per fish) were mounted on 200-Mesh copper grids. Grids were stained with lead citrate and saturated uranyl acetate (both from Aldrich) and examined under a Phillips 500 transmission electron microscope. Five randomiy selected lamellar

regions were photographed. Mean lamellar distance (l_h) was then determined by randomly placing a circular grid (see Weibel and Knight, 1964 for demonstration) of equidistant parallel lines over each micrograph. The digitizer tablet was calibrated to account for magnification, and the length of the lines crossing completely between the blood space and water were measured. The grid was superimposed four times per picture, so that approximately 100 measurements were made per fish .

Lamellar Surface Area

Lamellar surface area was evaluated using light microscopy. Tissue was excised, fixed, and embedded as for the TEM samples. Blocks were trimmed and cut into successive 1μm semi-thin sections, starting where the lamellae first appeared. Every tenth section (10 μm) was mounted on a glass slide, etched using a sodium methoxide/methanol bath, stained with Toluidene Blue (BDH, Toronto), and dehydrated in an alcohol series. A lamellar profile was created for each fish by plotting the average lamellar height of every collected section *versus* the distance along the lamellae (approximately 30 sections), and the area of this profile was then determined with the digitizer. This method is equivalent to that of Laurent and Hebibi (1989), in which the lamellar surface area was calculated utilising a formula which would represent the profile line used here.

Interlamellar area

A light micrograph from the lamellar surface area study was randomly selected from each of the trailing edge end, middle, and leading edge end of the lamellae. The area of the water channel between each pair of lamellae on the picture was determined by drawing a line from adjacent tips of each lamella and evaluating the surface area between each lamellaeusing a calibrated digitizer.

Statistical Analysis

All values are presented as means ± 1 standard error of the mean (SE). Results were analysed by one-way analysis of variance (ANOVA). In cases where significant differences between groups were indicated, Fisher's Least Significant Difference test was used to make comparisons between individual means. The fiducial limit of confidence was 5%.

RESULTS

Chloride cell surface parameters

Three chloride cell surface morphometric parameters were assessed (Figure 2.1). The individual cortisol and oGH treatments caused significant elevations in the average cell surface area of exposed cells (Figure 2.1A) without affecting their density (Figure 2.1B). Consequently, the fractional surface area of chloride cells on the filament epithelium was increased to a similar extent (~2X; Figure 2.1C) by cortisol or oGH. The combined cortisol and oGH treatment was the most effective in increasing the chloride cell fractional surface area (~6X; Figure 2.1C) because of its additional stimulatory effect on chloride cell density (Figure 2.1B). The chloride cell surface morphometric parameters were unaffected by sham injections.

Representative scanning electron micrographs from control and the combined cortisol/oGH treated fish are shown in Figure 2.2.

Ion uptake

The effects of individual and combined cortisol and oGH treatments on whole body Cl^- and Na^+ influxes ($J_{in}Cl^-$; $J_{in}Na^+$) are illustrated in Figure 2.3. Owing to the absence of any effects of vehicle injections on chloride cell surface morphology (see above), non-injected fish were not utilised in this part of the study. In general, cortisol and oGH caused significant increases in $J_{in}Cl^-$ and $J_{in}Na^+$ when compared to the corresponding sham groups. An exception was the lack of an effect of oGH on $J_{in}Cl^-$ when compared to the cortisol/oGH shams. The greatest rates of $J_{in}Cl^-$ and $J_{in}Na^+$ were achieved in the fish treated with the combination of cortisol and oGH.

$J_{in}Cl^-$ and $J_{in}Na^+$ were significantly correlated with chloride cell fractional area (r^2 = 0.91 and 0.88, respectively; data not shown).

Figure 2.1. The effects of cortisol (CORT), growth hormone (oGH), and a combined treatment (oGH/CORT) on surface morphometry: (A) average chloride cell area; (B) chloride cell density; and (C) chloride cell fractional area. For each study n = 4. Values shown are mean ± 1 SE. * indicates significantly greater than control/sham. + indicates significantly greater than all other treatments ($p \leq 0.05$).

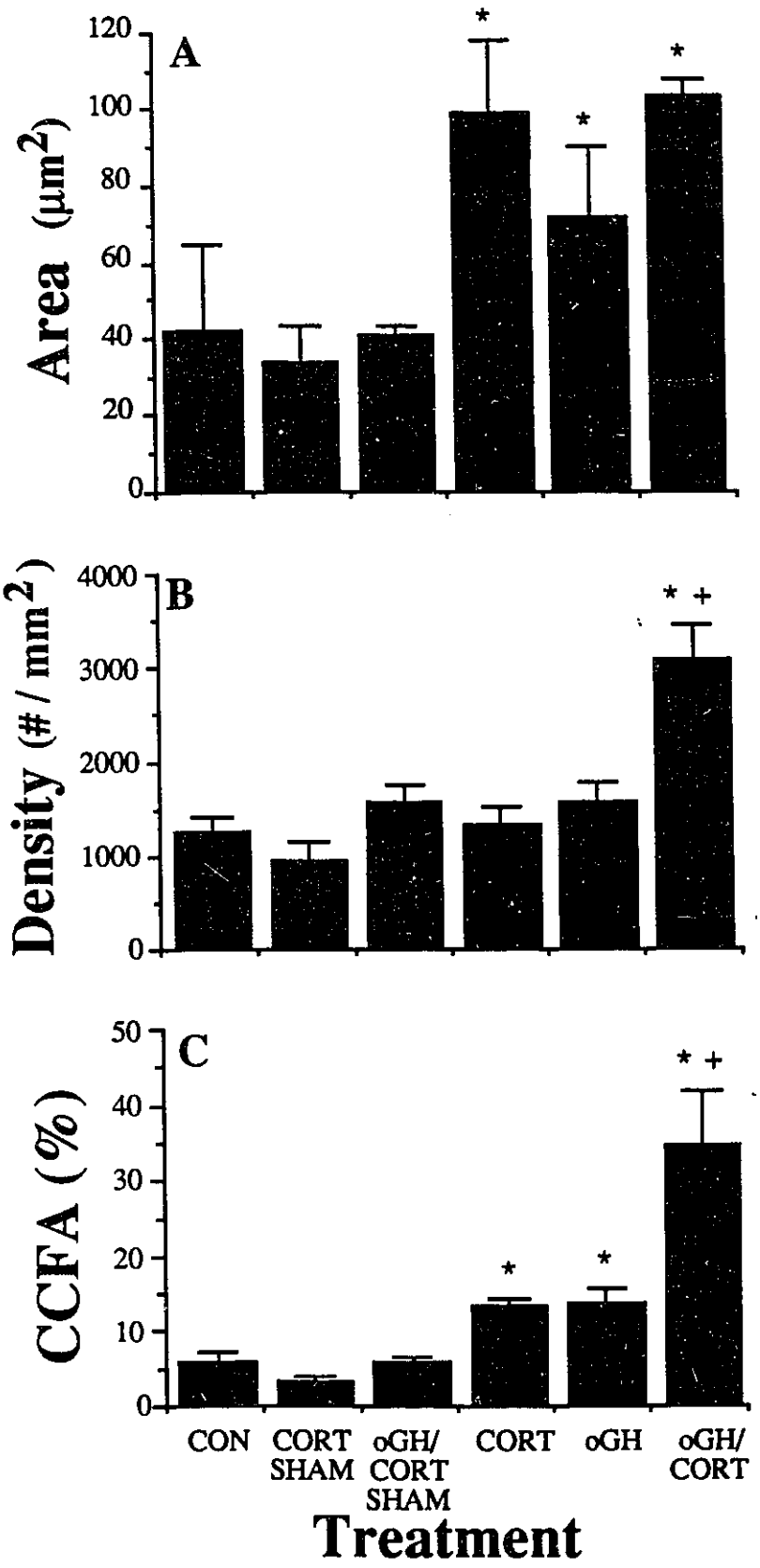

A
Area (μm²)
120
100
80
60
40
20
0
*
*
*
B
Density (# / mm²)
4000
3000
2000
1000
0
* +
C
CCFA (%)
50
40
30
20
10
0
* +
*
*
CON
CORT SHAM
oGH/ CORT SHAM
CORT
oGH
oGH/ CORT
Treatment

Figure 2.2. Representative scanning electron micrographs of trout gill filament and lamellar epithelia under control conditions (A and C) or after 10 days of treatment with combined cortisol and ovine growth hormone injections (B and D). CC = chloride cell; PC = pavement cell, M = mucous cell. Bar = 10 μm. Note the obvious proliferation of lamellar chloride cells (panel B) and the thickening of the lamellar base (panel D); for clarity only a few chloride cells and pavement cells are labelled.

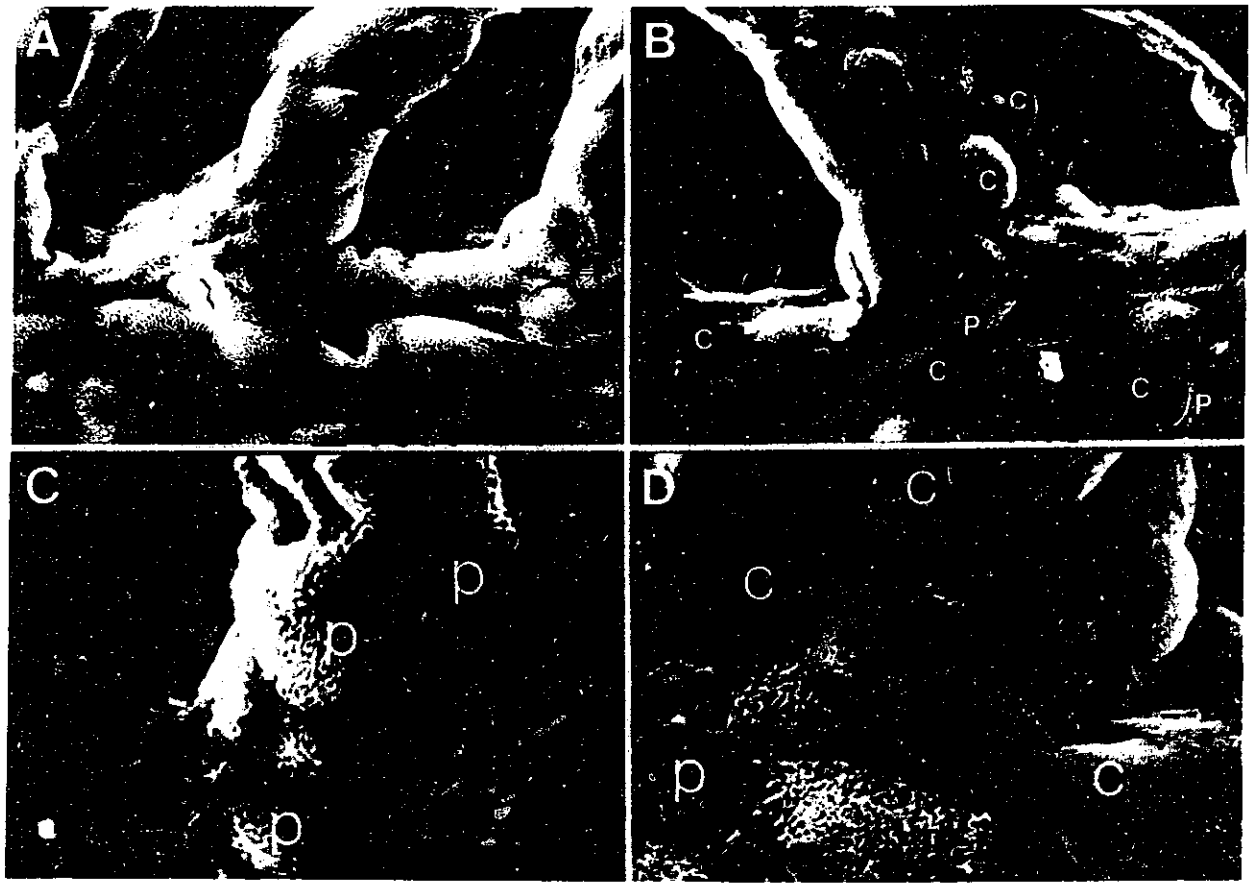
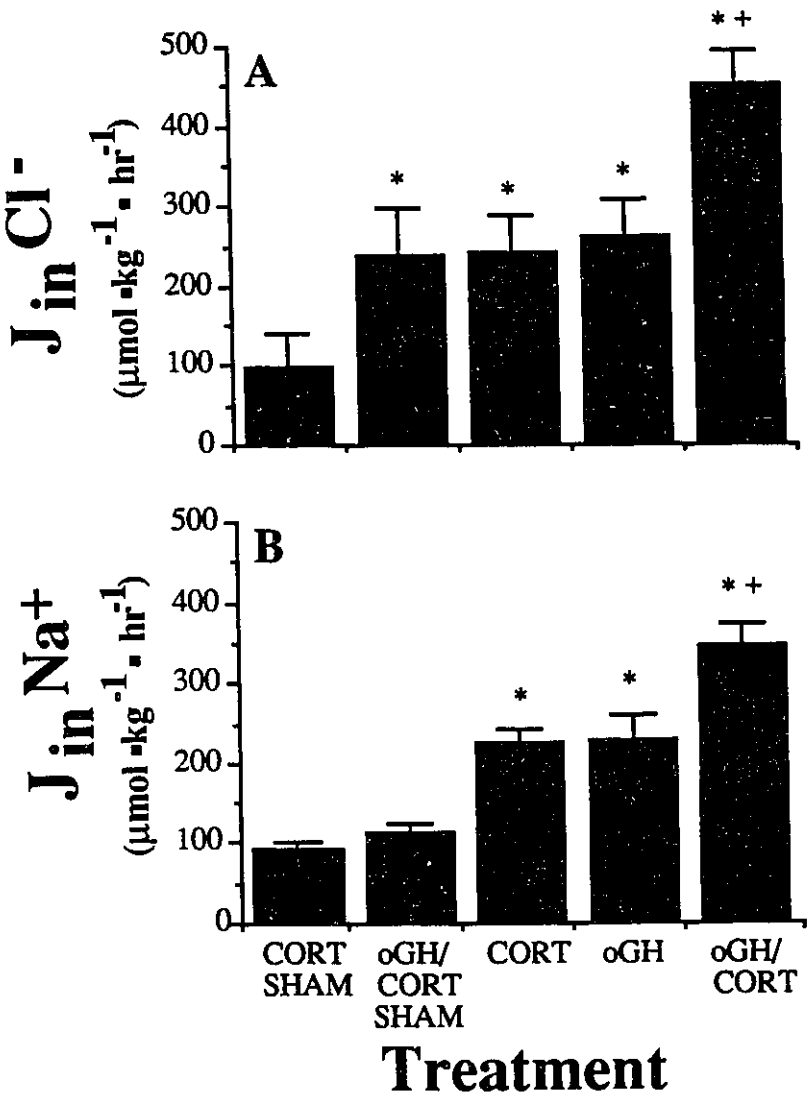

