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

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Summary

The objectives of this study were to induce chloride cell (CC) proliferation on the gill lamellae of rainbow trout *Oncorhynchus mykiss* and to evaluate the consequences for respiratory function. Chronic elevation of hormone levels was used to induce CC proliferation; fish were injected with a combination of cortisol ($8\,mg\,kg^{-1}$ intramuscularly every day for 10 days) and ovine growth hormone ($2\,mg\,kg^{-1}$ intraperitoneally every second day for 10 days). The extent of CC proliferation was quantified using scanning electron microscopy and a two-dimensional analysis. An extracorporeal preparation in combination with environmental hypoxia was used to assess the effects of CC proliferation on respiratory function. Arterial blood was routed from the coeliac artery through an external circuit in which pH (pHa), partial pressure of oxygen (P_{aO_2}) and partial pressure of carbon dioxide (P_{aCO_2}) were monitored continuously. Environmental hypoxia was imposed by gassing a water equilibration column supplying the experimental chamber with N_2.

The hormone treatment increased the average CC surface area by 2.7-fold and CC density by 2.2-fold; the combined effect was a fivefold increase in CC 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 $P_{wO_2}\leqslant 12.0\,kPa$. In comparison with 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 CC proliferation on blood gas variables in hormone-treated fish were accompanied by a significantly elevated ventilation amplitude and a lowered ventilation frequency.

The results of this study demonstrated (i) that impairment of respiratory gas transfer coincides with CC proliferation, (ii) that O_2 and CO_2 transfer are influenced differently and (iii) that partial compensation is achieved through physiological adjustments.

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Key words: rainbow trout, *Oncorhynchus mykiss*, gill, chloride cell, lamella, diffusion, cortisol, growth hormone, gas transfer, oxygen, carbon dioxide.

Introduction

The maintenance of osmotic, ionic and respiratory homeostases are important functions of the teleost fish gill and are contingent, at least in part, on morphological adjustments of the gill (Laurent and Perry, 1991). One of the most often studied and physiologically significant of such morphological adjustments involves variations in the number and/or size of the presumptive ion-regulating cell, the chloride cell (CC) (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 Na^+ and Cl^- from the dilute external medium (Laurent, 1984). Consequently, proliferation of branchial CCs (Laurent *et al.* 1985; Avella *et al.* 1987; Perry and Laurent, 1989) is utilized by fish as a mechanism for enhancing the ion-transporting capacity of the gills (Perry and Laurent, 1989, 1993; Laurent and Perry, 1991) in challenging environments such as ion-poor (soft) water.

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

It was recently demonstrated (Bindon *et al.* 1994) that CC proliferation, induced by chronic treatment of trout with cortisol and ovine growth hormone (Madsen, 1990*b*), 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 CC proliferation and the accompanying increase in the blood-to-water diffusion distance. Surprisingly few studies, however, have evaluated the relationship between branchial CC 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 than did 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 CCs, 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 not measured.

The goal of the present study was to induce CC proliferation on the gill lamellae of rainbow trout and to assess the consequences for respiratory function. CC proliferation was induced through chronic hormone elevation by injecting fish with cortisol and ovine growth hormone (Madsen, 1990*b*); this protocol has previously been shown to cause a thickening of the blood-to-water diffusion barrier (Bindon *et al.* 1994). Respiratory function was evaluated by continuously monitoring arterial blood P_{O_2}, P_{CO_2} and pH, under normoxic and hypoxic conditions, using an extracorporeal arterial blood circulation.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of either sex and of mean mass 893±198 g (S.E.M.) were obtained from a local supplier (Linwood Acres Trout Farm, Campbellcroft, Ontario) and transported in oxygenated water to the University of Ottawa fish facility. Fish were transferred to, and maintained in, large fibreglass aquaria (Living Stream, Dayton, Ohio) and supplied with continuously flowing City of Ottawa dechlorinated tapwater ($[Na^+]$=0.12 mmol l^{-1}; $[Cl^-]$=0.15 mmol l^{-1}; $[Ca^{2+}]$= 0.45 mmol l^{-1}; pH=7.5–7.7; temperature range 10–12 °C). Fish were acclimated for at least 4 weeks prior to experimentation. The 12 h:12 h photoperiod was kept constant over the course of the acclimation and experimental periods. Fish were fed to satiation three times weekly using a commercial trout food (Martin); feeding was discontinued 24 h prior to experimentation.

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 (CC) (Bindon *et al.* 1994). 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 kg^{-1} of ovine growth hormone; NIH-NIADDK, Bethesda, Maryland) every second day over the 10 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 neutralized solution of 0.1 % MS-222 and placed onto an operating table, where the gills were continuously irrigated with oxygenated anaesthetic solution. An indwelling catheter was implanted into the dorsal aorta (Soivio *et al.* 1975) using flexible polyethylene tubing (PE 50, Clay-Adams, i.d. 0.580 mm, o.d. 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; i.d. 1.14 mm; o.d. 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 00 gauge surgical silk. Fish were revived on the operating table with oxygenated water and then transferred to the experimental apparatus. All cannulae were flushed with heparinized (50 i.u. ml^{-1}) saline to prevent clotting. Fish were allowed to recover for 24 h prior to experimentation.

