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Gill epithelial cells kinetics in a freshwater teleost, *Oncorhynchus mykiss* during adaptation to ion-poor water and hormonal treatments

Pierre Laurent, Suzanne Dunel-Erb, Claudine Chevalier and Jacques Lignon
Laboratoire de Morphologie Fonctionnelle des Adaptations Centre d'Ecologie et de Physiologie Energétiques, C.N.R.S., 23, Rue du Loess, 67037 Strasbourg Cedex, France

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Abstract

The aim of this work was to determine the kinetics of the dramatic development of the gill chloride cells (CCs) during adaptation of the salmonid *Oncorhynchus mykiss* to an ion-poor environment.

To monitor cell division, the incorporation in the mitotic cell DNA of bromo-deoxyuridine (BrdUrd) was visualized with a monoclonal antibody. The density of labelled nuclei was used as an index of cellular division (proliferation), concomitantly with morphometry of phenotypic changes monitored with SEM.

In the filament epithelium, a phase of CC differentiation occurred within 12h after the transfer, followed by a delayed phase of cell proliferation (48h). In the lamellar epithelium, the present study demonstrates the absence of cell proliferation after ion-poor water transfer. The conclusion is that proliferation (mitosis) is important in the primary filament whereas differentiation and migration (from the filament) is the main mechanism for the appearance of CCs on the secondary lamellae.

The present study suggests that cortisol promoted differentiation, but not division, of cells. CCs, presumably premature, were stained by anti-cortisol monoclonal antibody indicating the presence of cortisol. No mature CCs were stained.

Growth hormone (oGH, ratGH) increased the rate of cell division both in lamellar and filament epithelium.

Introduction

The surface of the teleost gill epithelia displays three main types of cells: the chloride cells (CCs), the pavement cells and the mucus cells (see review: Laurent 1984). The CCs numerical density and external exposition are well known to be dependent on physiological and environmental factors (see, for instance: Laurent and Hebibi 1989).

[^{3}H]thymidine autoradiography has been used to study the kinetics of CCs during adaptation of euryhaline fish to salt-water (Chrétien and Pisam 1986). More recently, morphology and morphometry studies have shown that CCs undergo wide variations in relation to the external conditions (see review: Laurent and Perry 1991; Perry and Laurent 1993). One of the most significant cases concerns the wild populations of *Salmo fario* inhabiting poorly mineralized streams (Laurent *et al.* 1985) and other ecologically similar systems (Laurent and Perry 1991). This is also the case when salmonids are experimentally transferred from natural ion-

Correspondence to: Dr P. Laurent, 18, Rue de la Scierie, F-67117 Ittenheim, France; Tel: 33 88 28 59 24; Fax: 33 88 69 08 13.

rich fresh water (Strasbourg tapwater, STW) and artificial hard fresh water to natural soft water and artificial ion-poor water (IPW) (Laurent and Dunel 1980; Laurent *et al.* 1985; Avella *et al.* 1987; Leino *et al.* 1987; Perry and Laurent 1989; Laurent and Perry 1991; Perry, Goss and Laurent 1992). From these studies, it has been concluded that when a teleost fish has to face an environment that lacks the major ions, Na^+ and Cl^-, the number and size of CC's, visible on the external epithelial surface, are considerably augmented in comparison with control fish. Moreover, the CCs which in ion-rich fresh water (Laurent *et al.* 1985) are located in the filament epithelium, dramatically "invade" the lamellae after transfer to IPW. In *Oncorhynchus mykiss* morphometrical analysis coupled with ion flux measurements have shown that an adaptational response to lowered external NaCl concentration, as for instance by exposure to artificial, IPW consists of an increase of the total apical surface area of branchial CC's exposed to the external environment. Influxes of Cl^- and Na^+ are significantly correlated with individual and fractional CC apical surface areas (Perry and Laurent 1989). Concomitantly, in these IPW-transferred fish, the plasma cortisol level rises several-fold and later returns slowly to the control value (Perry and Laurent 1989), an observation suggesting that these phenotypic changes could be cortisol-dependent.

The mechanism underlying these adaptational responses are unknown. Does the increased number of CC result from an acceleration of cell division, or from an increased rate of cellular differentiation of resident stem cells which finally migrate onto the gill surface? In other words, can one speak of CC proliferation in these adaptational responses? This question is particularly relevant in the case of lamellar epithelium (see above). In addition, are some hormonal factors implicated such as cortisol or growth hormone in these adaptational responses?

To address these questions, we have compared the rates of gill cells division in salmonid fish transferred from a "control" environment, represented by Strasbourg tap water (STW), to artificial, mimicking some natural soft waters.

Materials and methods

To assess the rate of cell division, labelling of the newly synthesized DNA is the most commonly used method particularly since the introduction of the immunological method according to Grantzer *et al.* (1975). This method utilizes a monoclonal antibody against bromo-deoxyuridine in order to monitor the pulse labelled cells. 5-bromo-2′-deoxyuridine (BrdUrd) is incorporated into DNA during its process of replication (S-phase) in place of endogenous thymidine. BrdUrd incorporation was increased by addition of 5-fluoro-2′ – deoxyuridine, an inhibitor of thymidilate synthetase, an enzyme which lowers endogenous thymidine (Ellwart and Dormer 1985). In our study, the density of labelled nuclei was used as an index of cellular division.

Section 1: Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex, but none in breeding condition, weighing between 90 and 150g, were obtained from a local fish farm and held indoors in large fibreglass tanks (500 l), supplied with flowing, aerated, dechlorinated Strasbourg tapwater (STW, composition in mequiv l^{-1}: Ca^{2+} 4.48; Na^+, 0.59; Cl^-, 0.68; SO_4^{2+}, 1.27; HCO_3^-, 4.24; pH, 8.3, temperature: 11–13°C, 12L:12D artificial photoperiod). Each control and experimental group held in similar conditions consisted of 5 individuals.

Section 2: Effect of IPW on cell proliferation

Adaptation to ion-poor water (IPW) was performed in lucite tanks containing 100l of well-aerated water renewed twice daily with a mixture of 9 volumes of deionized water (DW) provided by an ion-exchange column and 1 volume of STW. Batches of fish were transferred to IPW for different periods of time, from 2 to 72h. At the end of these periods, lightly anaesthetized fish (MS 222 Sigma, 0.5g 10,000ml^{-1} IPW or STW) were injected with a solution containing 3mg of 5-bromo-2′-

deoxyuridine (Sigma) plus 5-fluoro-2′-deoxyuridine (quantity not communicated by the supplier) in 1ml of saline. Injections were made slowly through the Cuvier's canal (*ductus Cuvieri*), to allow a direct access of the solution to the gill circulation. One hour after the injection, the fish were sacrificed by stunning and bleeding. Small pieces of gill filaments were processed for immunohistochemistry (Section 5). Identical procedure was used with the control fish. IPW was only replaced by STW without admixture of DW, in the 100l tanks.

Section 3: Effect of cortisol on cell proliferation

Two methods were used to administer cortisol:

i. Kinetics studies. Batches of fish acclimated to STW were injected with cortisol (hydrocortisone 21-phosphate, Sigma), 4mg 0.4ml saline kg^{-1} of fish, intramuscularly and left for 0, 6, 12 and 24h before injection of BrdUrd. Control fish were injected at the same time with 0.4ml kg^{-1} of saline alone. BrdUrd was injected as above (Section 2) and pieces of gill filaments were processed for BrdUrd immunohistochemistry (Section 5).

ii. Qualitative studies. A multi-injection method is often used to reinforce the effect of cortisol (Laurent and Perry 1990). In the present studies, two injections at 48h interval were made intramuscularly (4mg $0.4ml^{-1}$ saline kg^{-1} of fish). The fish were sacrificed on the day after, 1h after injection of the labelling agent.