A
B
C
C
P
C
C
P
C
p
p
p
D
C
C
p
C

Figure 2.3. The effects of cortisol (CORT), ovine growth hormone (oGH), and a combined treatment on (A) chloride and (B) sodium uptake ($J_{in}Cl^-$; $J_{in}Na^+$) across the gill. For each study n = 8. * indicates significantly greater than control/sham. + indicates significantly greater than all other treatments ($p \leq 0.05$).


A
B
J_{in} Cl^-
J_{in} Na^+
(µmol·kg^{-1}·hr^{-1})
500
400
300
200
100
0
* +
*
CORT SHAM
oGH/ CORT SHAM
CORT
oGH
oGH/ CORT
Treatment

Lamellar Morphometric Parameters

Figure 2.4 shows the effect of each treatment on the harmonic mean thickness (τ_h) of the lamellar blood-to-water diffusion barrier. The thickness of the lamellar epithelium was significantly greater in cortisol and oGH treated fish than in control and sham treated fish. The combined cortisol and oGH treatment caused a further increase in the barrier thickness. The harmonic mean thickness of the lamellar epithelium was significantly correlated (r^2 = 0.88) with gill filament chloride cell fractional area (Figure 2.5). Figure 2.6 is a representative TEM micrograph showing the differences in water-to-blood barrier thickness between untreated and oGH/control treated fish.

Lamellar surface area was unaffected by the sham or any of the hormone treatments (Table 2.1). The number of lamellae per filament was also unaltered by hormone treatments and thus, it would appear that overall gill surface area was not increased as a morphological adjustment to counter the increased blood to water barrier thickness.

Treatment with cortisol significantly decreased the inter-lamellar space at the trailing and leading edge ends of the lamellae (Figure 2.7). Fish treated with oGH displayed a significantly reduced inter-lamellar space. The combined treatment of cortisol and oGH together also led to significant decreases of the inter-lamellar space at all three regions. This latter decrease was significantly greater than that induced by the individual hormone treatments in both the trailing edge and middle regions (Figure 2.7). Figure 2.8 is a representative light micrograph showing the differences in inter-lamellar space between untreated and oGH/cortisol treated fish.

Figure 2.4. Effect of cortisol (CORT), growth hormone (oGH), and a combined treatment (oGH/CORT) on the harmonic mean blood-to-water barrier distance (τ_h). * indicates significantly greater than control/sham. + indicates significantly greater than all other treatments ($p \leq 0.05$).

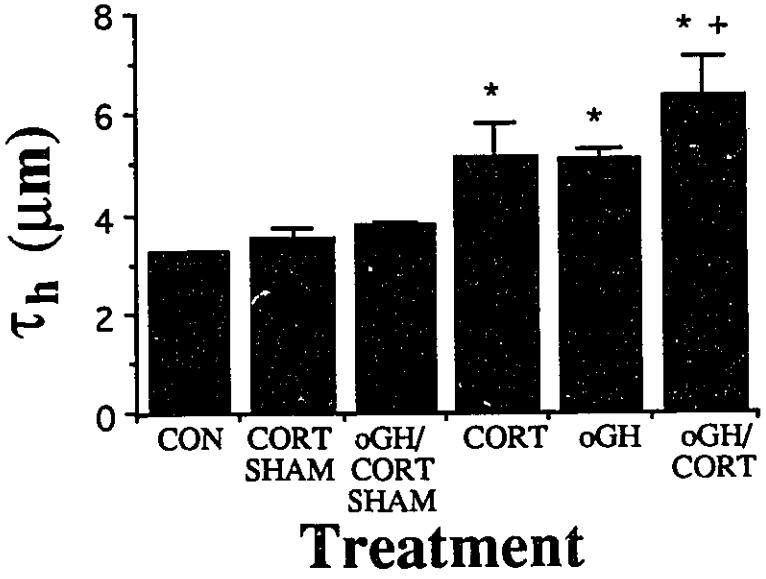

8
6
4
2
0
τ_h (μm)
* +
*
*
CON
CORT SHAM
oGH/ CORT SHAM
CORT
oGH
oGH/ CORT
Treatment

Figure 2.5. The relationship between chloride cell fractional area (CCFA) and τ_h. τ_h = (10.72 x CCFA) + 3.25. r^2 = 0.88. For each study n = 4. Values shown are mean ± 1 SE.

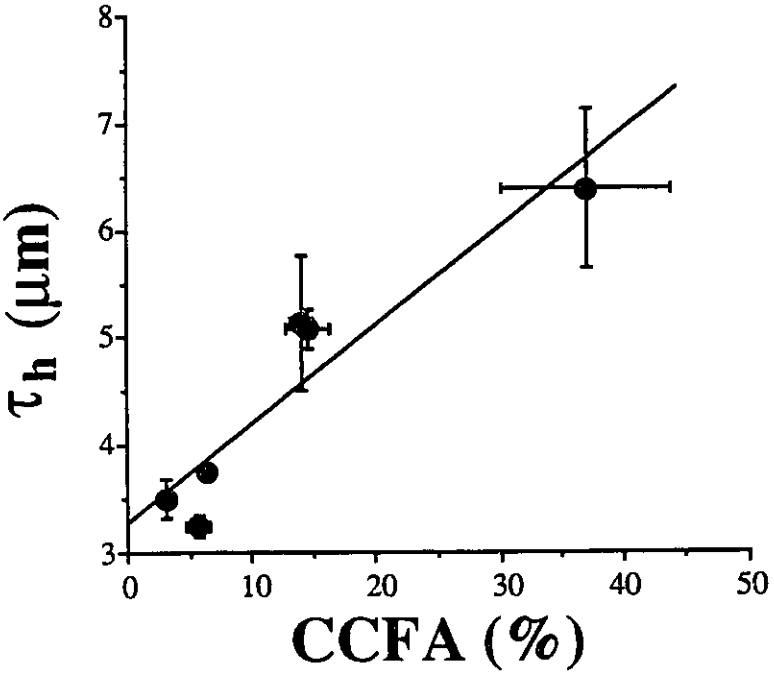

8
7
6
5
4
3
τ_h (μm)
0
10
20
30
40
50
CCFA (%)

Figure 2.6. Representative transmission electron micrographs of gill lamellar cross sections of (A) control and (B) growth hormone/cortisol treated trout. Important to note is the significant difference in the distance of the space separating the water (W) and blood space (annotated as R representing the red blood cells). Magnification = 3000X.

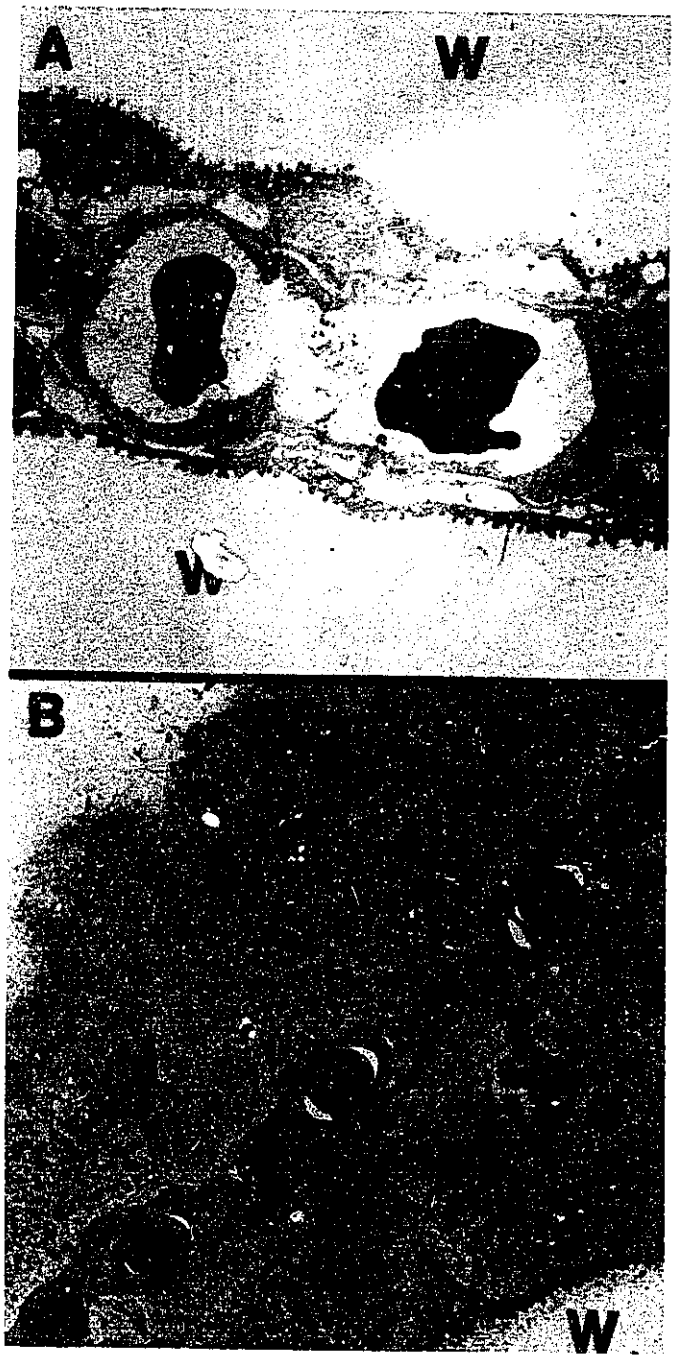

A
W
W
B
W

Table 2.1. Effect of cortisol, ovine growth hormone, and a combination of both treatments on lamellar surface area (A_{ls}) as measured by light microscopy. For each group, n = 4. No significant differences were observed amongst treatments.

Treatment	A_{IS} (μm^2)
Control	3312 ± 41
Cortisol Sham	3551 ± 93
Cortisol/Growth Hormone Sham	3493 ± 39
Cortisol	3353 ± 158
Growth Hormone	3479 ± 208
Cortisol/Growth Hormone	3660 ± 116

Figure 2.7. The effect of cortisol (CORT), growth hormone (oGH), and a combined treatment (oGH/CORT) on interlamellar space. For each study n = 4. Values shown are mean ± 1 SE. * indicates a significant decrease from control/sham treatments. + indicates significantly decreased over all other treatments ($p \leq 0.05$).