Experimental apparatus

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 arrangement allowed continuous 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 was initiated by connecting the two coeliac cannulae in series with three measuring cells containing P_{O_2}, P_{CO_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 maintained them 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 i.u. ml^{-1}) prior to starting the flow of blood. This arrangement allowed continuous monitoring of arterial P_{O_2} (Pa_{O_2}), P_{CO_2} (Pa_{CO_2}) and pH (pHa) over the course of the experiment.

Pa_{O_2} and Pa_{CO_2} were measured using Radiometer P_{O_2} and P_{CO_2} electrodes (models E-5046 and E-5036, respectively) 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 P_{O_2} and P_{CO_2}) or commercially supplied buffer solutions (for pH).

P_{O_2} of the water (Pw_{O_2}) was continuously measured using a second Radiometer P_{O_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 digitized using a commercial analog–digital interface (Data Translation Incorporated). Digital output was fed to a computer utilizing customized data acquisition software (AD-DATA; P. Thoren, Göteborg, Sweden). Mean values for each variable were captured and stored at 5 s intervals.

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 stabilize and to obtain control (pre-hypoxia) readings; Pw_{O_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 $P_{W_{O_2}}$. The rate of $P_{W_{O_2}}$ decrease was about 0.80 kPa min^{-1}; thus, hypoxia was imposed over a 20 min period. During this stage, $P_{W_{O_2}}$ 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

CC 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 by a blow to the head and a sample consisting of several pairs of filaments joined at the septum was removed from the second gill arch on the left side. Under a stereo microscope, several pairs of filaments were separated from the gill arch tissue. Tissues were kept submerged in buffer (see below) to prevent dehydration and then incubated in a gluteraldehyde-based fixative (5 % in 0.15 mol l^{-1} sodium cacodylate buffer, pH 7.4) for 1 h at 4 °C. Following this primary fixation, the tissue was rinsed in cacodylate buffer and post-fixed in 1 % osmium tetroxide. The filament pairs were rinsed again to remove any fixative residue and were subsequently dehydrated in a graded ethanol series (40, 50, 60, 70, 85, 95 and 2×100 %) followed by consecutive 2 min baths in 1,1,1,3,3,3-hexylmethyldisilizan (Aldrich). Tissue was then allowed to dry in air overnight. Dried filament pairs were mounted using silver paint (EMS, Fort Washington, PA) on SEM specimen stubs suitable for a Phillips 500 SEM. The filament pairs were oriented so that the area of interest, namely the inner (trailing) edge of the filament close to the septum, was parallel with the stub plate.

The SEM was focused on the inner edge where the filament meets the lamellae on both the anterior and posterior filaments. Five, randomly selected pictures per fish were taken (at a magnification of 1000×) for analysis.

Three morphological CC variables were evaluated using the SEM photographs: CC fractional area (CCFA), individual apical surface area and CC density. Variables were assessed by using a digitizer tablet (Jandel Scientific) attached to a computer fitted with commercial morphometry software (Sigmascan) to trace the outer edges of all visible CCs in a picture. For the determination of mean cell area, only whole cells were measured, while all cells (complete and partial) were used for the determination of CCFA and CC density. CCFA and density were calculated as follows:

$$\text{CCFA} = \frac{\sum \text{area of whole cells}}{10^6 \times \text{picture area}},$$

$$\text{Cell density} = \text{CCFA}/\text{average whole-cell area}.$$

Data and statistical analysis

Data are presented as means ±1 standard error of the mean (S.E.M.). For the extracorporeal experiments, statistical analysis was performed at the normoxic $P_{W_{O_2}}$ of 21.3 kPa and subsequently at every decrease of 1.3 kPa to the final $P_{W_{O_2}}$ of 5.3 kPa. Extracorporeal experiments were analyzed using two-way analysis of variance (ANOVA) with $P_{W_{O_2}}$ and treatment as factors. In cases where significant differences