Section 4: Effect of oGH on cell proliferation

The procedure was the same as for cortisol. Ovine-GH, supplied by NHPP (NIADDK-oGH-15), was injected intramuscularly at a concentration, 2mg $0.4ml^{-1}$ saline kg^{-1} fish.

Section 5: Fixation procedure for immunohistochemistry

Pieces of gill filaments (1 mm long) were immersed for 24h in sublimate Bouin-Hollande fixative (35% formaldehyde (4ml), picric acid (4g), copper acetate (2.5g), saturated solution of mercuric chloride, completed to 100ml with distilled water). After fixation, pieces were immersed for at least 12h in 70% ethanol followed by treatments with 1% iodine in 70% ethanol for 4h and 3.5% aqueous sodium disulphide for a few minutes. Finally pieces were dehydrated, embedded in paraffin and sectioned.

Section 6: Immunohistochemical procedure

The procedure for BrdUrd was similar to that proposed by the supplier of the anti-BrdUrd antibody (Amersham International plc, Amersham U.K. 1992).

The procedure for cortisol consisted of incubating deparaffinated sections in rabbit anti-cortisol-21-thyroglobulin antibody (Sigma, ref C8409), diluted 1:5 in PBS, 9 vol + NSS, 1 vol, overnight at 4°C according to a method described previously (Rahim *et al.* 1988).

The same procedure was used for oGH. Anti-ovine GH (NIDDK-anti-oGH, NIH, dilution: 1:10) or anti-rat GH antibodies (NIH, dilution: 1:10) were used.

Section 7: Cellular localization of cortisol in control and experimental conditions

Rainbow trout, referenced as STW-cortisol (Section 3), were held for 2 days in 100l of running aerated STW prior to daily IM injections of cortisol for 3 days. On day 4, the fish were sacrificed and pieces of gill were fixed and processed as described in Section 5. Rainbow trout, referenced as IPW-cortisol, were kept in IPW for the duration of the experiment, injected with cortisol (as above) and the pieces of gill processed as in Section 5. Cellular cortisol was finally visualized with the procedure

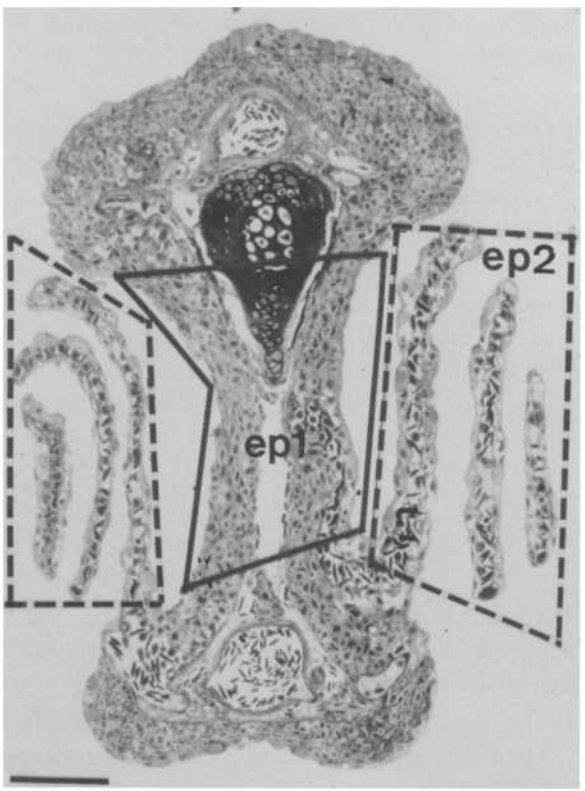

Fig. 1. Filament cross-section of trout gill showing the counting areas in the filament epithelium (ep1, solid line) and in the lamellar epithelium (ep2, dashed line). It is worth mentioning that the counts resulting from this method are expressed as density per cross-section (of filament or lamellae) and has no absolute value. In other words, this value is not related to constant tissular volume or epithelial surface area. Bar: 0.1 mm.

described in Section 7. To eliminate the possibility that immunostaining reflected only a cellular uptake, in some fish (referenced as IPW-saline), injection of cortisol was omitted or replaced by injection of saline. In this condition, only endogenous cortisol, of which the plasma concentration is known to increase during IPW transfer (Perry and Laurent 1989) was present in the gill cells.

Section 8: Definition of the cell proliferation loci

Two loci were selected among the possible sites of cellular proliferation according to the internal organization of the gill filament in salmonids (see Laurent 1984). These loci are delineated in Figure 1 and concern (a) filament epithelium (epi). This multilayered epithelium is the main location of the CCs. Cells of the innermost layer are poorly differentiated and some are presumably CCs (see review of Pisam and Rambourg 1991). A small number of mucus cells are also present in the part of the filament delineated on Figure 1, whereas most of them are located on the edges of the filament (upper and lower area of the filament section shown on Figure 1). The uppermost layer of this epithelium comprises the apical part of CCs and pavement cells; (b) lamellar epithelium (ep 2). This epithelium overlaps the lamellae. This epithelium is usually bilayered. The innermost layer is made of non-differentiated cells; the uppermost layer mostly comprises pavement cells, a variable number of CCs according to the environmental conditions and a few mucus cells.

Section 9: Determination of mitotic cell densities

Labelled nuclei (mitotic cells in S-phase) were separately counted in both loci, on 5μm thick histological sections taken at random. The portions of filament examined were situated near the interfilamental septum, according to the method described previously (Laurent and Hebibi 1989). Five fish from each group were examined; 10 filaments were counted for each fish. The mean numbers of mitotic cells were calculated per fish and per group of fish with their standard error (SEM). Statistical comparisons were made with one-way ANOVA test.

Results

Effect of acclimation to IPW

Figure 2 shows the number of labelled nuclei 6, 12, 24, 48 and 72h after transfer to IPW. Two days after the onset of the transfer, a significant increase of labelled nuclei was observed in the filament epithelium. Before and after 48h, the number of