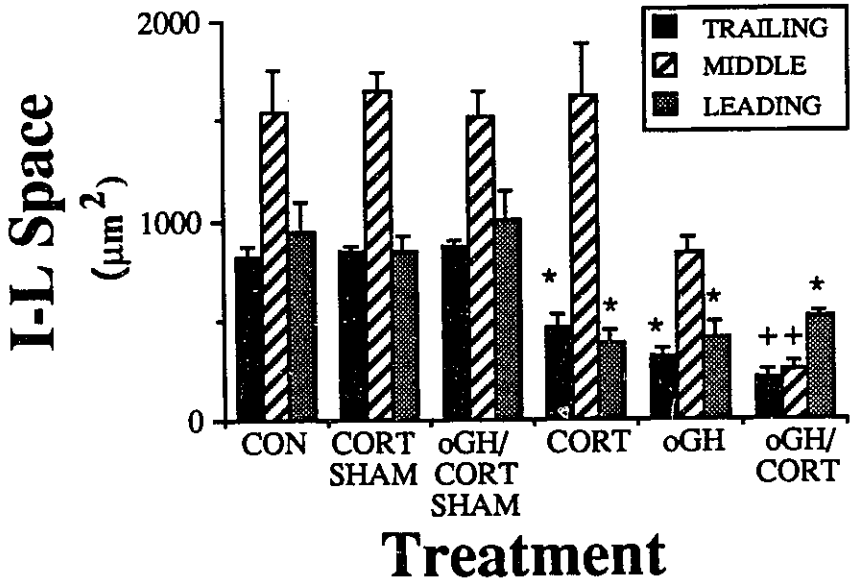
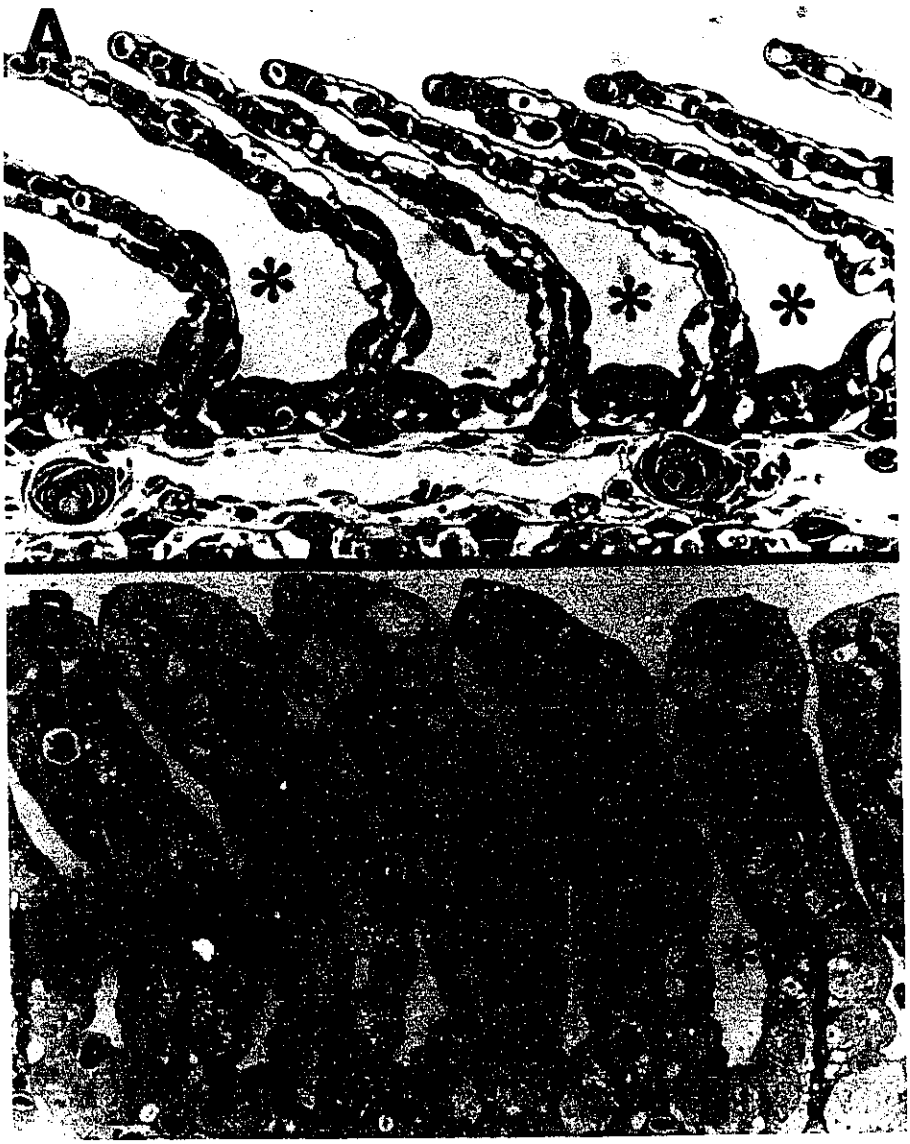

I-L Space
(μm²)
2000
1000
0
TRAILING
MIDDLE
LEADING
CON
CORT SHAM
oGH/ CORT SHAM
CORT
oGH
oGH/ CORT
Treatment

Figure 2.8. Representative light micrographs of gill lamellar cross sections of (A) control and (B) growth hormone/cortisol treated trout. * indicates interlamellar space. Magnification = 200X.


A

DISCUSSION

Methods

The goal of this study was to evaluate the effects of branchial chloride cell proliferation/enlargement on ionic uptake and morphological diffusing capacity and to determine whether the enhancement of ionic uptake known to be associated with chloride cell proliferation is accompanied by concomitant negative effects on gas transfer. The experimental approach was to treat fish with hormones (cortisol and/or oGH) using protocols known to elicit increases in chloride cell fractional surface area (Doyle and Epstein, 1972; Perry and Walsh, 1989; Laurent and Perry, 1990; Madsen, 1990c,d; Perry *et al.*, 1992b). The hormones were used simply as tools to elicit a wide range of gill chloride cell morphologies so as to permit the determination of relationships between chloride cell fractional surface area, ionic uptake, and blood-to-water diffusion distance. It is likely that the doses used (i.e. 8 mg kg^{-1} for cortisol and 2 mg kg^{-1} for oGH) produced pharmacological, rather than physiological, concentrations in the blood. Moreover, the use of non-homologous growth hormone was an additional confounding factor. Thus, although it is clear that cortisol and oGH treatments had marked physiological and morphological effects, we make no attempt to assign physiological roles to these hormones on the basis of the present experimental protocol. It should be noted that the results of numerous previous studies, however, have implied that both cortisol and growth hormone are important regulatory hormones (see below).

In this study, chloride cell fractional surface area was determined using a two-dimensional analysis and the data were used as an index of chloride cell proliferation and/or enlargement. This approach unquestionably underestimates the true surface area of exposed apical membranes because it does not account for the presence of microvilli and other surface projections. Nevertheless, since it is likely that only the

exposed (uncovered) chloride cells are involved in ionic uptake, such an analysis was considered to be an appropriate and a reliable technique. Other methods which utilise specific stains for chloride cells (e.g. anthroyloubain; McCormick, 1990a,b; DASPMEI; Karnaky *et al.*, 1984; OsO_4/zinc iodide; Madsen and Korsgaard, 1991) in conjunction with light microscopy are also reliable but cannot readily discern between uncovered and covered chloride cells.

The quantification of epithelial chloride cell morphometric parameters using SEM requires that the analysis be performed on regions that, by virtue of their flatness, can be readily photographed without distorting the surface morphology. For this reason, the trailing edge of the filament near the septum was chosen for all morphometric analyses. Clearly, it would have been optimal to quantitatively assess lamellar epithelial chloride cell morphology because the lamellae, rather than the filaments, are the predominant sites of gas transfer, but, this was not practical nor absolutely necessary as it was previously demonstrated that increases in chloride cell surface area on the filament are mirrored by increases on the lamella (Laurent and Perry, 1990). The qualitative analysis of scanning electron micrographs in this study (Figure 2.2) clearly demonstrates the simultaneous proliferation of chloride cells on both the filament and lamellar epithelia.

The effects of hormone treatment on gill morphology and ionic uptake

Cortisol treatment has been used in numerous previous studies as a method of eliciting proliferation/enlargement of chloride cells or increases in Na^+/K^+-ATPase levels in both gills (Doyle and Epstein, 1972; Perry and Wood, 1985; Richman and Zaugg, 1987; McCormick and Bern, 1989; Perry and Walsh, 1989; Laurent and Perry, 1990; McCormick *et al.*, 1991; Perry *et al.*, 1992a,b) and opercular epithelia (Foskett *et al.*, 1983; McCormick, 1990a). The increase in chloride cell fractional surface area reported here is in agreement with prior studies although in the present study it was

achieved by the enlargement of individual cells rather than by the usual combined effects of cell enlargement and increased cell numbers (Laurent and Perry, 1990; Perry *et al.*, 1992a,b).

The effects of cortisol injections on chloride cell morphology can be attributed specifically to the cortisol because vehicle injections were without effect. However, the daily injections of cortisol (8 mg kg^{-1}) likely caused the circulating levels to exceed normal physiological limits (e.g. see Perry and Wood, 1985) and thus we recognise that it is not possible to establish a physiological role for cortisol from these results. On the other hand, previous studies demonstrated that plasma cortisol levels rise in trout during exposure to ion-poor environments (Perry and Wood, 1985; Perry and Laurent, 1989) and that their increases coincide with increases in chloride cell fractional surface area. On the basis of these correlative data, it was suggested that cortisol is involved in the hormonal regulation of ionic transport in freshwater fishes (see reviews by Laurent and Perry, 1991; Perry and Laurent, 1993) in addition to its well known role as an osmoregulatory hormone in seawater fishes (Foskett *et al.*, 1983).

Fewer studies have examined the effects of growth hormone on gill chloride cell morphology. Richman and Zaugg (1987) reported that treatment of freshwater acclimated Coho salmon (*Oncorhynchus kisutch*) with bovine growth hormone increased gill Na^+/K^+-ATPase levels without affecting branchial chloride cell density. On the other hand, the results of the present, and a previous study (Madsen, 1990d), clearly demonstrate a stimulatory effect of ovine growth hormone on branchial chloride cell surface area in trout. It is possible that the differences between the studies reflect the origin of the growth hormone (bovine *versus* ovine). The increase in chloride cell surface area and/or the rise in gill Na^+/K^+-ATPase levels induced by growth hormone are believed to enhance the hypo-osmoregulatory ability of euryhaline species transferred from freshwater to seawater (e.g. Madsen, 1990d).

The enhanced hypo-osmoregulatory ability of anadromous fishes treated with exogenous growth hormone together with the elevation of endogenous levels of growth hormone during smoltification or after transfer to seawater (e.g. Sakamoto *et al.*, 1991; Yada *et al.*, 1991; Yada and Hirano, 1991) suggests an important involvement of this hormone in the adaptation of salmonids to seawater.

The relationship between chloride cell morphology and ionic uptake

The increase in chloride cell surface area was accompanied by increases in the rates of Na^+ and Cl^- uptake across the gill. Such positive correlations between chloride cell surface area and rates of Na^+ or Cl^- uptake were reported previously (Avella *et al.*, 1987; Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.*, 1992b) and have provided supportive, albeit indirect, evidence that the chloride cell is the site of branchial NaCl uptake in freshwater teleosts. The contention, however, that the chloride cell is the site of both Na^+ and Cl^- uptake may be incorrect (Goss *et al.*, 1992). The results of the study of Goss *et al.* (1992) demonstrated that during hypercapnic acidosis in brown bullhead (*Ictalurus nebulosus*), Cl^- uptake was correlated positively with chloride cell fractional surface area whereas Na^+ uptake was not. On the basis of these results, it was suggested that the chloride cell is the site of Cl^- uptake whereas the pavement cell is the site of Na^+ uptake. The simultaneous increases in Na^+ and Cl^- uptake caused by chloride cell proliferation could alternatively be explained by a secondary stimulation of Na^+ uptake by pavement cells in response to the initial stimulation of Cl^- uptake (otherwise an internal acid-base disturbance would develop); it is not possible to distinguish between these two options. Regardless, the proliferation of branchial chloride cells is an important physiological response of teleost fish to ion-poor water and other environments that challenge its ability to maintain ionic balance. Either directly or

indirectly, this response is associated with an increased capacity of the gills to absorb Na^+ and Cl^- ions from the water.

The relationship between chloride cell morphology and gas transfer

The results of this study demonstrate conclusively that cortisol and/or oGH-induced proliferation of lamellar chloride cells caused a marked thickening of the blood-to-water diffusion barrier as well as a decrease in the area of the inter-lamellar water channels. In the absence of any compensatory adjustments (see below), the approximate twofold increase in the blood-to-water diffusion distance would be expected to decrease the diffusive conductance by a similar factor and thus significantly impair oxygen uptake and carbon dioxide excretion across the gills. In addition, the reduction in the area of the inter-lamellar water channels would probably impede the flow of ventilatory water which would also impair gas transfer.

The decrease in the morphological diffusing capacity that accompanies lamellar chloride cell proliferation is consistent with low arterial PO_2 values and the lowered resistance to hypoxia occurring in trout naturally acclimated to ion-poor water and displaying abundant lamellar chloride cells (Thomas *et al.*, 1988). Thus, the hypothesis proposed by Thomas *et al.* (1988) that gas transfer is compromised by chloride cell proliferation is consistent with the results of the present study. Conversely, Laurent and Hebibi (1989) reported that branchial diffusive conductance was increased by a factor of 4.4 in rainbow trout acclimated to ion-poor water because of the combined effects of a thinning of the blood-to-water diffusion barrier and an increase in lamellar surface area. As lamellar surface chloride cell proliferation occurred in each study (Thomas *et al.*, 1988; Laurent and Hebibi, 1989; present study), it is difficult to reconcile the apparent discrepant effects on the morphological diffusing capacity of the gill.