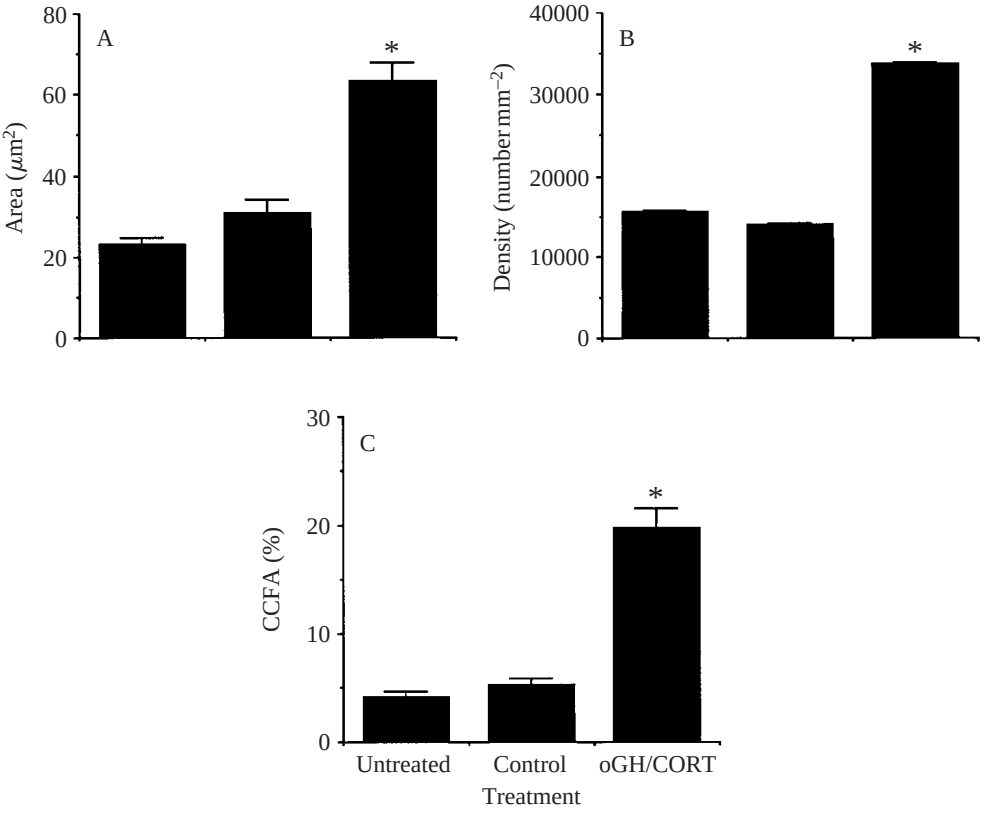

Fig. 1. Effects of ovine growth hormone and cortisol (oGH/CORT) and control (saline vehicle) treatments on surface morphometric variables. (A) individual chloride cell (CC) surface area, (B) chloride cell density and (C) chloride cell fractional area (CCFA). For each group, N=6. An asterisk indicates a value significantly different from those of both the untreated and control groups (P<0.05). Error bars indicate +1 S.E.M.

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 Fig. 1. There were no significant differences between untreated and control fish for any of the variables examined (cell density, average chloride cell area or CCFA). However, there were significant differences between the untreated and control groups and the hormone-treated group. Average CC surface area was increased approximately 2.7-fold (from 23.0±1.7 μm^2 in the untreated fish to 63.2±4.6 μm^2 in the hormone-treated fish; Fig. 1A), whereas CC density increased