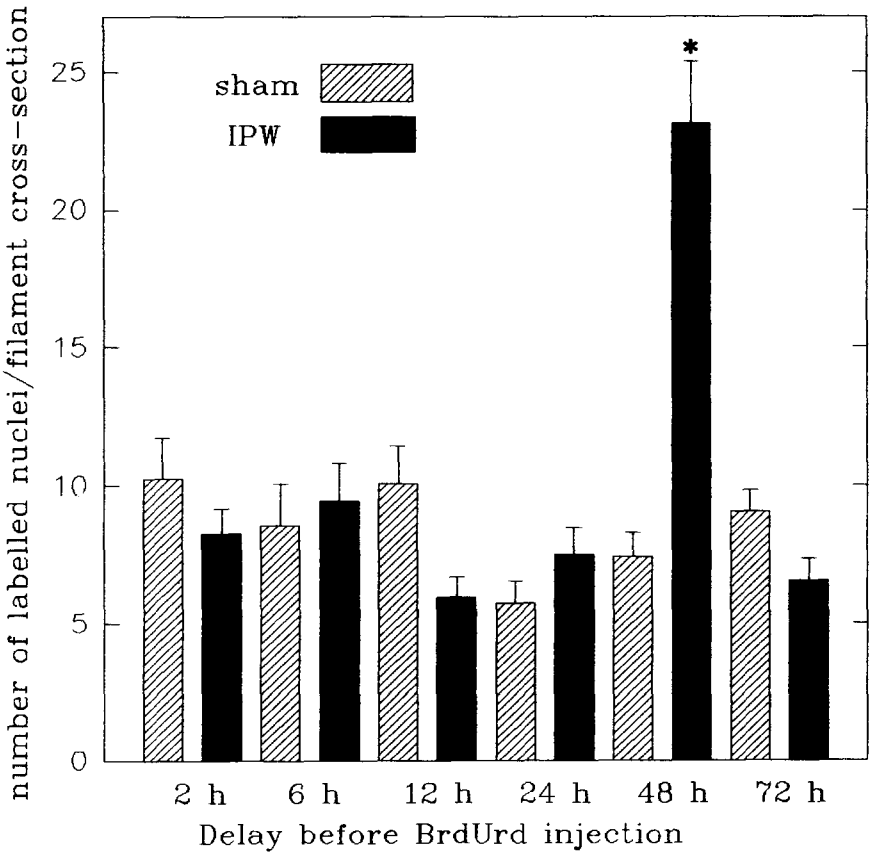

Fig. 2. Effect of the transfer to IPW on the number of labelled nuclei in the filament epithelium. Note a significant increase at 48h after the transfer.

labelled nuclei remained statistically constant and close to control values. The lamellar epithelium showed a contrasted and complex response but indicating the absence of labelling increase.

Figures 3a, b show representative cross-sections of the BrdUrd labelled, in control (a) and in 48h acclimated trout to IPW (b). The higher labellings were obtained in the filament epithelium of IPW acclimated trout and the lower in the lamellar epithelium. In the filament, the labelled nuclei belong to cells located in the innermost or intermediate epithelial layer. In the lamella the labelled nuclei belong to cells indifferently located in the inner, the outer layer and to the pillar cells.

SEM morphometry and IPW

The study of CCs with SEM morphometry on the filament epithelium showed that changes became significant 24h after transfer of trout to IPW. Three parameters were calculated (Fig. 4): i. the individual apical surface area of CCs, ii. the fractional surface area of CCs (per unit surface area of filament), iii. the density of CCs in contact with the external milieu. These parameters were significantly increased in IPW transferred trouts. After 72h, numerous CCs were visible on the surface of the lamellae (Fig. 5b) in contrast with control fish (Fig. 5a).

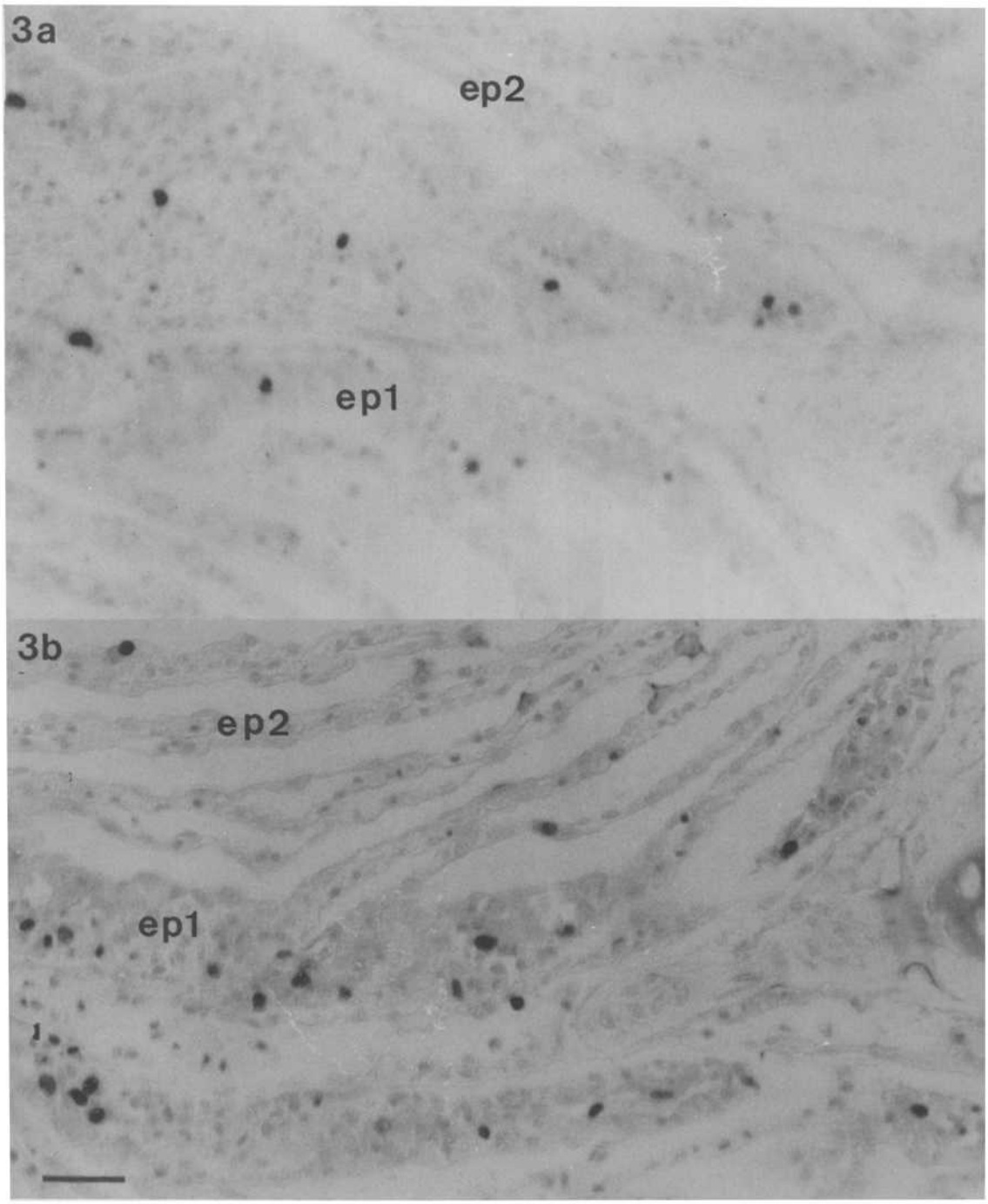

Fig. 3a. Representative LM photos of the filament cross section after BrdUrd labelling in control trout. Note the absence of labelled nuclei in lamellae (ep2), whereas a few are present in the filament (ep1).

Fig. 3b. The same as in Fig. 3a but after 48h in IPW. Note the presence of numerous labelled nuclei in the filament. A few are visible in the lamellae. Bar: 25μm.