The thickening of the blood-to-water diffusing barrier and the restriction of the inter-lamellar channels that were associated with the proliferation of lamellar chloride cells in the present study would be expected to limit gas transfer in the absence of other compensatory adjustments. Potential physiological adjustments include hyperventilation and the concomitant elevation of the convection requirements for O_2 and CO_2 transfer, increases in haematocrit and/or cardiac output, and an increase in haemoglobin-O_2 binding affinity (see review by Perry and McDonald, 1993). Clearly, further studies are required to directly assess the impact of chloride cell proliferation on gas transfer and to determine if any secondary compensatory adjustments are invoked.

CHAPTER 3

THE EFFECTS OF BRANCHIAL CHLORIDE CELL PROLIFERATION ON RESPIRATORY FUNCTION IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

INTRODUCTION

As I discussed in detail in the introduction, the maintenance of osmotic, ionic and respiratory homeostasis are important functions of the teleost fish gill. The mechanics of these functions are contingent, at least in part, on morphological adjustments of the gill (Laurent and Perry, 1991). One of the most frequently studied and physiologically significant of such morphological adjustments involves variations in the number and/or size of the chloride cell (Laurent, 1984; Laurent and Perry, 1991). In freshwater teleosts, these cells are thought to perform an essential role in maintaining a balanced internal ionic environment by absorbing Ca^{2+}, Na^{+} and Cl^{-} from the dilute external medium (Laurent, 1984). The proliferation of branchial chloride cells (Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989) is consequently utilised by fish as a mechanism for enhancing the ion-transport capacity of the gills (Perry and Laurent, 1989; Laurent and Perry, 1991) in challenging environments such as ion-poor (soft) water.

The movement of respiratory gases across the gill epithelium is also affected by surface and sub-surface morphological changes. The diffusive movement of O_2 or CO_2 across the gill is influenced by at least two morphometric parameters: the functional lamellar surface area and the thickness of the blood-to-water diffusion barrier (Hughes and Morgan, 1973; Randall and Daxboeck, 1984).

As demonstrated in Chapter 2, chloride cell proliferation, induced by chronic treatment of trout with cortisol and ovine growth hormone (Madsen, 1990b), caused an increase in the blood-to-water diffusion distance across the lamellar epithelium without a concurrent adjustment (increase) in lamellar surface area. From this observation, it was predicted that gas transfer would be impaired in fish displaying excessive chloride cell proliferation. Surprisingly few studies, however, have evaluated the relationship between branchial chloride cell morphology and gas transfer. Thomas *et al.* (1988) showed that rainbow trout acclimated to an ion-poor environment had a lower resistance

to acute hypoxia. Specifically, they reported that trout naturally acclimated to ion-poor water ([NaCl] = 0.10 mmol l^{-1}) maintained markedly lower arterial blood O_2 partial pressures when compared to fish that were naturally adapted to higher salt levels ([NaCl] = 1.0 mmol l^{-1}). It was suggested that this apparent impairment of branchial O_2 uptake resulted from the proliferation of lamellar chloride cells, thereby reducing the effective surface area for gas transfer. In contrast, Laurent and Hebibi (1989) reported that exposure of trout to ion-poor water increased the gill diffusive conductance for gas transfer as the result of a thinning of the blood-to-water diffusion barrier and an increase in lamellar surface area; blood respiratory variables were, however, not measured.

The goal of this study was to induce chloride cell proliferation on the gill lamellae of rainbow trout and to assess the consequences on respiratory function. Chloride cell proliferation was again induced through chronic hormone elevation by injecting fish with cortisol and ovine growth hormone utilising the techniques and procedures described in Chapter 2. Respiratory function was evaluated by continuously monitoring arterial blood PO_2, PCO_2 and pH, under normoxic and hypoxic conditions, using an extracorporeal arterial blood circulation.

MATERIALS AND METHODS

Experimental animals

Information on the rainbow trout used in this series of experiments can be found in Chapter 2.

Injection protocol

Fish were divided into three groups: untreated, control and treatment. Fish selected for treatment were given multiple injections of cortisol and growth hormone to induce proliferation of branchial chloride cells (chloride cell) (Chapter 2). Cortisol (hydrocortisone-21-hemisuccinate sodium salt; Sigma) was injected daily (8 mg kg^{-1} intramuscularly) for 10 days. Growth hormone was injected intraperitoneally (2 mg g^{-1} of ovine growth hormone; NIH-NIADDK, Bethesda, Maryland) every second day over the ten day period. All injections were given in 0.5 ml of Cortland saline (0.9%; pH 7.8; Wolf, 1963). Control fish were given 0.5 ml of the saline only. Injected fish were first lightly anaesthetised (i.e. to the point of losing equilibrium) in a 0.1% (w/v) solution of aminobenzoic acid ethyl ester (MS-222; Sigma) adjusted to pH 7.5 with $NaHCO_3$.

Surgical procedure

Fish were anaesthetised in a neutralised solution of 0.1% MS-222 and placed onto an operating table, so that the gills were continuously irrigated with oxygenated anaesthetic solution. An indwelling catheter was implanted into the dorsal aorta (Soivo *et al.*, 1975) using flexible polyethylene tubing (PE 50, Clay-Adams, ID = 0.580 mm, OD = 0.965 mm). This catheter allowed for measurement of dorsal aortic blood pressure (P_{da}) during the experimental procedure (see below). A second catheter (PE 160 tubing; ID = 1.14 mm; OD = 1.57 mm) was placed in the opercular cavity to allow the measurement of ventilation amplitude.

The fish was then placed on its side and a small incision was made close to and parallel to the outer edge of the operculum. The coeliac artery was isolated and two cannulae (PE 50) were inserted in opposite (orthograde and retrograde) directions (Thomas and Le Ruz, 1982) and were secured in place with two ligatures of 2-O surgical silk. Fish were revived on the operating table with oxygenated water and then transferred to the experimental set-up. All cannulae were flushed with heparinized saline (50 units per ml) to prevent clotting. Fish were allowed to recover for 24 h prior to experimentation.

Experimental set-up

Fish were kept in black Perspex boxes fitted with an insert to limit, but not completely restrain their movement (i.e. they could not turn in the box). The dorsal aortic and opercular catheters were connected to pressure transducers (Bell and Howell 4-327-I). Dorsal aortic and ventilation pressures were monitored through a chart recorder connected to each transducer. Transducers were calibrated against a static column of water. This set-up allowed for a continual measurement of both pressure values: dorsal aortic pressure was recorded directly, while ventilation amplitude was recorded as the difference between the inspiratory and expiratory opercular pressures. In addition, ventilation frequency was measured periodically by visually counting buccal movements.

The extracorporeal circulation (Figure 3.1) was initiated by connecting the two coeliac cannulae in series with three measuring cells containing PO_2, PCO_2, and pH electrodes. Blood was removed from the main circulation of the fish through the retrograde cannula, passed through the three cells, and returned to the fish through the orthograde cannula. Blood flow (approximately 1.2 ml min^{-1}) through the measuring cells was maintained using a small peristaltic pump. All three cells were supplied with constantly flowing water which allowed them to remain at the ambient water

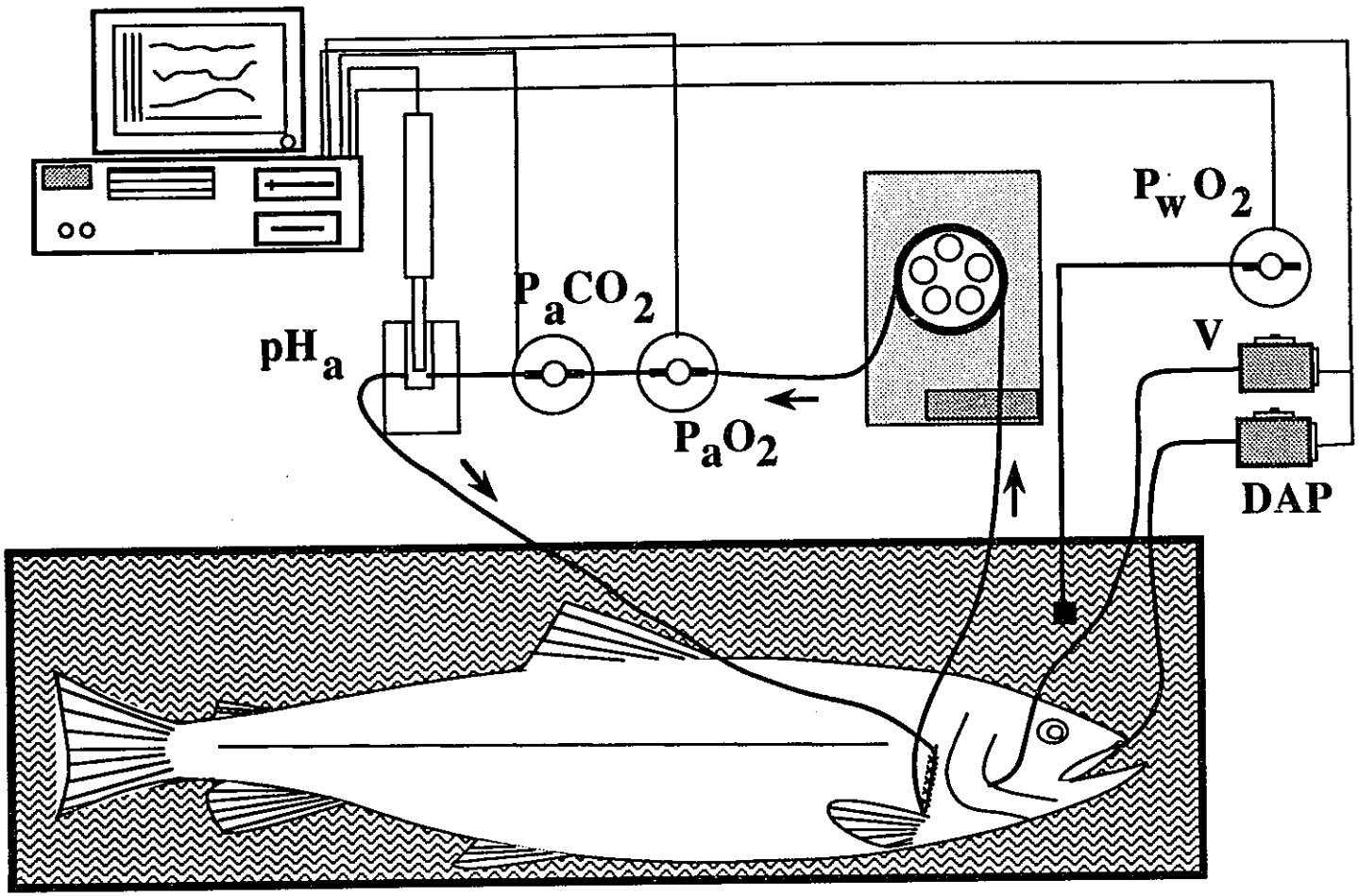
temperature (10-12°C). The total volume of blood in the external loop at any one time was approximately 1 ml, which represents less than 2% of the total blood volume of the fish. The external loop tubing was flushed with heparinized saline (540 IU per ml) prior to starting the flow of blood. This set-up allowed for continuous monitoring of arterial PO_2 (P_aO_2), PCO_2 (P_aCO_2), and pH (pH_a) over the course of the experiment.

P_aO_2 and P_aCO_2 were measured using Radiometer PO_2 and PCO_2 electrodes (models E-5046 and E-5036) connected to a PHM-73 meter. Arterial pH was measured using a pH glass electrode (Metrohm) also connected to the PHM-73 meter. Calibration of the electrodes was accomplished using either saline equilibrated with appropriate gas mixtures (for PO_2 and PCO_2) or commercially supplied buffer solutions (for pH).

PO_2 of the water (P_wO_2) was continuously measured using a second Radiometer PO_2 electrode connected to a PHM-72 Mk2 meter. Water was siphoned through a measuring cell containing the electrode.

All electrode/meter and transducer/recorder combinations produced analog outputs that were converted to digital outputs using a commercial analog-digital interface (Data Translation Incorporated). Digital output was fed to a computer utilising customised data acquisition software (AD-DATA; P. Thoren, Göteborg, Sweden). Mean values for each parameter were captured and stored at 5 sec intervals.

Figure 3.1. Schematic diagram of the extracorporeal circulation preparation. A peristaltic pump maintains blood flow through the external loop which passes through several electrodes. The output of these electrodes are collected by a data acquisition program on a computer. Additionally, water and ventilatory parameters (that do not form part of the external loop) are also monitored and collected by the program. P_aO_2 = partial pressure of oxygen in arterial blood, P_aCO_2 = partial pressure of carbon dioxide in arterial blood, P_wO_2 = partial pressure of oxygen in water, DAP = dorsal aortic pressure, pH_a = arterial pH.