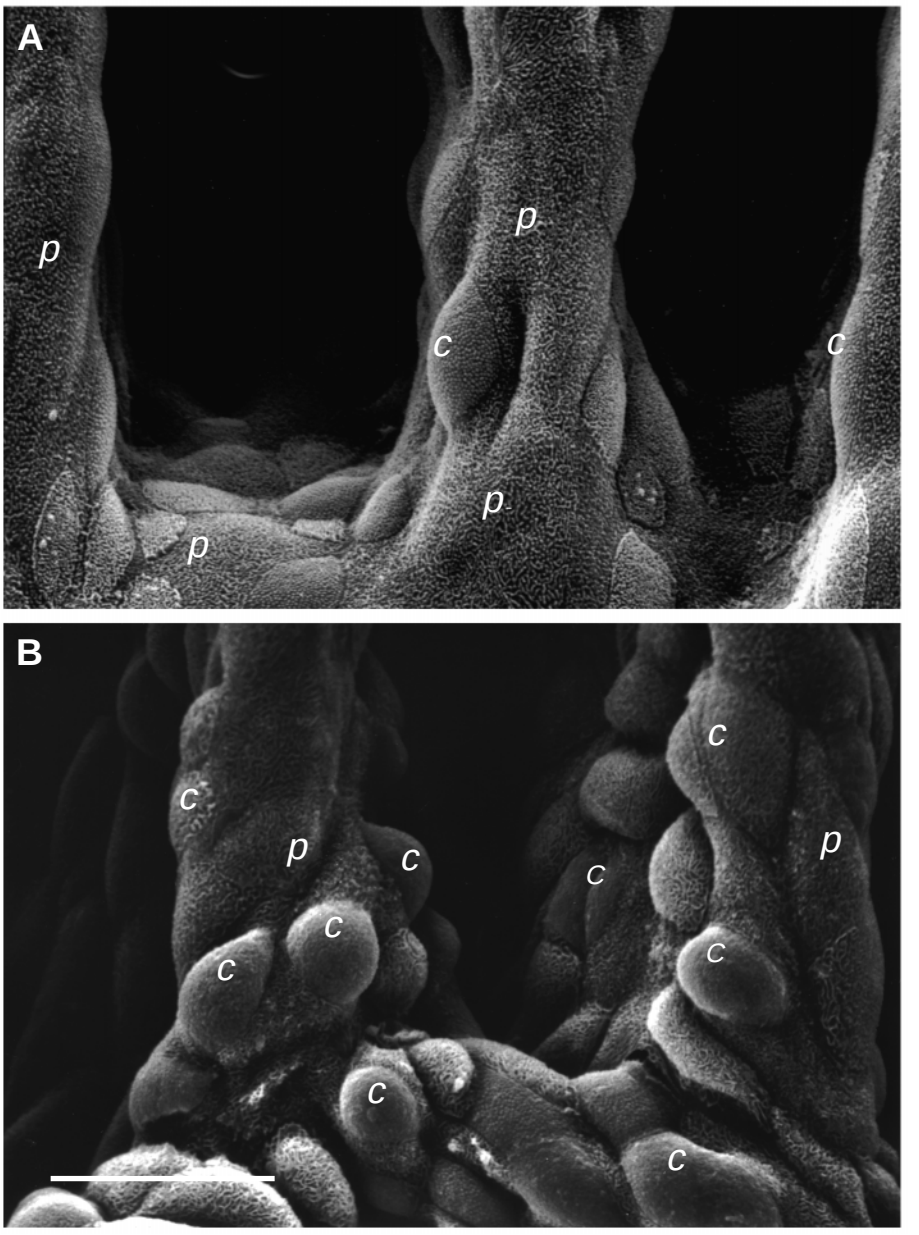

Fig. 2. Representative scanning electron micrographs of trout gill filaments and lamellae from (A) control fish and (B) from fish treated with a combined ovine growth hormone and cortisol regimen. *c*, chloride cell; *p*, pavement cell. Scale bar, 10 μm.

roughly 2.2-fold (from 1559±101 cells mm^{-2} to 3372±252 cells mm^{-2}; Fig. 1B). As a result, the CC fractional area was elevated approximately fivefold (from 4.10±0.75 % to 19.96±2.24 %; Fig. 1C) by the hormone injections.

Fig. 2 shows representative SEM micrographs of gills from control and hormone-treated

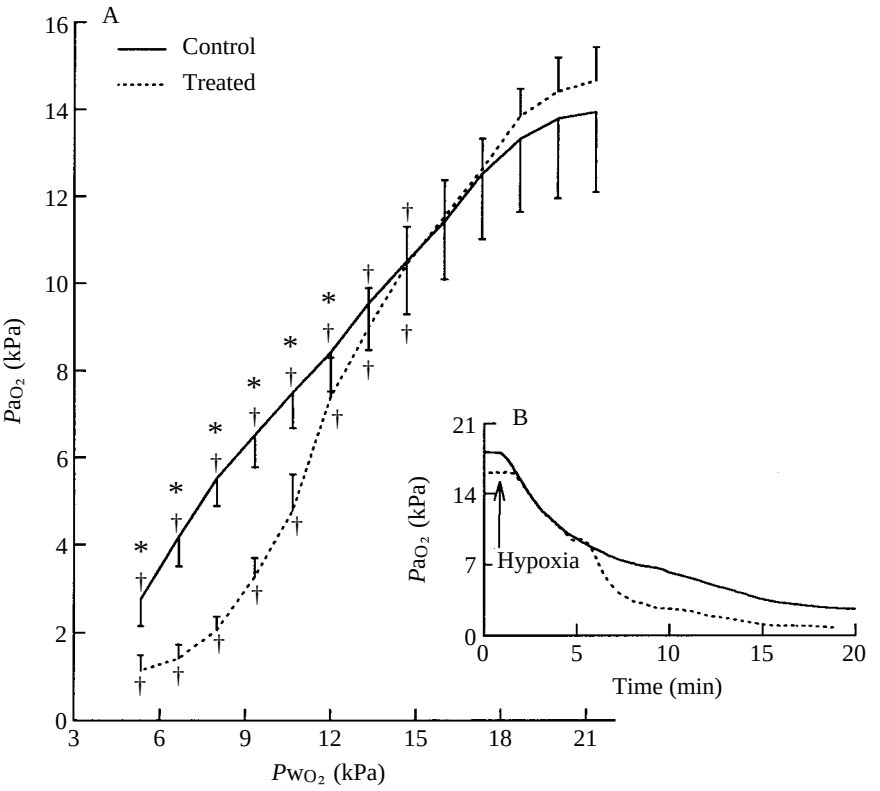

Fig. 3. (A) Effect of environmental hypoxia on arterial oxygen partial pressure (P_{aO_2}) in control (solid line) fish and those treated with a combined ovine growth hormone and cortisol regimen (dashed line). An asterisk indicates a significant difference between treatment groups; a dagger indicates a P_{aO_2} level significantly different from the normoxia value (at a P_{wO_2} of 21.3 kPa) ($P<0.05$). Error bars indicate ± 1 S.E.M. (B) A representative recording of P_{aO_2} for control and treated fish during the period over which hypoxia was induced.

fish. Note, in particular, the differences in CC number and size and, additionally, in the location of CCs between the two groups. Lamellar CCs were larger and more prominent in the treated fish (see Fig. 2B) and clearly coincided with 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. Therefore, 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 Fig. 3. Both groups experienced significant decreases in P_{aO_2} at all levels of $P_{wO_2} \leqslant 14.7$ kPa. Under normoxic conditions and mild hypoxia (i.e. $P_{wO_2} > 12.0$ kPa), P_{aO_2} was not affected by hormone treatment, but at $P_{wO_2} \leqslant 12.0$ 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 Fig. 3B.