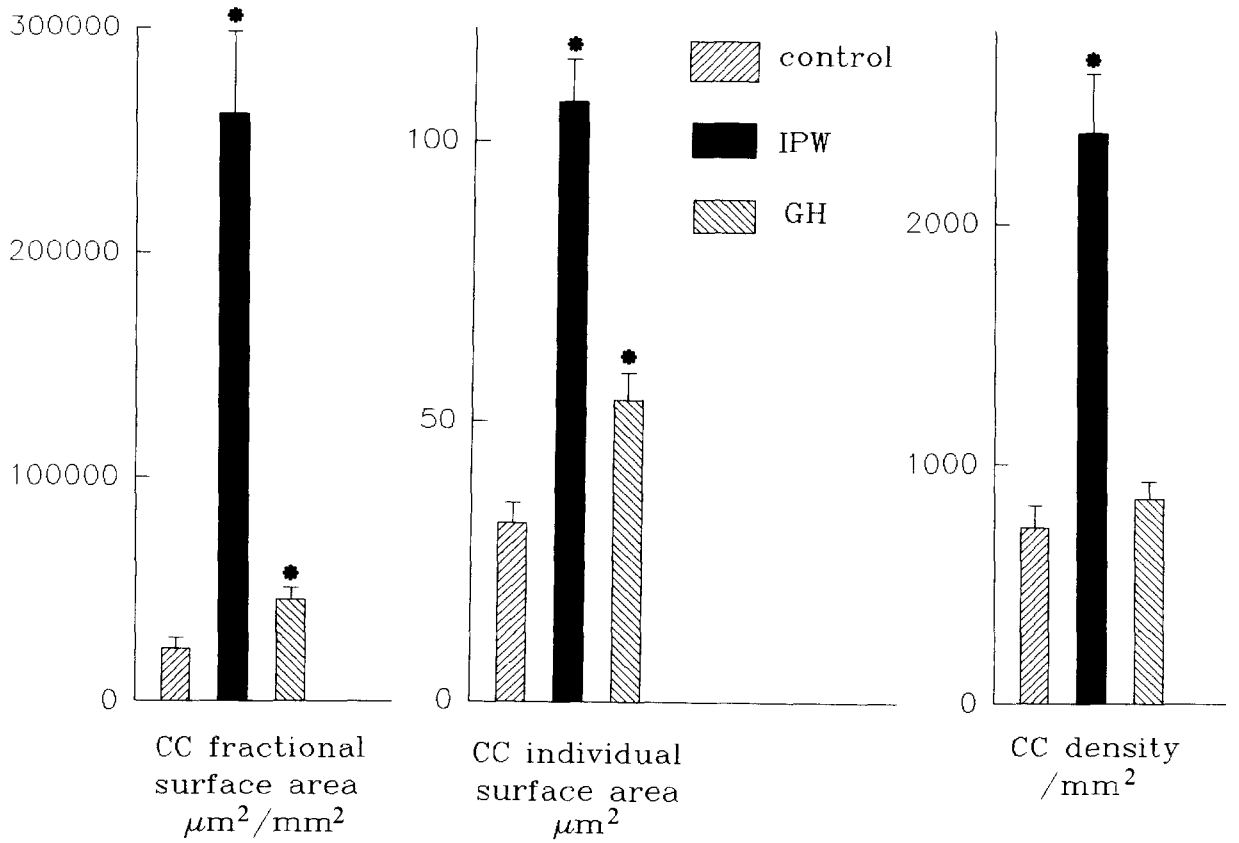

Fig. 4. SEM measurement of chloride cell morphometry on the filament epithelium (epl) in control trout, trout transferred in ion poor water for 24h (IPW) and trout treated with ovGH (2 injections, 5 days). Note the huge effect of IPW in comparison with growth hormone.

Effect of cortisol

The density of labelled nuclei 5 days after two intraperitoneal injections of cortisol (see Material and Methods, Section 3) was significantly decreased in the filament epithelium (Fig. 6). In the lamellar epithelium, though decreased, the labelling was not significantly affected (Fig. 6). The study of the labelling kinetics, in spite of a progressive increase of labelling in control fish (see Discussion), showed that the effect of cortisol began between 12 and 24h after a single injection (see Material and methods, Section 3) and was significant for both filament (Fig. 7) and lamella.

SEM morphometry and cortisol

In parallel with the determination of labelled nuclei density after the 5 days treatment with cortisol, the gill surface was examined with the SEM (Fig. 8). Both fractional and individual apical surface area increased significantly. Conversely the chloride cell density did not change significantly.

LM localization of cortisol

This study revealed the existence of an homogenous population of strongly immunoreactive cells located within the filament epithelium (Fig. 9a, c), but rarely observed in the lamellae. These cells were morphologically distinct from CCs which were un-

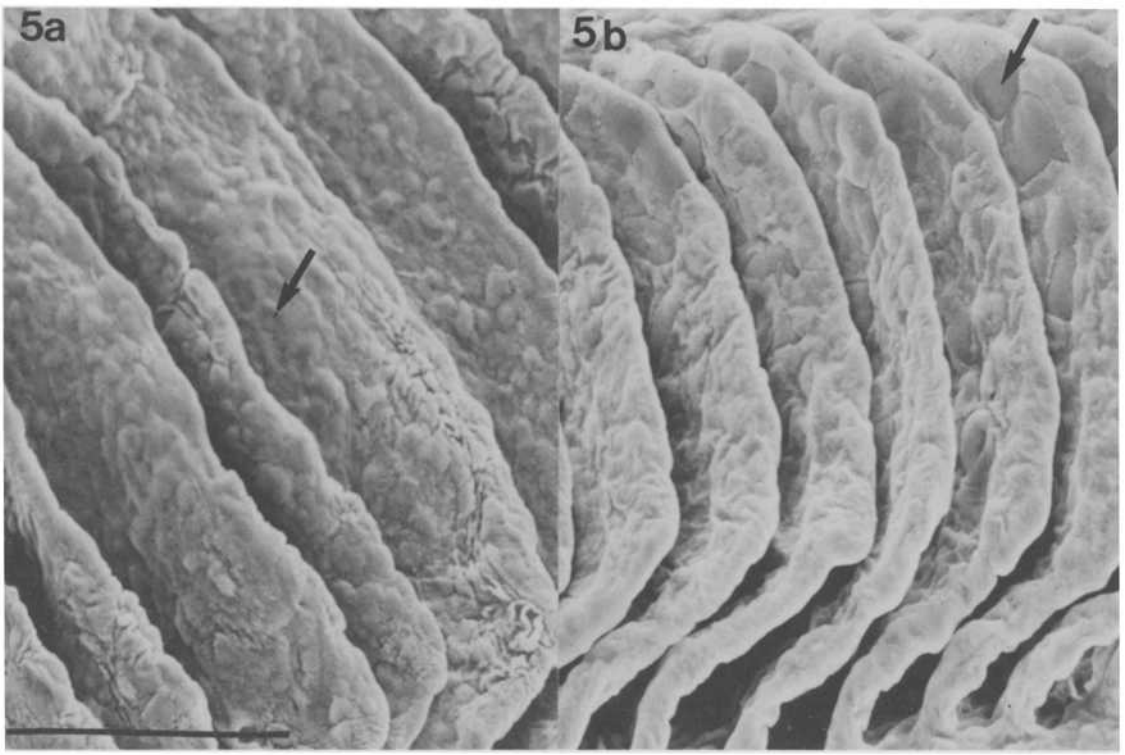

Fig. 5a. Representative SEM photo of the lamellar epithelium in control trout.

Fig. 5b. Representative SEM photo of the lamellar epithelium in trout transferred in IPW. In both photos arrows point to the apical surface of CCs. Note that in trout transferred in IPW, the CCs are numerous and have a large apical surface. Bar: 100μm.

stained, fully developed, and largely open to the outside. Immunoreactive cells were often observed on the side of non-immunoreactive CCs. These stained cells were identified, with the TEM owing to their shape and their situation, as elongated cells, often seen open to the external milieu by a narrow apical aperture. These cells contained a numerous population of mitochondria and a poorly developed network of CC type tubules (Fig. 10). In trout held in STW, not treated with cortisol, sham injected with saline or not injected (control), these immunostained cells were still present (Fig. 9b). In addition, it is worth noting that acclimation of trout in IPW which increased both the morphometry parameters of the CCs (Fig. 9d) and the BrdUrd labelling, enhanced the numerical density of these immunoreactive cells (Fig. 9e).