P_wO_2
P_aCO_2
pH_a
V
P_aO_2
DAP

Experimental protocol

The experimental protocol consisted of two distinct stages: normoxia and passage through graded hypoxia. The normoxic period was used to allow all variables to stabilise and to obtain control (pre-hypoxia) readings; P_wO_2 during this period was constant at approximately 21.3 kPa. System stability generally occurred within 10-20 min of rerouting the blood to the external loop. During the second stage, fish were subjected gradually to hypoxia by bubbling compressed N_2 through a water equilibration column supplying the experimental holding box. The flow rate of N_2 was monitored and adjusted in order to produce an approximately linear decrease in P_wO_2. The rate of P_wO_2 decrease was about 0.80 kPa min^{-1}; thus, hypoxia was imposed over a 20 min period. During this stage, P_wO_2 was reduced from the normoxic value of 21.3 kPa to an end value of 5.3 kPa. All blood gas and ventilatory variables were continually monitored throughout the hypoxic period.

Morphometric analysis

Chloride cell proliferation was confirmed using scanning electron microscopy (SEM). Following completion of the experimental protocol, each fish was returned to a state of environmental normoxia. Fish were killed and a sample consisting of several pairs of filaments joined at the septum were removed from the second gill arch on the left side. The exact protocol for preparing and measuring the surface morphometric parameters is shown in Chapter 2.

Data and statistical analysis

Data are presented as means ± 1 standard error of the mean (SE). For the extracorporeal experiments, statistical analysis was performed at the normoxic P_wO_2 level of 21.3 kPa and subsequently at every decrease of 1.3 kPa to the final P_wO_2 of 5.3 kPa. Extracorporeal experiments were analysed using two-way analysis of variance

(ANOVA) with P_wO_2 and treatment as factors. In cases where significant differences between groups were indicated, multiple comparison of means was accomplished using Tukey's test. Results of morphometric analyses were compared using Student's unpaired t-tests. The fiducial limit of significance was 5%.

RESULTS

Morphometric analysis

Results of the surface morphometric analysis of the untreated, control (vehicle only), and growth hormone/cortisol treated fish are shown in Figure 3.2. There were no significant differences between untreated and control fish for any of the parameters examined (cell density, average chloride cell area, or CCFA). However, there were significant differences between the untreated/control and hormone-treated groups. Average chloride cell surface area was increased approximately 2.7 x (from 23.0 ± 1.7 mm^2 in the untreated fish to 63.2 ± 4.6 mm^2 in the hormone treated fish; Figure 3.2A), whereas chloride cell density increased roughly 2.2 x (from 1559 ± 101 cells/mm^2 to 3372 ± 252 cells/mm^2; Figure 3.2B). As a result, the chloride cell fractional area was elevated approximately 5 x (from 4.10 ± 0.75 % to 19.96 ± 2.24 %; Figure 3.2C) by the hormone injections.

Figure 3.3 shows representative SEM micrographs of gills from control and hormone-treated fish. Note in particular the differences in chloride cell number and size and, additionally, in the location of chloride cells between the two groups. Lamellar chloride cells were larger and more prominent in the treated fish (see Figure 3.3B) and clearly contributed to an overall thickening of the lamellar epithelium and a decrease in the thickness of the inter-lamellar water channel.

Extracorporeal experiments

There were no consistent differences in data obtained from the untreated and control fish. Thus, for clarity of presentation, the results from untreated fish are not shown. The effects of acute graded hypoxia on P_aO_2 are illustrated in Figure 3.4. Both groups experienced significant decreases in P_aO_2 at all levels of $P_wO_2 \leq 14.7$ kPa. Under normoxic conditions and mild hypoxia (i.e. $P_wO_2 > 12.0$ kPa), P_aO_2 was not

Figure 3.2. Effects of ovine growth hormone and cortisol (oGH/CORT) and sham (saline vehicle) treatments on surface morphometric parameters: (A) individual chloride cell (chloride cell) surface area, (B) chloride cell density, and (C) chloride cell fractional area (CCFA). For each group, n = 6. * indicates significantly different from untreated and sham groups ($p < 0.05$). Error bars indicate ± 1 SE.

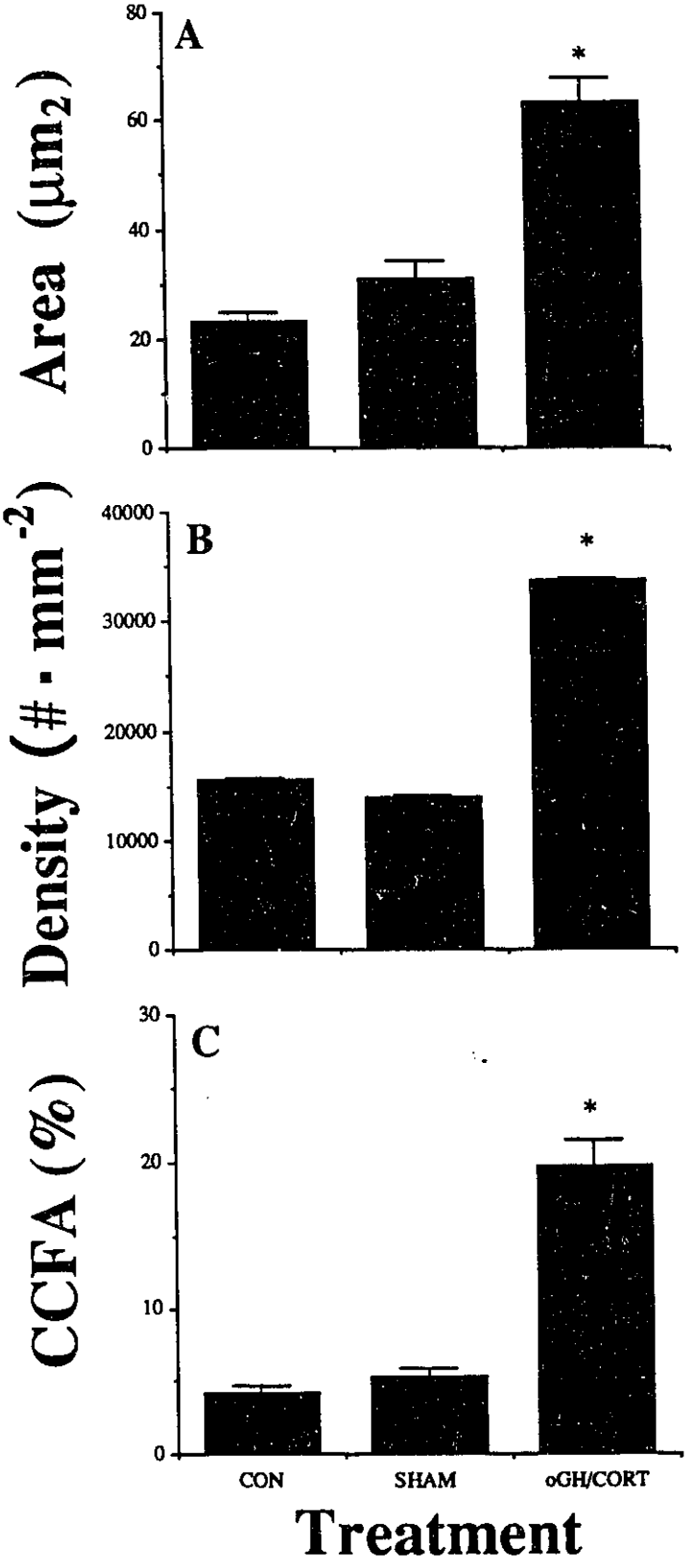

A
B
C
Area (μm2)
Density (# · mm-2)
CCFA (%)
80
60
40
20
0
40000
30000
20000
10000
0
30
20
10
0
*
CON
SHAM
oGH/CORT
Treatment

Figure 3.3. Representative scanning electron micrographs of trout gill filaments and lamellae from (A) control fish and (B) from fish treated with a combined ovine growth hormone and cortisol regimen . Arrows indicate chloride cells; PC = pavement cell. Bar = 10μm.

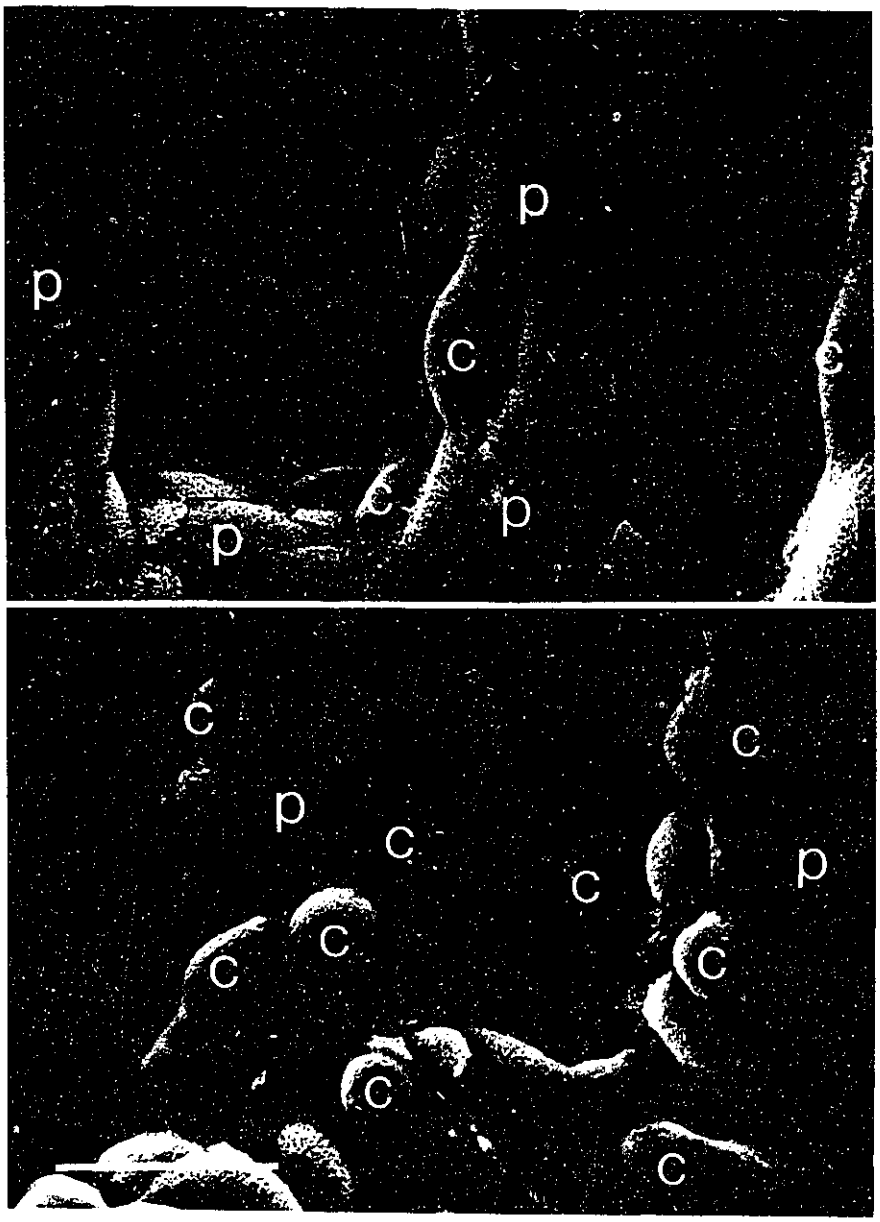
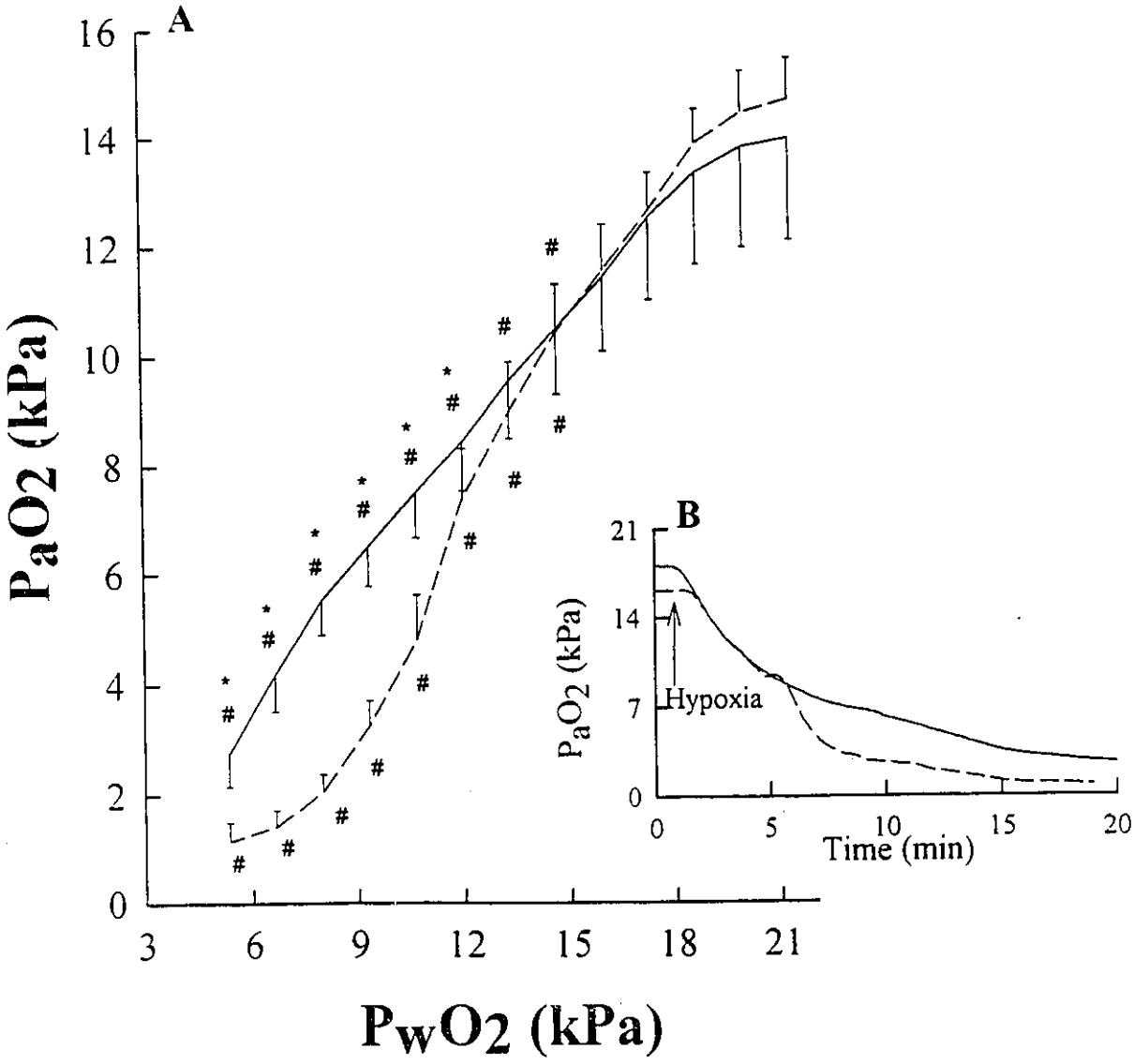