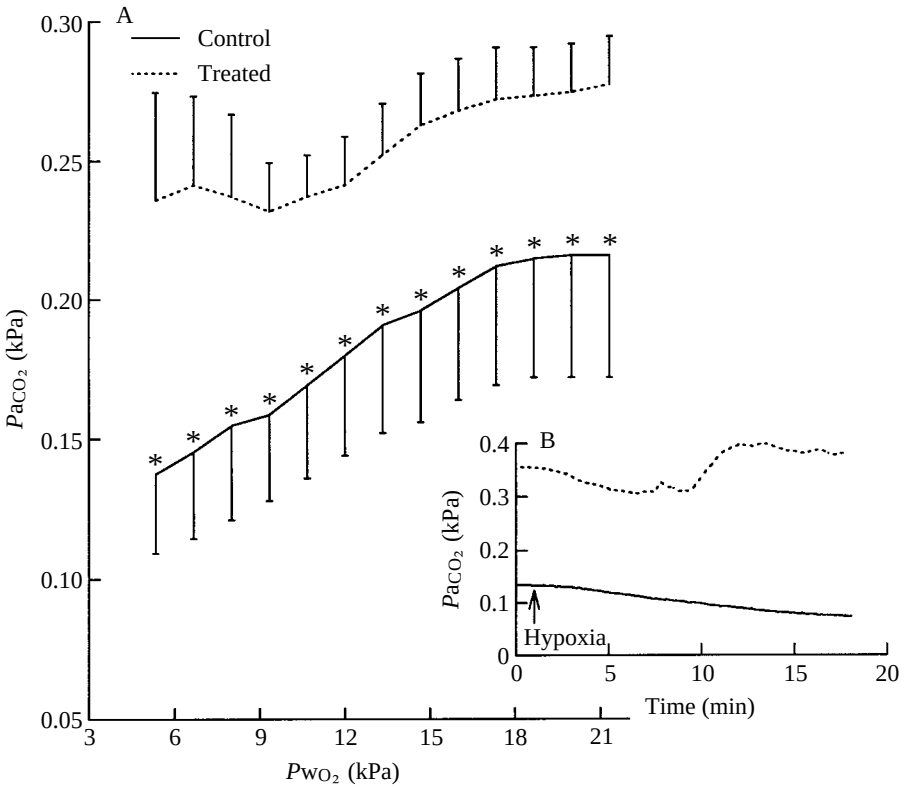

Fig. 4. (A) Effect of environmental hypoxia on arterial carbon dioxide partial pressure (P_{aCO_2}) in control fish (solid line) and those treated with a combined ovine growth hormone and cortisol regimen (dashed line). An asterisk indicates a significant difference between treatment groups. Regression 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. Error bars indicate ±1 S.E.M. (B) A representative recording of P_{aCO_2} for control and treated fish during the period over which hypoxia was imposed.

Despite the clear tendency in both control and treated fish for P_{aCO_2} to decrease during the acute hypoxia (Fig. 4), the two-way ANOVA indicated that the changes were not significant, probably because of 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). Hormone-treated fish had significantly higher P_{aCO_2} values than control fish at all P_{wO_2} levels (Fig. 4A). Fig. 4B 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 (pHa; Fig. 5); 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 pHa. This drop in pHa, which was generally preceded by a brief period (approximately 5 s) of intense struggling by the fish,