Effect of growth hormone

Two injections of oGH significantly increased the density of labelled nuclei in the filament epithelium and in the lamellar epithelium (Fig. 11). In the kinetics study (1 injection), the density of labelled nuclei increased in filament, 48h after the single injection (Fig. 12). In other words, the rate of cell division was significantly accelerated, an effect which was still present after 4 days. However, in contrast with the 2 injections experiment, there was no significant effect in the lamellar epithelium.

Morphometry of surface chloride cells after GH treatment

Figure 4 shows morphometry data after a 5 days treatment (2 injections). A small but significant increase was observed with the individual and frac-

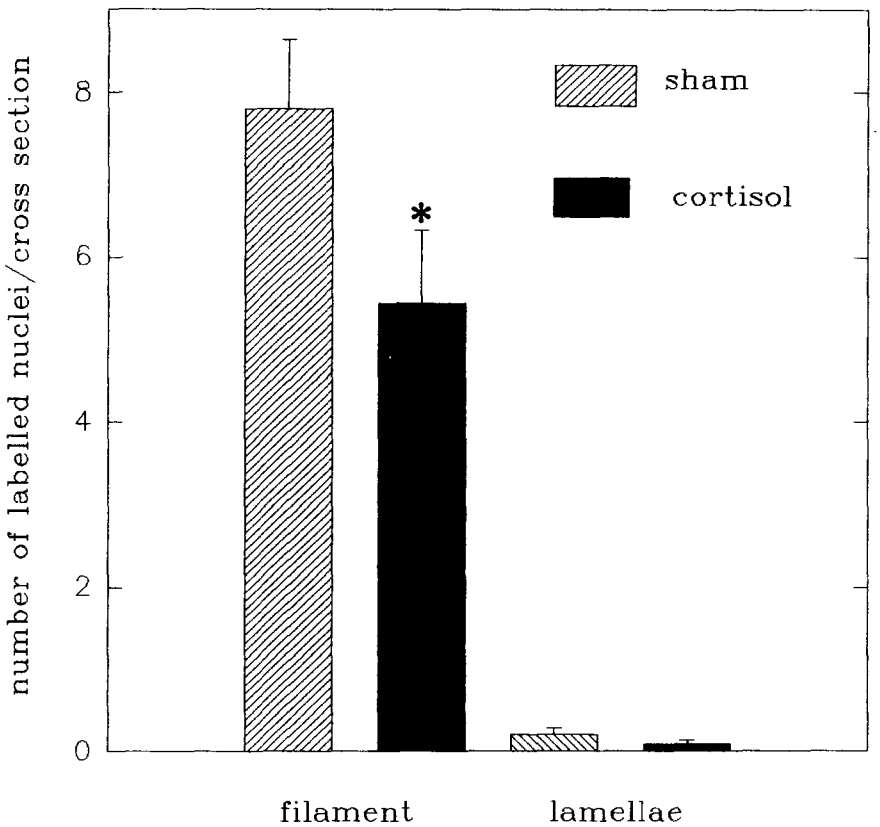

Fig. 6. Effect of injection of cortisol on the number of labelled nuclei in the filament epithelium and the lamellar epithelium.

tional apical surface area of the chloride cell. The numerical density of the chloride cells externally open was not affected by GH.

Discussion

Methodology

The primary target of this study was to determine if the changes in the gill epithelial surface relating to environmental conditions, resulted solely from a process of cell proliferation or solely of cell differentiation, or both (see review of Laurent and Perry 1991; Perry and Laurent 1993). These changes concern the numerical density of CCs which increases markedly in certain conditions (Laurent and Dunel 1980; Laurent *et al.* 1985; Avella *et al.* 1987; Perry and Laurent 1989; Laurent and Perry 1990) and, to a less extent, the numerical density of mucus cells (Laurent and Hebibi 1989).

BrdUrd cell labelling is presently considered as a reliable method to study cell proliferation. In cell kinetic studies this method is much less time consuming than the classic [^{3}H]-thymidine autoradiography (Kikuyama *et al.* 1988; Ricardi *et al.* 1988; Silvestrini *et al.* 1988). This method has been widely used to study cell kinetics in various tissues since the development of a monoclonal antibody against BrdUrd as a reagent to detect DNA-incorporated BrdUrd immunohistochemically (Gratzner 1982). The main drawback of this method is that it cannot be used in transmission electron microscopy, in contrast with [^{3}H]-thymidine autoradiography (Chrétien and Pisam 1986), and therefore, the labelled cells cannot be classified to types.

Uridine incorporates into the nuclear DNA in the S-phase. At this time, it is impossible, with the LM, to predict the cellular type of the future newborn cells. Unfortunately, our attempt to discriminate

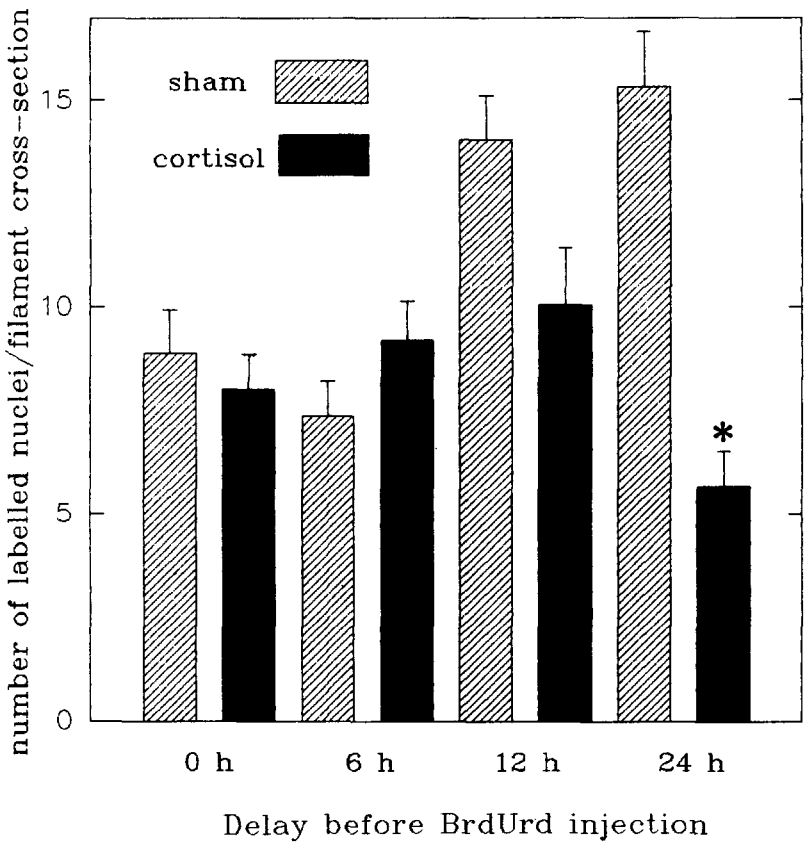

Fig. 7. Effect of cortisol on the number of labelled nuclei in the filament epithelium. Note that in control fish the number of nuclei increases at 12h after the saline injection whereas in the injected fish the number decreases significantly.

the cellular types by the size and the shape of cells containing labelled nuclei, brought about inconclusive results. Therefore we came to the conclusion that we should limit our study to determine: (i) if the changes in the numerical density of labelled cell divisions (considered as an index of cell mitosis) were concomitant or not with the phenotypic changes, (ii) how much each epithelium was respectively affected by those changes of labelling density.