p
p
c
c
p
c
p
c
c
p
c
c
c
c
p
c
c

Figure 3.4. (A) Effect of environmental hypoxia on arterial oxygen partial pressure (P_aO_2) in control (solid line) fish and those treated with a combined ovine growth hormone and cortisol regimen (dashed line). * indicates significant differences between treatment groups; # indicates P_aO_2 levels significantly different from normoxia values (at P_wO_2 21.3 kPa) ($p < 0.05$). (B) shows a representative recording of P_aO_2 values for control and treated fish during the period over which hypoxia was induced. Error bars indicate ± 1 SE.


A
B
P_aO_2 (kPa)
P_wO_2 (kPa)
Hypoxia
Time (min)
0
2
4
6
8
10
12
14
16
3
6
9
12
15
18
21
7
5
10
15
20

affected by hormone treatment, but at $P_wO_2 \leq 12.0$ kPa, P_aO_2 values were significantly lower in hormone treated fish. Representative P_aO_2 recordings are shown for control and treated fish in Figure 3.4B. Despite the clear tendency in both control and treated fish for P_aCO_2 to decrease during the acute hypoxia (Figure 3.5), the two-way ANOVA indicated that the changes were not significant, probably due to the high degree of variability in the P_aCO_2 data. However, linear regression analysis demonstrated that P_aCO_2 was related to P_wO_2 in both groups (slope = 0.005, p = 0.001 for control group; slope = 0.003, p = 0.001 for treated group). Also, hormone-treated fish had significantly higher P_aCO_2 values than control fish at all P_wO_2 levels (Figure 3.5A). Figure 3.5B shows representative P_aCO_2 traces for control and treated fish. The elevated P_aCO_2 in treated fish was accompanied by a reduced arterial pH (pH_a; Figure 3.6); pHa values for treated fish were significantly lower than for the control group at all P_wO_2 values.

In response to hypoxia, both groups exhibited an initial, non-significant alkalosis which was frequently followed by a rapid decrease in pH_a. This drop in pH_a was generally preceded by a brief period (~5 sec) of intense struggling by the fish, and occurred at higher P_wO_2 levels and to a greater extent in hormone treated fish. Representative pH_a recordings are presented in Figure 3.6B.

The effects of environmental hypoxia on ventilation amplitude are illustrated in Figure 3.7. In comparison with the control group, the ventilation amplitude of hormone treated fish was significantly elevated at most P_wO_2 values, while their ventilation frequency was significantly reduced (Table 3.1). Both groups displayed a significant increase in ventilation amplitude under severely hypoxic conditions (Figure 3.7A); representative recordings of ventilation amplitude for both a control and a hormone treated fish are shown in Figure 3.7B. The pressure spikes visible in both traces, but more prevalent in the recording of the hormone-treated fish, arose from a variety of causes, including coughs, head movements and struggling. Ventilation frequency was not significantly altered by hypoxia (Table 3.1).

Figure 3.5. (A) Effect of environmental hypoxia on arterial carbon dioxide partial pressure (P_aCO_2) in control (solid line) fish and those treated with a combined ovine growth hormone and cortisol regimen (dashed line). * indicates significant differences between treatment groups. Regression analysis (not shown) indicated that P_aCO_2 was linearly related to P_wO_2; control group, slope = 0.005, p = 0.001; hormone-treated group, slope = 0.003, p = 0.001. (B) shows a representative recording of P_aCO_2 values for control and treated fish during the period over which hypoxia was imposed. Error bars indicate ± 1 SE.

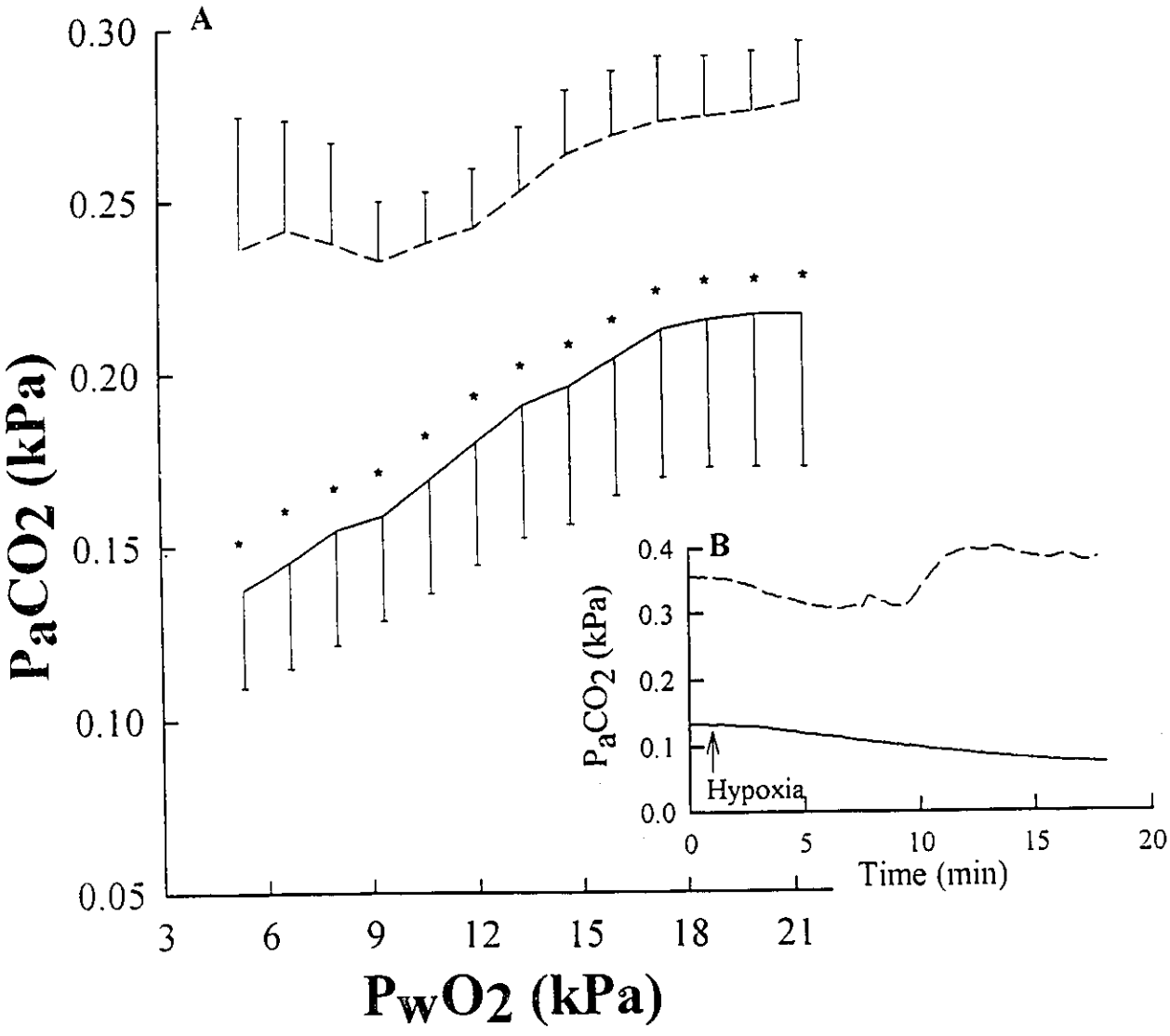

A
0.30
0.25
0.20
0.15
0.10
0.05
PaCO2 (kPa)
3
6
9
12
15
18
21
PwO2 (kPa)
B
0.4
0.3
0.2
0.1
0.0
PaCO2 (kPa)
Hypoxia
0
5
10
15
20
Time (min)

Figure 3.6. (A) Effect of environmental hypoxia on arterial pH (pH_a) in control (solid line) fish and those treated with a combined ovine growth hormone and cortisol regimen (dashed line). * indicates significant differences between treatment groups; # indicates pH_a levels significantly different from normoxia values (at P_wO_2 21.3 kPa) ($p < 0.05$). (B) shows a representative recording of pH_a values for control and treated fish during the period over which hypoxia was imposed. Error bars indicate ± 1 SE.

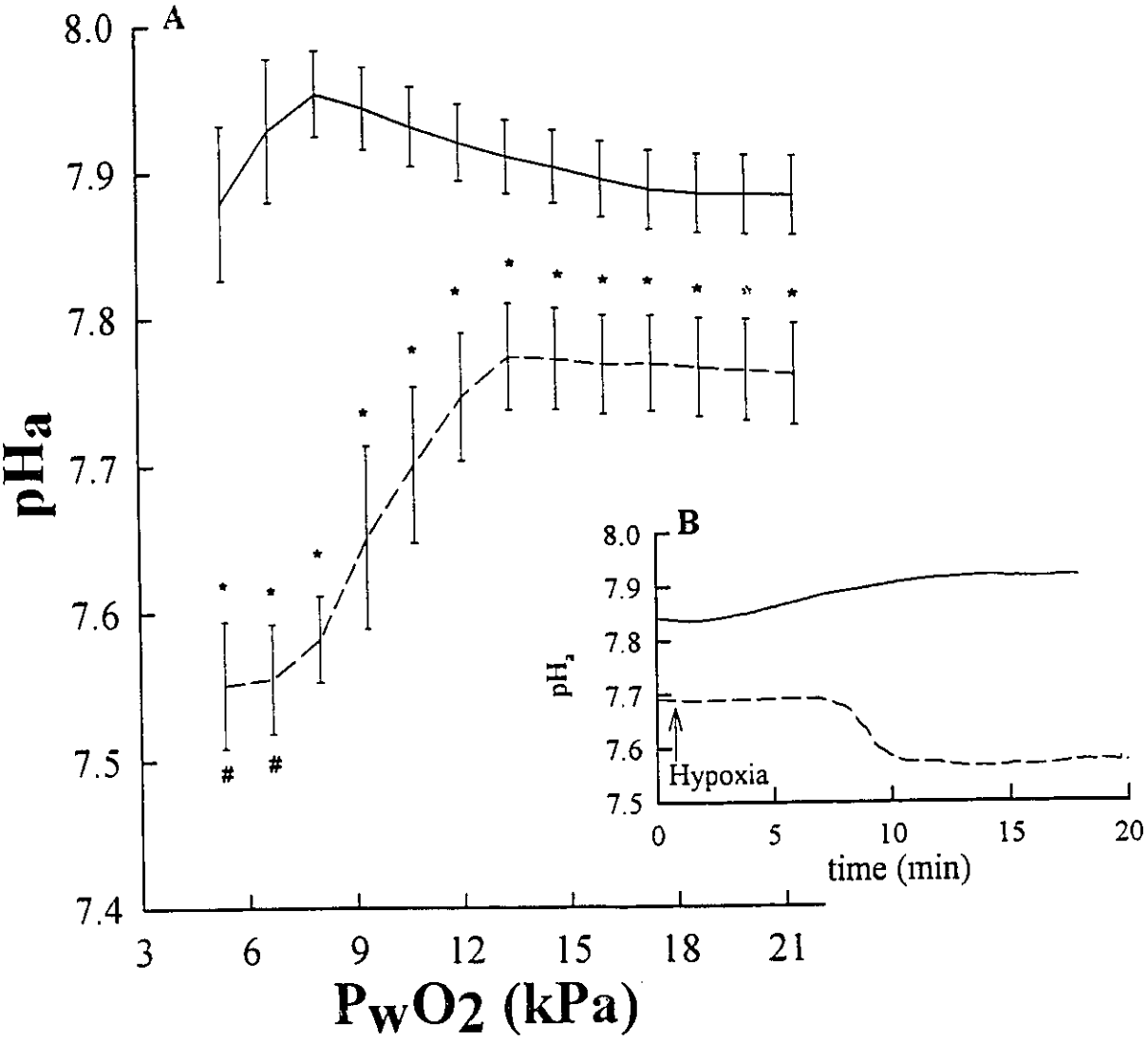

A
8.0
7.9
7.8
7.7
7.6
7.5
7.4
pHa
3
6
9
12
15
18
21
PwO2 (kPa)
B
8.0
7.9
7.8
7.7
7.6
7.5
pHa
Hypoxia
0
5
10
15
20
time (min)

Figure 3.7. (A) Effect of environmental hypoxia on mean ventilation amplitude in control fish (solid line) and those treated with a combination of ovine growth hormone and cortisol (dashed line). * indicates significant differences between treatment groups. # indicates significant differences from severely hypoxic values (5.3-8.0 kPa) ($p < 0.05$). Inset (B) shows representative recordings for each group during the time period over which hypoxia was imposed. The pressure spikes visible in these traces were caused by coughs, head movements and struggles. Error bars indicate ± 1 SE.