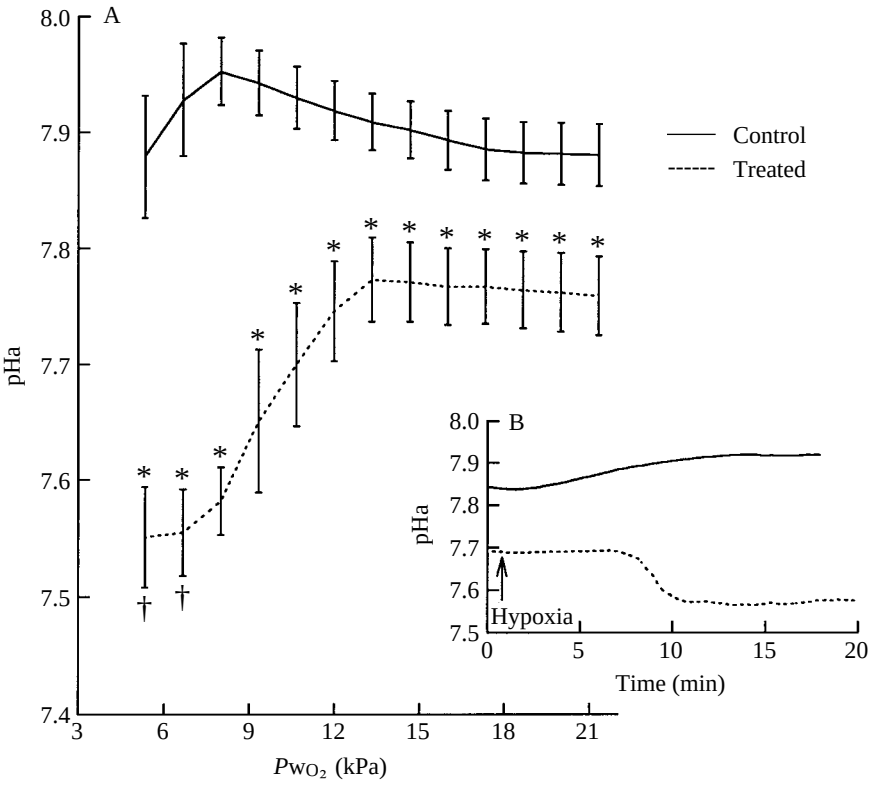

Fig. 5. (A) Effect of environmental hypoxia on arterial pH (pHa) in control (solid line) fish and those treated with a combined ovine growth hormone and cortisol regimen (dashed line). An asterisk indicates a significant difference between treatment groups; a dagger indicates a pHa value significantly different from the normoxia value (at a P_{WO_2} value of 21.3 kPa) ($P<0.05$). Error bars indicate ±1 S.E.M. (B) A representative recording of pHa values for control and treated fish during the period over which hypoxia was imposed.

occurred at higher P_{WO_2} levels (approximately 13 kPa) and to a greater extent in hormone-treated fish and near the end of the hypoxic period (approximately 8 kPa) in control fish. Representative pHa recordings are presented in Fig. 5B.

The effects of environmental hypoxia on ventilation amplitude are illustrated in Fig. 6. 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 1). Both groups displayed a significant increase in ventilation amplitude under severely hypoxic conditions (Fig. 6A); representative recordings of ventilation amplitude for both a control and a hormone-treated fish are shown in Fig. 6B. 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 1).