Transfer to IPW

The effect on nuclear labelling of the transfer of trout to IPW was consistent with the results of chloride cell counting by LM: increases of both chloride cell numerical density (Laurent and Hebibi 1989, see Table 3) and of labelled nuclear density (this study, Fig. 2) both measured using the same method. Therefore, it can be concluded that the transfer to IPW provoked a CC proliferation. Moreover, as the rate of cell division increased significantly 48h after the transfer to IPW, whereas the phenotypic changes occurred as early as 12h after transfer (Perry and Laurent 1989, see Figure 3), a delayed cell proliferation probably occurred to replenish the stock of stem cells. Some contamination of the data by labelling of future mucus cells in epl is possible. However, the magnitude of this problem is likely small and cannot significantly alter the conclusion because of the rather specific localization of mucus cells on the edges of the filament. However, it is worth mentioning that the numerical density of mucus cells increases after transfer of trout to IPW (Laurent and Hebibi 1989).

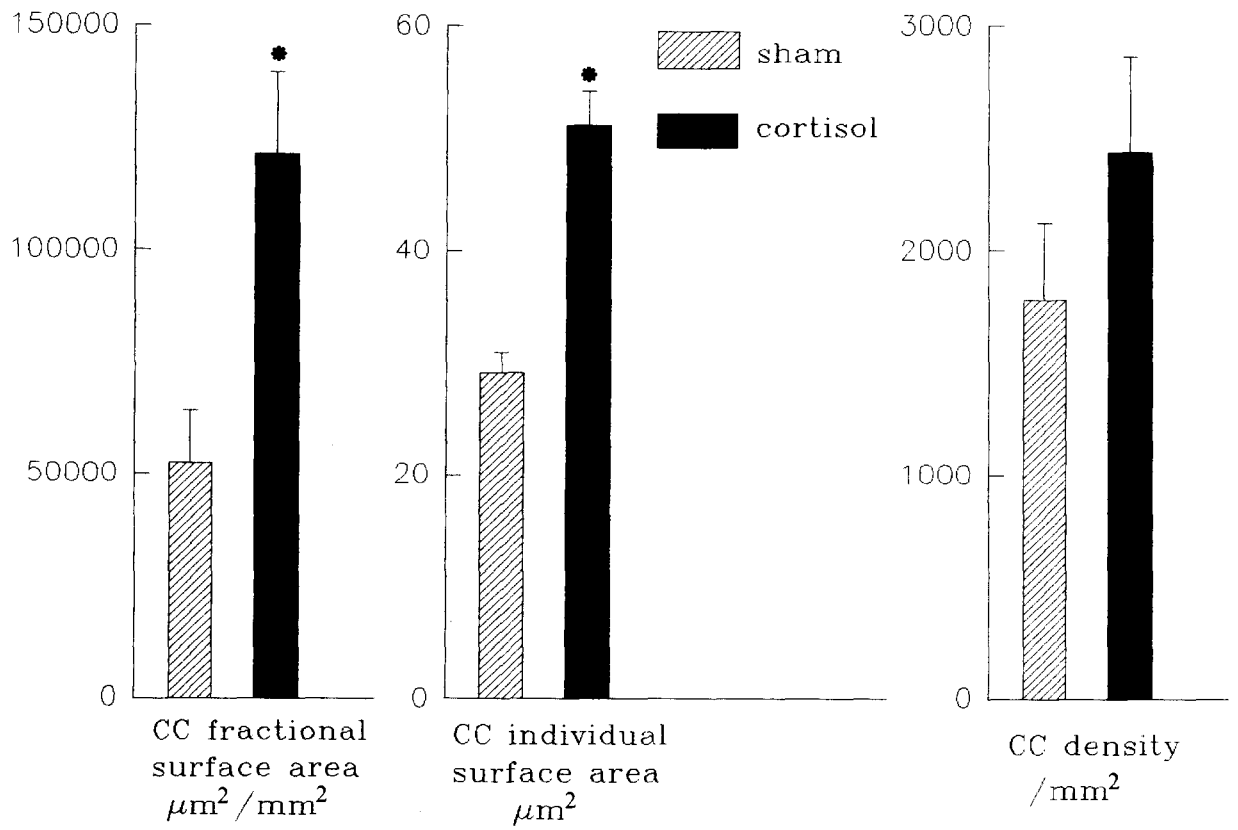

Fig. 8. SEM measurement of chloride cell morphometry in control trout, and trout injected with cortisol

Concerning the lamellae, it is worth noting that the labelling density was very low in control fish. This observation confirmed the data of Chrétien and Pisam (1986) which have shown that the labelled cells, after a comparable time of contact (1·5h) with the marker (^{3}H-thymidine), were located in the primary epithelium and "occasionally" in the lamellar epithelium. At a first glance, the results of the present study were not consistent with the large increase of the chloride cell density observed with the SEM in the lamellar epithelium after transfer to IPW (Fig. 5b). Indeed, our data did not show any increase of the rate of cell division. Even more, the figures of experimental fish were significantly lower at 12 and 72h. This inconsistency might have several causes.

As a first possibility, new chloride cells could be the result of a very active process of *in situ* differentiation of stem cells. If this were so, these cells would be part of those described in the internal layer of the lamellar epithelium (see review by Laurent 1984). Eventually, they would expose their apical surface to the external environment, as shown with the SEM (Fig. 5b).

As a second possibility, CCs could develop in the primary epithelium and would migrate into the base of the lamellae. Actually, it is known that the lamellae grow up from their base contrary to the filaments which grow from their tip (Morgan 1974a, b; Dunel 1975). The lamellar epithelium lies in continuity with the interlamellar part of the filament epithelium which has the highest density of chloride cells (see: Laurent 1984). As there is no anatomical interruption, that is, no *basal lamina* between filament and lamellar epithelia (Laurent and Dunel 1978), there is no obstacle to the CC migration. On the other hand, the fact that the appearance of CCs on the surface of the secondary lamellae took much less time than the 48h necessary to obtain a labelling response in filament, suggested that the process is