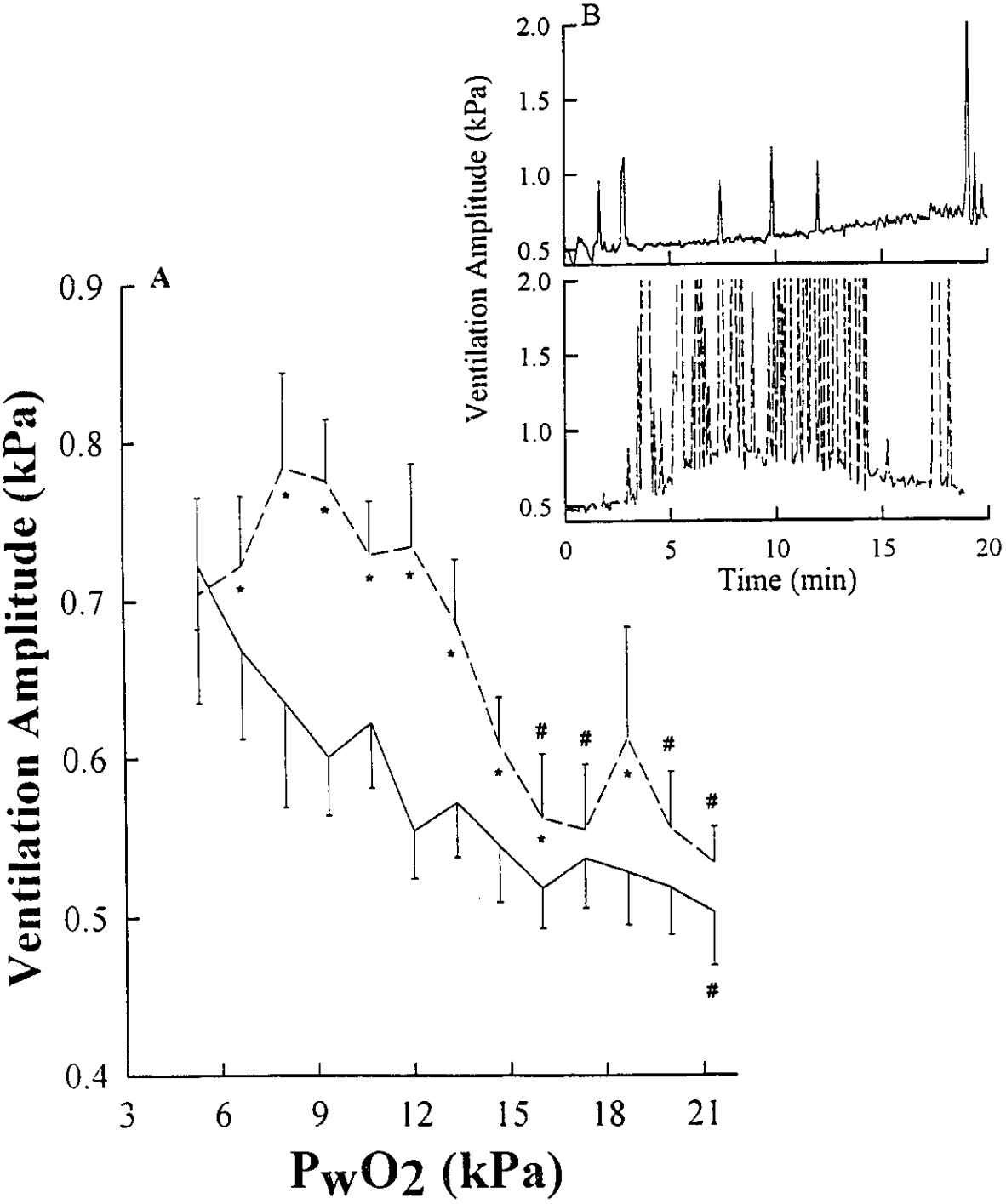

A
B
Ventilation Amplitude (kPa)
PwO2 (kPa)
Time (min)
0.9
0.8
0.7
0.6
0.5
0.4
3
6
9
12
15
18
21
2.0
1.5
1.0
0.5
0
5
10
15
20
#
*

Table 3.1. Effect of environmental hypoxia on ventilation frequency (V_f) in control fish and those treated with a combination of ovine growth hormone and cortisol. Values are means ± 1 SE. * indicates a significant difference between treatment groups ($p < 0.05$).

P_wO_2 (kPa)	Control V_f (min^{-1})	Treated V_f (min^{-1})
21.3	57±3	42±4 *
17.3	62±4	47±5 *
13.3	64±4	51±5 *
11.3	65±5	52±5 *
10.0	67±4	55±5 *
8.7	68±4	49±6 *
6.7	65±2	45±5 *
5.3	65±2	47±5 *

DISCUSSION

Chloride cell morphology

The objective of the present study was to analyse the consequences of lamellar chloride cell proliferation on the respiratory function of rainbow trout. Chloride cell proliferation was induced by chronic treatment of fish with cortisol and ovine growth hormone. Cortisol (Doyle and Epstein, 1972; Perry and Walsh, 1989; Laurent and Perry, 1990; Madsen, 1990a,b; Perry *et al.,* 1992a,b) and growth hormone (Madsen, 1990b) are independently effective in increasing both the size and/or number of lamellar chloride cells. The combined use of these hormones has an additive effect (Madsen, 1990b; see also Chapter 2) and thus was selected for the present study. Further, Chapter 2 clearly demonstrated that the chloride cell proliferation elicited by this combined hormone treatment produced an approximate two-fold increase in the thickness of the lamellar blood-to-water diffusion barrier without a concomitant and compensatory increase in lamellar surface area.

Chloride cell prevalence and size in untreated, control and hormone-treated fish were assessed in the present study using a two-dimensional analysis; the suitability of this technique was discussed in Chapter 2. Although the morphology of filament rather than lamellar epithelial chloride cells was quantified owing to the constraints of the SEM methods employed, increases in chloride cell surface area on the filament were shown previously to be paralleled by similar alterations on the lamella (Laurent and Perry, 1990; see also Figure 3.3). In agreement with prior studies (Doyle and Epstein, 1972; Perry and Walsh, 1989; Laurent and Perry, 1990; Madsen, 1990a,b; Perry *et al.,* 1992a,b), chloride cell fractional area (CCFA) was significantly elevated by treatment with cortisol and ovine growth hormone (Figure 3.2C), and the increase in CCFA was achieved by the combined effects of chloride cell enlargement (Figure 3.2A) and a rise in cell number (Figure 1B). Based on these results and those shown in Chapter 2, the

blood-to-water diffusion distance across the lamellar epithelium was almost certainly increased and the area of the inter-lamellar water channels decreased, while the lamellar surface area remained unchanged. The diffusion barrier thickness, water channel area and lamellar surface area were not measured in this study because the necessary morphometric analyses are extremely time-consuming, and because of the conclusive data presented by the previous study in Figure 2.1 of this thesis. However, by using the empirical correlation between CCFA and diffusion barrier thickness that was demonstrated previously [τ_h= (10.72 x CCFA) + 3.25; r^2 = 0.88], the barrier thickness probably increased from 3.7 to 5.4 μm in the present study.

Consequences on gas transfer - normoxia

The thickening of the blood-to-water diffusion barrier and restriction of inter-lamellar water channels associated with chloride cell proliferation would, in the absence of compensatory adjustments, be expected to impair gas transfer. An increase in the blood-to-water diffusion distance would cause a corresponding decrease in the diffusive conductance, while the reduced area of the inter-lamellar water channels could impede the flow of ventilatory water. Evidence to support the hypothesis that gas transfer is compromised by chloride cell proliferation was contributed by Thomas *et al.* (1988). These authors reported that, at a variety of environmental O_2 tensions, P_aO_2 values for fish naturally adapted to ion-poor water ([NaCl]= 0.10 mmol l^{-1}) were substantially lower than those of fish acclimated to a higher salt concentration ([NaCl] = 1.0 mmol l^{-1}). Although chloride cell morphology was not quantified, branchial chloride cells from the ion-deprived stock of fish appeared to be proliferated in comparison to the higher salt-acclimated group (Thomas *et al.*, 1988).

The results of the present study demonstrate conclusively that i) respiratory gas transfer is impaired by chloride cell proliferation, ii) O_2 and CO_2 transfer are affected differently, and iii) partial compensation is achieved through physiological adjustments.

CO_2 excretion was probably restricted in hormone-treated fish since these fish exhibited elevated P_aCO_2 levels (Figure 3.5) and a significant acidosis (Figure 3.6) in comparison to the control group. Similarly, it is likely that O_2 uptake was compromised by hormone treatment even though the P_aO_2 values of treated fish were significantly lower than control levels only at environmental O_2 tensions of 12.0 kPa or less (Figure 3.4). These findings support the notion of a compromise between ionic regulation and gas transfer as proposed by Thomas *et al.* (1988), but are in conflict with the morphological study of Laurent and Hebibi (1989). The results of the latter study demonstrated an increase in the morphological diffusing capacity of the trout gill after proliferation of chloride cells in fish kept in ion-poor water. At the present, I have no explanation for the discrepancy.

Physiological adjustments that could potentially compensate for the decrease in diffusive conductance and restriction of convective water flow caused by chloride cell proliferation were reviewed by Perry and McDonald (1993), and rely mainly on variations of the surface area for gas transfer or the diffusion gradient for gas movement. The increase in diffusion distance resulting from chloride cell proliferation could be counterbalanced by a corresponding increase in lamellar surface area. Although **morphological** modification of lamellar surface area has been eliminated (see Table 2.1), an increase in the **functional** surface area for gas exchange could be achieved through hyperventilation, causing the recruitment of normally unused water channels in the distal area of the filaments (Perry and McDonald, 1993). Thus, the elevated ventilation amplitude displayed by the hormone-injected fish (Figure 3.7) could have yielded a functional increase in the surface area for gas transfer, despite the reduced ventilation frequency in this group (Table 3.1). Additionally, the recruitment of normally unperfused distal lamellae by neurohumoral mechanisms or by increases in blood pressure also may have increased the functional lamellar surface area (Farrell *et al.*, 1979). It is unclear whether hyperventilation occurred in the hormone-treated fish

because of the opposite effects on ventilation amplitude (increase) and frequency (decrease).

Hyperventilation, if occurring, could improve gas transfer through its influence on the diffusion gradients for O_2 and CO_2. The mean diffusion gradient is a function of the inspired (P_IO_2 or P_ICO_2) and expired (P_EO_2 or P_ECO_2) O_2 or CO_2 tensions as well as the post-branchial (arterial; P_aO_2 or P_aCO_2) and pre-branchial (venous; P_vO_2 or P_vCO_2) tensions, as follows (Wood and Perry, 1985): $\Delta PO_2 = 0.5(P_IO_2 + P_EO_2) - 0.5(P_aO_2 + P_vO_2)$. Thus, the increased ventilation amplitude in treated fish should increase P_EO_2 and decrease P_ECO_2, resulting in both cases in an increase in the diffusion gradients. Owing to the greater solubility in water of CO_2 over O_2 (30-fold difference), however, the fish gill is normally hyperventilated with respect to CO_2 excretion and therefore hyperventilation has less impact on P_aCO_2 than P_aO_2 (Perry, 1986; Iwama *et al.*, 1987). This could conceivably explain why P_aO_2 was maintained at control levels in normoxic hormone-treated fish, while P_aCO_2, and hence pH_a, were not (Figures 3.4, 3.5 and 3.6). An alternate explanation for the different sensitivities of P_aO_2 and P_aCO_2 to changes in the diffusion distance is that under normoxic conditions, the trout gill is principally perfusion limited with respect to oxygen transfer (Daxboeck *et al.*, 1982; Randall and Daxboeck, 1984; Malte and Weber, 1985), but diffusion limited with respect to carbon dioxide transfer (Cameron and Polhemus, 1974; Malte and Weber, 1985). Thus branchial O_2 uptake does not appear to be limited by the thickness of the diffusion barrier during normoxia, when the water-to-blood PO_2 gradient is high, but is rendered diffusion limited during hypoxia owing to the reduction in the water-to-blood PO_2 gradient. Although the diffusivity of CO_2 across the gill is greater than O_2, the overall process of CO_2 excretion may be more sensitive to changes in the diffusion distance because of the requirement to dehydrate plasma HCO_3^- (see Piiper, 1989). The conversion of plasma HCO_3^- to CO_2 probably is limited by the slow velocity of red blood cell Cl^-/HCO_3^- exchange in relation to other steps in the pathway, including CO_2

diffusion and the catalysis of H_2CO_3 dehydration by red cell carbonic anhydrase (Perry, 1986; Perry and Gilmour, 1993; see also Piiper, 1989).