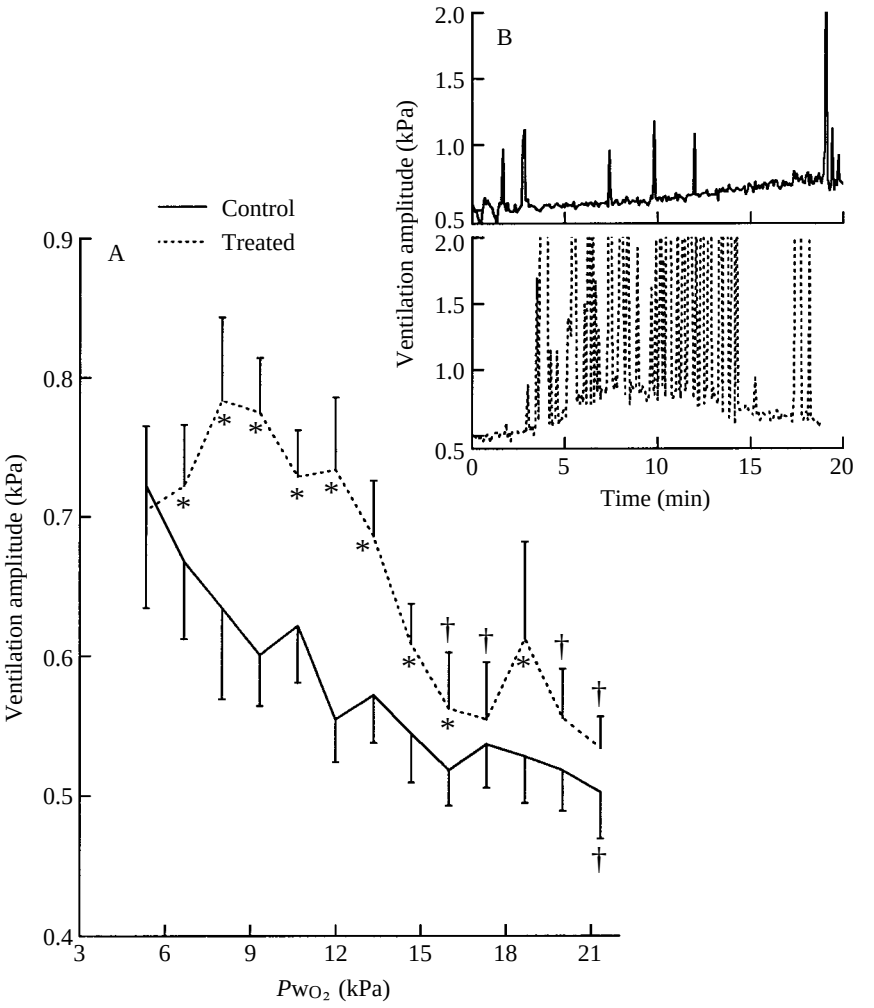

Fig. 6. (A) Effect of environmental hypoxia on mean ventilation amplitude in control fish (solid line) and those treated with a combination of ovine growth hormone and cortisol (dashed line). An asterisk indicates a significant difference between treatment groups. A dagger indicates a significant difference from severely hypoxic values (5.3–8.0 kPa) (P<0.05). Error bars indicate ±1 S.E.M. (B) Representative recordings for each group during the period over which hypoxia was imposed. The pressure spikes visible in these traces were caused by coughs, head movements and struggles.

Discussion

Chloride cell morphology

The objective of the present study was to analyze the consequences of lamellar chloride cell (CC) proliferation on the respiratory function of rainbow trout. CC 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,

Table 1. *Effect of environmental hypoxia on ventilation frequency in control fish and those treated with a combination of ovine growth hormone and cortisol*

$P_{W_{O_2}}$ (kPa)	Control frequency (min^{-1})	Treated frequency (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*

Values are means ± 1 S.E.M. An asterisk indicates a significant difference between treatment groups ($P<0.05$).

1990*a*,*b*; Perry *et al.* 1992*a*,*b*) and growth hormone (Madsen, 1990*b*; Bindon *et al.* 1994) are independently effective in increasing both the size and/or number of lamellar CCs. The combined use of these hormones has an additive effect (Madsen, 1990*b*; Bindon *et al.* 1994) and was therefore selected for the present study. Further, Bindon *et al.* (1994) clearly demonstrated that the CC proliferation elicited by this combined hormone treatment produced an approximate twofold increase in the thickness of the lamellar blood-to-water diffusion barrier without a concomitant and compensatory increase in lamellar surface area.

CC 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 by Bindon *et al.* (1994). Although the morphology of filament rather than lamellar epithelial CCs was quantified, owing to the constraints of the SEM methods employed, increases in CC surface area on the filament have previously been shown to be paralleled by similar alterations on the lamella (Laurent and Perry, 1990; Bindon *et al.* 1994; see also Fig. 2). In agreement with prior studies (Doyle and Epstein, 1972; Perry and Walsh, 1989; Laurent and Perry, 1990; Madsen, 1990*a*,*b*; Perry *et al.* 1992*a*,*b*), CC fractional area (CCFA) was significantly elevated by treatment with cortisol and ovine growth hormone (Fig. 1C), and the increase in CCFA was achieved by the combined effects of CC enlargement (Fig. 1A) and a rise in cell number (Fig. 1B). On the basis of these results and the study of Bindon *et al.* (1994), 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 Bindon *et al.* (1994). However, by using the empirical correlation between CCFA and diffusion barrier thickness that has been demonstrated previously (blood-to-water diffusion barrier thickness = 10.72CCFA + 3.25; r^2=0.88), we calculate that the barrier thickness probably increased from 3.7 to 5.4 μm in the present study.

Consequences for gas transfer

Normoxia

The thickening of the blood-to-water diffusion barrier and restriction of inter-lamellar water channels associated with CC 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 CC proliferation was contributed by Thomas *et al.* (1988). These authors reported that, at a variety of environmental O_2 tensions, $P_{a_{O_2}}$ 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 CC morphology was not quantified, branchial CCs for the ion-deprived fish appeared to be more numerous than in the group acclimated to a higher salt concentration (Thomas *et al.* 1988).

The results of the present study demonstrate conclusively (i) that respiratory gas transfer is impaired by CC proliferation, (ii) that O_2 and CO_2 transfer are affected differently, and (iii) that partial compensation is achieved through physiological adjustments. CO_2 excretion was probably restricted in hormone-treated fish since these fish exhibited elevated $P_{a_{CO_2}}$ levels (Fig. 4) and a significant acidosis (Fig. 5) in comparison with the control group. Similarly, it is likely that O_2 uptake was compromised by hormone treatment even though the $P_{a_{O_2}}$ values of treated fish were significantly lower than control levels only at environmental O_2 tensions of 12.0 kPa or less (Fig. 3). These findings support the hypothesis 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 CCs in fish kept in ion-poor water. We have no explanation for the discrepancy.

Physiological adjustments that could compensate for the decrease in diffusive conductance and restriction of convective water flow caused by CC proliferation have been reviewed by Perry and McDonald (1993) and rely mainly on changes in the surface area for gas transfer or the diffusion gradient for gas movement. The increase in diffusion distance resulting from CC proliferation could be counterbalanced by a corresponding increase in lamellar surface area. Although *morphological* modification of lamellar surface area has been ruled out (Bindon *et al.* 1994), an increase in the *functional* surface area for gas exchange could be achieved through hyperventilation, which could recruit 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 (Fig. 6) could have yielded a functional increase in the surface area for gas transfer, despite the reduced ventilation frequency in this group (Table 1). Additionally, the recruitment of normally unperfused distal lamellae by neurohumoral mechanisms or by increases in blood pressure may also have increased the functional lamellar surface area (Farrell *et al.* 1979). It is unclear whether hyperventilation occurred in the hormone-treated fish because the treatment had opposite effects on ventilation amplitude (increase) and frequency (decrease).