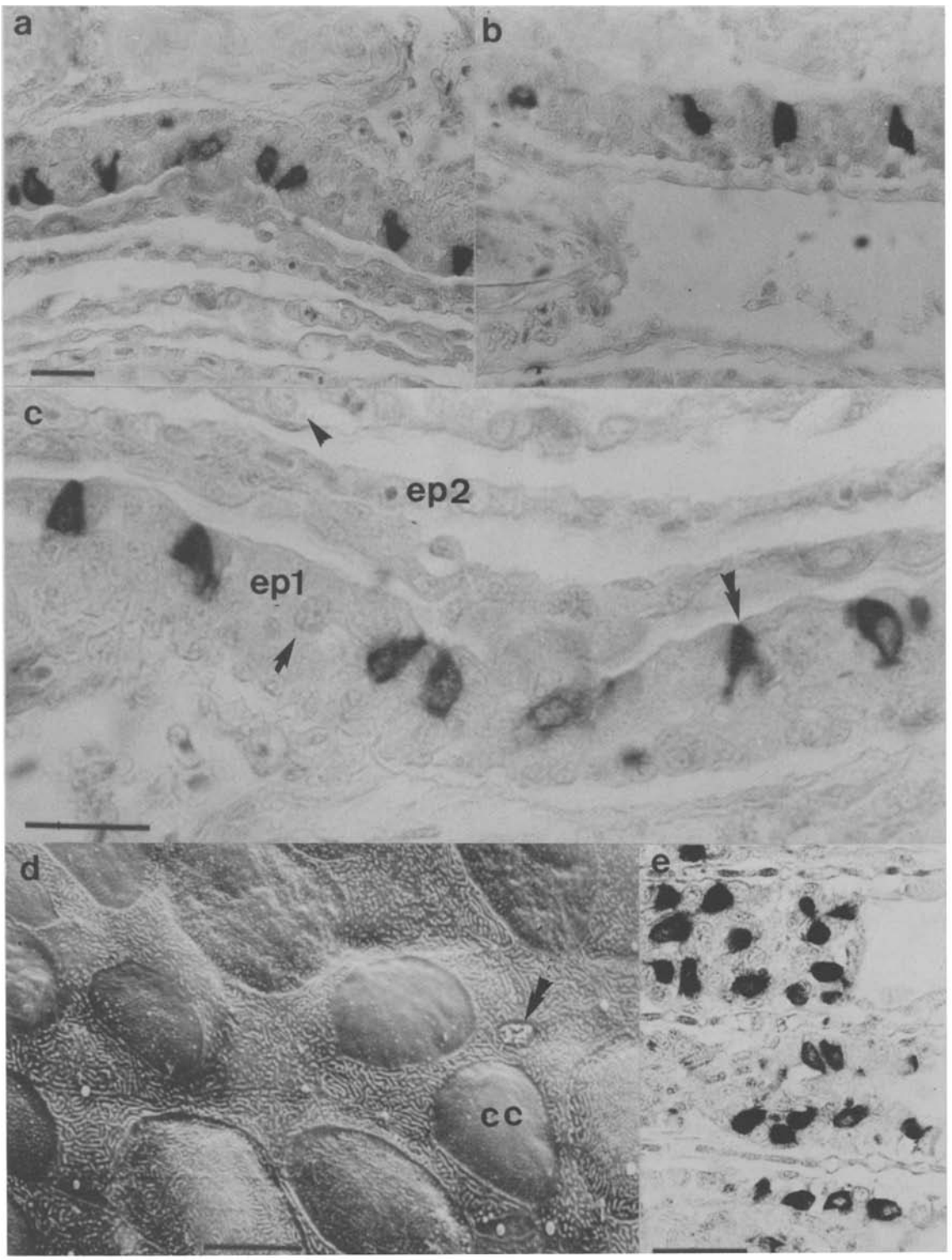

Fig. 9. Immunohistochemical staining of cortisol with anti-cortisol antibody (C8409, Sigma). a) in a trout injected with cortisol (bar: 100μm); b) in a trout injected with saline (bar: 100μm); c) close up of Fig. 9a (upside down), arrow points to a fully developed chloride

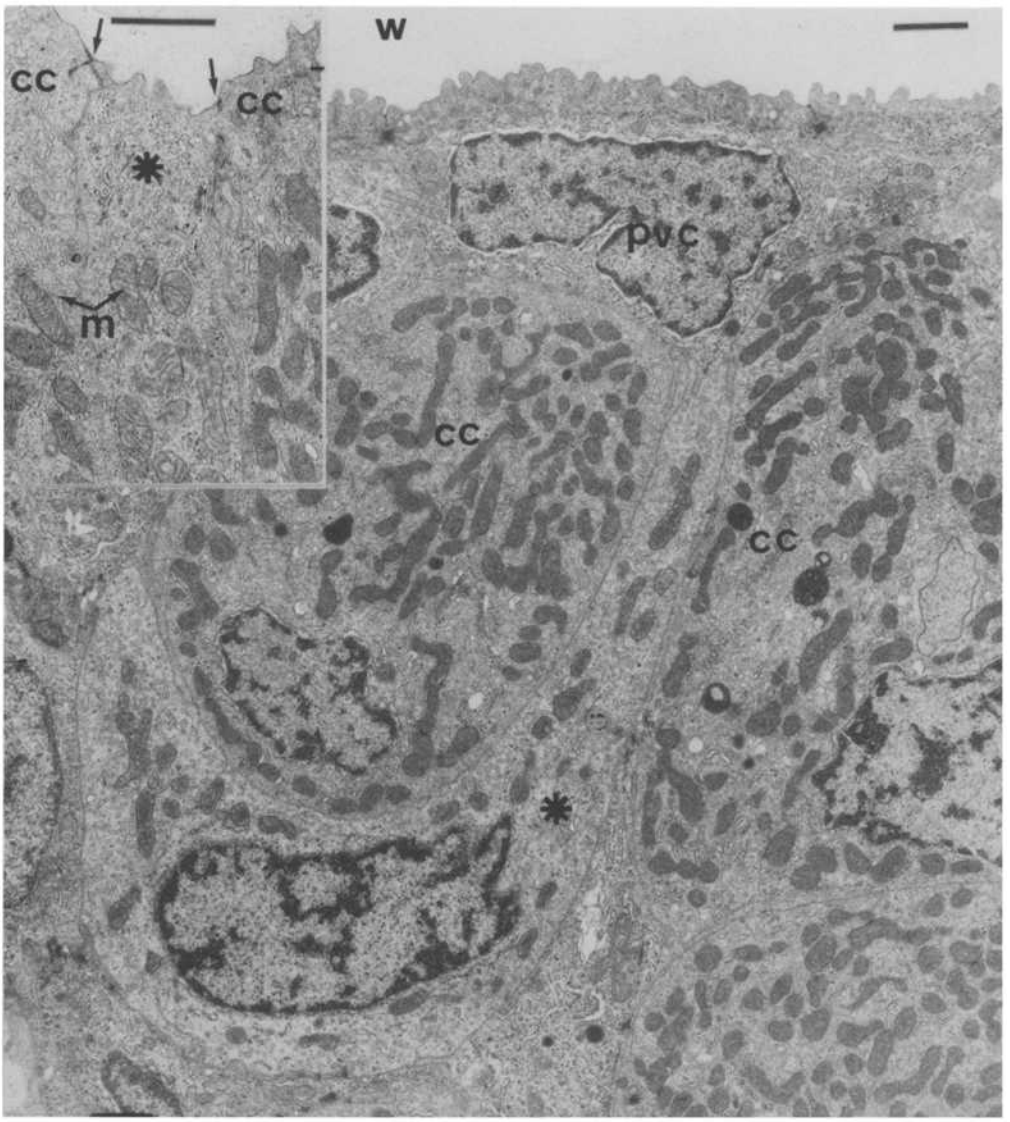

Fig. 10. TEM photo of a group of CCs in a trout showing a precursor CC (asterisk) located underneath a fully matured CC. Note the relative poor network of tubules. Bar: 5μm. Insert: a close up of the apical part of this group of CCs (same series of ultrathin sections, a few microns deeper in the block). Note that the asterisk marked cell of Fig. 10a has the same morphology as stained cells of Fig. 9c. Bar: 2μm.

dual. In addition to CC migration from the epithelium, a process which, according to Chrétien and Pisam (1986), takes 4 days to reach the surface of the epithelium, a rapid differentiation of some lamellar resident stem cells should occur to explain the rapid phenotypic changes.