The diffusion gradients for O_2 and CO_2 are also determined by factors affecting the arterial and venous gas tensions. Increases in haematocrit, cardiac output or haemoglobin-O_2 (Hb-O_2) binding affinity will enhance O_2 uptake (Perry and McDonald, 1993); these factors were not investigated in the present study. It is likely, however, that the hypercapnia and acidosis experienced by the hormone-treated fish in this study would have an adverse impact on haemoglobin-O_2 binding affinity and capacity because of the large Bohr and Root shifts in rainbow trout (Cameron, 1971; Eddy, 1971).

Consequences on gas transfer - hypoxia

Lowering of the environmental O_2 tension elicits a set of characteristic responses in rainbow trout that are dependent upon the intensity of the hypoxia (see review by Thomas and Motais, 1990). Moderate hypoxia typically results in increases in ventilation frequency and amplitude accompanied by a hypocapnic alkalosis. These responses were observed in this study; ventilation amplitude (Figure 3.7) and frequency (Table 3.1) displayed increasing trends in both control and hormone-treated fish in the P_wO_2 range of 18.7 to 9.3 kPa. Correspondingly, P_aCO_2 decreased (Figure 3.5) and in control fish, pH_a tended to rise (Figure 3.6).

Exposure of rainbow trout to severe hypoxia is known to elicit the mobilisation of catecholamines (see review by Randall and Perry, 1992). These hormones initiate a series of integrated physiological responses that serve to optimise cardiovascular and respiratory functions (reviewed by Perry and Wood, 1989; Randall and Perry, 1992; Thomas and Perry, 1992). Stimulation of red blood cell (rbc) ß-adrenoceptors leads to the activation of a rbc membrane Na^+/H^+ antiporter which extrudes protons in exchange for plasma Na^+ (reviewed by Nikimma, 1992; Perry and McDonald, 1993); thus,

catecholamine release can be recognised *in vivo* by a sudden and marked decrease in pH_a (e.g. Fievet *et al.*, 1987). A recent model (Perry and Reid, 1992) proposed that catecholamines are released into the circulation when an arterial blood O_2 content corresponding to ~50-60% Hb-O_2 saturation is reached.

The pH_a drop characteristic of catecholamine release occurred at a much higher P_wO_2 in hormone-treated fish than in control fish (Figure 3.6); the difference was probably due to a combination of factors. First, hormone-treated fish were unable to maintain their P_aO_2 at control levels as the environmental O_2 tension was lowered (Figure 3.4). At P_wO_2 values of 12.0 kPa or less, the P_aO_2 of treated fish was significantly lower than that of the control group, and this would result in a corresponding reduction in Hb-O_2 saturation. Secondly, the right Bohr shift of the Hb-O_2 dissociation curve associated with the relative acidosis and hypercapnia in hormone-treated fish would lower the Hb-O_2 binding affinity and hence elevate the P_aO_2 at which 50-60% Hb-O_2 saturation was achieved. It is likely that the earlier catecholamine release in hormone-treated fish was responsible for the slight difference in the trace patterns of P_aCO_2 versus P_wO_2 for control and treated fish (Figure 3.5). The P_aCO_2 values for treated fish tended to increase during the period of deepest hypoxia, presumably as a result of the titration of plasma HCO_3^- by protons extruded from the rbc by the Na^+/H^+ antiporter. Consequently, the slope of the relationship of P_aCO_2 against P_wO_2 was lower for hormone-treated fish than for the control group, in which P_aCO_2 decreased throughout the hypoxic period.

Conclusions

The results of this study clearly demonstrated that the cortisol and ovine growth hormone induced proliferation of lamellar chloride cells has a negative impact on respiratory gas transfer. The effects of chloride cell proliferation on gas exchange are presumably due to a thickening of the blood-to-water diffusion barrier and a restriction of

ventilatory water flow through the diminished inter-lamellar areas. While secondary compensatory adjustments, such as an increased ventilation amplitude, apparently were sufficient to preserve arterial O_2 tensions at control values during normoxic environmental conditions, CO_2 excretion was compromised at all P_wO_2 levels. Further, hormone-treated fish were unable to maintain control P_aO_2 values as hypoxia was imposed, and released catecholamines (as indicated by the abrupt reduction of pH_a) at substantially higher P_wO_2 levels than control fish. The results of this study imply that morphological modifications to preserve ionic and/or osmotic homeostasis may have deleterious consequences for respiratory homeostasis.

CHAPTER 4

GENERAL DISCUSSION

THE INTERRELATIONSHIP BETWEEN IONOREGULATORY AND RESPIRATORY HOMEOSTASIS

The purpose of this research was to test the hypothesis that morphological changes resulting from an increased chloride cell prevalence on the gill would adversely affect gas exchange at the lamellae. Specifically, the prediction was that an increase in chloride cell number and fractional area would impair gas transfer, primarily as a result of an increase in the mean lamellar water-to-blood diffusion distance. Cortisol and growth hormone were used as tools to simulate the lamellar morphological state during periods of intense ionoregulation. The data presented in Chapters 2 and 3 have provided some initial insight into some of the possible physiological compromises that occur when fish experience periods of ionic imbalance and hence intensive ionoregulatory activity. The use of cortisol and ovine growth hormone at pharmacological levels to induce the proliferation of chloride cells also allowed a greater understanding of the effect of this now common laboratory technique on lamellar and filamental morphology.

Morphological studies

The majority of the morphological and morphometric predictions presented in Chapter 1 were correct. Scanning electron microscopy confirmed the prediction that, in addition to their positive effects on Na^+/K^+-ATPase levels (Madsen, 1990a,c,d), both cortisol and growth hormone increased chloride cell surface parameters. Additionally, the results confirmed a synergistic effect of the two hormones.

The transmission electron microscopic studies showed that the proliferation of apically exposed chloride cells increased the water-to-blood barrier thickness of the lamellae. Treatment with cortisol and ovine growth hormone alone produced significantly increased barrier thicknesses over untreated fish. The combined treatment with cortisol and growth hormone produced a doubling in the mean barrier

thickness. In terms of the variables associated with Fick's Law discussed in Chapter 1, the TEM results presented in Chapter 2 showed an increase in the diffusion barrier variable (D). It can be predicted that without any other compensatory adjustments this alone would lead to a decrease in O_2 and CO_2 diffusion across the lamellae. The simplest compensatory morphological adjustment, namely an increase in lamellar surface area, did not occur. This is antithetic to the prediction made in the introduction to this thesis and contrary to the conclusions made by Laurent and Hebibi (1989). Thus, unless the chloride cells themselves altered the Krogh's constant to increase the diffusibility of gases, it would be predicted that there would be an overall decrease in O_2 uptake and CO_2 excretion rates.

Physiological studies

An increased prevalence of chloride cells on the gill significantly affected both the ionoregulatory and respiratory functions. While not an important aspect of this thesis, it is important to note the ion uptake results which helped confirm that the additional chloride cells induced by the hormone treatments were physiologically active.

The main focus of this thesis was to describe changes that occurred in respiratory parameters following the induced morphological changes. According to Thomas *et al.* (1988), the ionic condition of the water is an important determinant in the ability of the gills to transfer gas and hence to resist hypoxic exposure. This is due mainly to the morphological variation that occurs with altered ion content. The results presented in Chapter 3 of this thesis do not refute this hypothesis. In comparison to untreated fish, hormone treated fish which experienced chloride cell proliferation exhibited significant alterations to gas transfer, namely a heightened PCO_2 and lowered PO_2, and alterations to arterial pH under both normoxic and hypoxic conditions. At high P_wO_2, plasma oxygen levels (P_aO_2) were maintained. The ability

to compensate for the increased barrier thickness was, however, lost during the subsequent exposure to hypoxia. These results are similar to those of Thomas *et al.* (1988), with the exception that the present study did not see a significant difference in P_aO_2 at normoxic levels. The present study suggests that there was insufficient lamellar recruitment (i.e. an increase in functional surface area) to overcome the effects of the morphological changes. Additionally, there were no obvious compensatory morphological variations such as an increase in anatomical surface area. In terms of the effect of chloride cell proliferation on PCO_2, the results confirmed the predictions made for both normoxia and hypoxia, namely there were significant increases in plasma PCO_2 levels. It is believed that this CO_2 retention was the primary reason for the associated decrease in pH_a, which became increasingly more significant during both mild and deep hypoxia. The acidosis witnessed was consistent with the results of Thomas *et al.* (1988). Although no direct evidence is presented to support this, it is plausible that, in part, the drop in pH could have resulted from catecholamine release leading to red blood cell Na^+/H^+ exchange and which would further acidify the blood (Thomas and Perry, 1992). The severity of the increased water-to-blood barrier distance was also evident upon examining the ventilatory data which showed that the changes in both frequency and strength of ventilation were insufficient to maintain blood gas homeostasis.

FUTURE DIRECTIONS

It was the intention of this thesis to relate chloride cell proliferation and respiratory function. The initial results present a basis for which further studies can more fully examine the relationships among the multiple physiological functions of the gill. Future experiments should attempt to broaden the general conclusion of this thesis that chloride cell proliferation impairs gas transfer. In particular, the following areas could be examined:

(a) **Physiological function** - the idea of how chloride cell proliferation would affect both respiratory and other physiological functions should continue to be studied. A central theme is whether increased prevalence is an overall detriment to physiological function (i.e. are fish exposed to irregular water conditions affected by the lack of oxygen more than other possible physiological changes such as acid-base disturbances?). Using the results of the respiratory studies in Chapter 3, further examination into how respiratory function is adjusted as a result of situations where an increased oxygen demand is required (i.e. such as during exercise) would help provide some additional insight into how respiratory gas transfer is affected. In addition, a comparison of acute versus chronic thickening of the gill should be examined to determine whether long term exposure to conditions leading to increased chloride cell proliferation affects long term physiological function (i.e growth and development). Possible further experiments could be performed to study the effect of chloride cell proliferation on other physiological parameters such as acid-base balance, gill metabolism, and catecholamine release (i.e. are the pH_a drops reported in Chapter 3 due to an earlier than normal catecholamine release into the blood when compared to P_wO_2 values?).

(b) **Morphological component** - continued morphological studies, with the overall goal being to examine the gill tissue itself, so as to describe detailed alterations within it. For example, the initial studies presented in Chapter 2 of this thesis led to a prediction that gas transfer should be halved, owing to the doubling of the water-to-blood gas diffusion barrier without any significant change in the anatomical surface area. The differences may lie in one of two areas, either (a) an undetected increase in the functional surface area, or (b) a dramatic change in the third variable component of Fick's Law, Krogh's constant of diffusion. A change in the K_x value could occur as a consequence or changes to membrane or cytoplasm composition of the gill cells (for example, owing to the abundance of chloride cells which should be described).

Additionally, an examination into other possible morphological variations at the gill, such as what happens to associated cell structures (i.e. gap junctions which are involved in ion movement), and to examine if the changes seen in one lamellae can be correlated to the gill as a whole (or are there adjustments in certain areas alone?).

(c) **Environmental concerns** - a major focus of future studies could be to see if the morphological and physiological conditions induced by hormones in the laboratory are actually seen when fish are exposed to simulated ionic challenges. Such studies should include the examination of fish exposed to a wide variety of water conditions (ion rich/poor and/or acidic/alkaline water, pollutants). Further, several unpublished observations appear to indicate a high degree of chloride cell proliferation in both natural lakes and fish hatcheries/farms. Using similar methods as were performed in this study, an investigation into possible compensatory adjustments made by these fish and, into their respiratory status, could also be done.

In conclusion, the results of the present study do not refute the original hypothesis that chloride cell proliferation would adversely effect the respiratory function of the gill. As an end to this thesis, and as a guide for future work, I would propose that the hypothesis be re-worded to state that: during periods of either intense ionoregulatory or gas transfer challenges, compensatory adjustments made are a compromise between these two required functions and, specifically, that gas transfer is affected during periods of increased ion flux.

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