Hyperventilation, if it occurs, could improve gas transfer through its influence on the diffusion gradients for O_2 and CO_2. The mean diffusion gradient (ΔP_{O_2}) is a function of the inspired ($P_{I_{O_2}}$ or $P_{I_{CO_2}}$) and expired ($P_{E_{O_2}}$ or $P_{E_{CO_2}}$) O_2 or CO_2 tensions as well as the post-branchial (arterial; $P_{a_{O_2}}$ or $P_{a_{CO_2}}$) and pre-branchial (venous; $P_{v_{O_2}}$ or $P_{v_{CO_2}}$) tensions, as follows (Wood and Perry, 1985):

$$\Delta P_{O_2} = 0.5(P_{I_{O_2}} + P_{E_{O_2}}) - 0.5(P_{a_{O_2}} + P_{v_{O_2}}) .$$

Thus, the increased ventilation amplitude in treated fish should increase $P_{E_{O_2}}$ and decrease $P_{E_{CO_2}}$, 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 so hyperventilation has less impact on $P_{a_{CO_2}}$ than on $P_{a_{O_2}}$ (Perry, 1986; Iwama *et al.* 1987). This could explain why $P_{a_{O_2}}$ was maintained at control levels in normoxic hormone-treated fish, while $P_{a_{CO_2}}$ and, hence, pHa were not (Figs 3, 4 and 5). An alternative explanation for the different sensitivities of $P_{a_{O_2}}$ and $P_{a_{CO_2}}$ 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 strongly 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 P_{O_2} gradient is high, but becomes diffusion-limited during hypoxia owing to the reduction in the water-to-blood P_{O_2} gradient. Although the diffusivity of CO_2 across the gill is greater than that of 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 is probably limited by the slow rate 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-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).

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 (Fig. 6) and frequency (Table 1) displayed increasing trends in both control and hormone-treated fish in the $P_{w_{O_2}}$ range

18.7–9.3 kPa. Correspondingly, $P_{a_{CO_2}}$ decreased (Fig. 4) and, in control fish, pHa tended to rise (Fig. 5).

Exposure of rainbow trout to severe hypoxia is known to elicit the mobilization of catecholamines (see review by Randall and Perry, 1992). These hormones initiate a series of integrated physiological responses that serve to optimize cardiovascular and respiratory functions (reviewed by Perry and Wood, 1989; Randall and Perry, 1992; Thomas and Perry, 1992). Stimulation of red blood cell (RBC) β-adrenoceptors leads to the activation of an RBC membrane Na^+/H^+ antiporter, which extrudes protons in exchange for plasma Na^+ (reviewed by Nikinmaa, 1992; Perry and McDonald, 1993); thus, catecholamine release can be recognized *in vivo* by a sudden and marked decrease in pHa (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 approximately 50–60 % Hb O_2-saturation is reached.

The pHa drop characteristic of catecholamine release occurred at a much higher $P_{w_{O_2}}$ in hormone-treated fish than in control fish (Fig. 5); the difference was probably due to a combination of factors. First, hormone-treated fish were unable to maintain their $P_{a_{O_2}}$ at control levels as the environmental O_2 tension was lowered (Fig. 3). At $P_{w_{O_2}}$ values of 12.0 kPa or less, the $P_{a_{O_2}}$ 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. Second, the rightward 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 $P_{a_{O_2}}$ at which 50–60 % Hb O_2-saturation was achieved. The $P_{a_{CO_2}}$ values for treated fish tended to increase at the lowest $P_{w_{O_2}}$ values, presumably as a result of the titration of plasma HCO_3^- by protons extruded from the RBCs by the Na^+/H^+ antiporter. Consequently, the slope of the relationship of $P_{a_{CO_2}}$ against $P_{w_{O_2}}$ was less steep for hormone-treated fish than for the control group, in which $P_{a_{CO_2}}$ decreased throughout the hypoxic period.

In conclusion, the results of this study clearly demonstrated that the cortisol and ovine growth hormone treatment induced proliferation of lamellar CCs and reduced respiratory gas transfer. The effects of CC 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, were apparently sufficient to preserve arterial O_2 tensions at control values during normoxic environmental conditions, CO_2 excretion was compromised at all $P_{w_{O_2}}$ levels. Further, hormone-treated fish were unable to maintain control $P_{a_{O_2}}$ values as hypoxia was imposed and released catecholamines (as indicated by the abrupt reduction of pHa) at substantially higher $P_{w_{O_2}}$ 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.

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