←

cell with a large apical surface not stained. Note the pear-shaped black stained cells in epl with a narrow surface of contact with the external milieu ↓ ↓. Stained cells are not present in the inner layers of the filament epithelium and are absent in lamellar epithelium, in spite of the presence of fully developed CCs (arrowhead) (bar: 100μm); d) representative SEM of the filament surface. Note the large apical surface area of the numerous chloride cells (bar: 15μm). A double arrow head points to the narrow surface of contact of a young chloride cell. e) Immunostaining of cortisol positive cells in epl of trout in IPW (bar: 120μm).

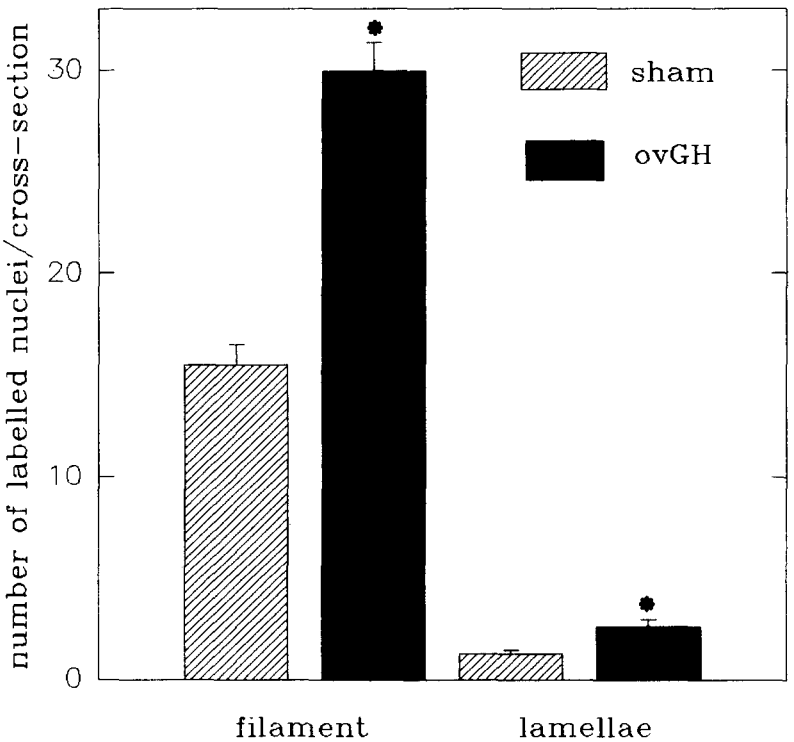

Fig. 11. Effect of injection of oGH on the number of labelled nuclei in the filament epithelium and in the lamellar epithelium. Note a significant increase in both cases.

Effect of cortisol

The plasma cortisol concentration has been demonstrated to raise transiently after transfer of trout to IPW (Perry and Laurent 1989). The effects of cortisol on CCs have been reported recently by several investigators (Laurent and Perry 1990; Madsen 1990c; McCormick 1990). In the study by Laurent and Perry (1990), a 10 days treatment consisting in daily injection of cortisol (4mg kg^{-1} b.w.) brought about two to threefold increases of the individual and fractional apical surface areas and of the density of CCs contacting the external milieu. These effects were concomitant with a significant increase of Na^+ and Cl^- influx. These data were confirmed by the present study in which two injections (interspersed by two days) caused less but still significant effects on CCs (Fig. 8). Thus, cortisol causes the same effect as IPW on the accessibility of CCs to the environment. That is why it has been suggested that the adaptational response to low environmental [NaCl] is *pro parte* cortisol-mediated (Perry and Laurent 1989). However, it is worth noting that cortisol was shown, in the present kinetic study, to significantly decrease the number of labelled nuclei in gill after a delay of 12–24h (Figs. 7), a depression still present after 5 days (Fig. 6). At a first glance, this response might be surprising as cortisol was demonstrated to increase the CC density. Thus if the response to low environmental NaCl is cortisol-mediated, other factors must be acting as for instance GH or any cell division stimulant. Even more, comparison of the effects of cortisol with those of IPW revealed a possible antagonism: cortisol engaging some epithelial cells more in a process of differentiation than replication. This remark is important as we know that the transfer to IPW causes a significant increase of plasma cortisol concentration (Perry and Laurent 1989). Therefore, the data presented in Figure 2 might be the result of two effects. A complementary aspect which should be emphasized is that simply handling

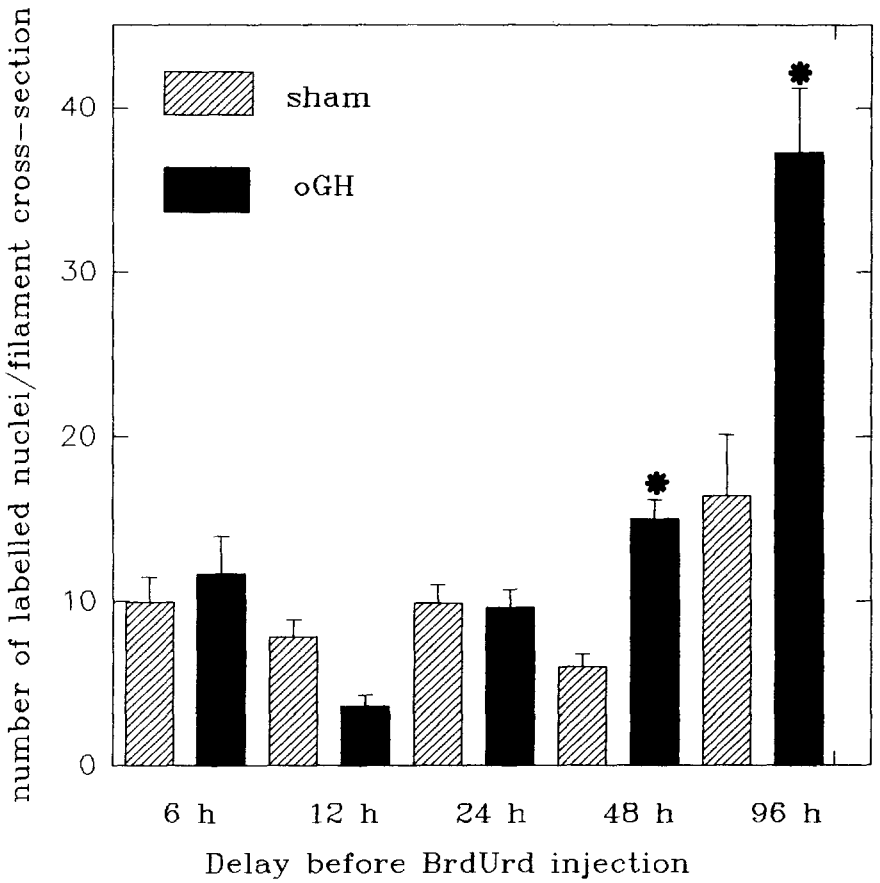

Fig. 12. Kinetics of oGH effect on the number of labelled nuclei in the filament epithelium. Note that in injected fish, the number of nuclei decreases at 12h after injection whereas it increases significantly at 48h and 96h.

trout and other teleosts significantly increases plasma cortisol levels (Barton *et al.* 1980, 1987). This might also account for a wide variability of control data. Indeed, according to Pickering *et al.* (1991), the elevation of plasma cortisol after a stress of handling in the rainbow trout is accompanied by a significant reduction in the concentration of circulating growth hormone.

If cortisol, *per se*, does not cause proliferation of gill cells including CCs, it certainly enhances the activity of CCs (see above; Madsen 1990; McCormick 1990). There is no direct cytological evidence, so far, showing that cortisol promotes or activates CC differentiation from quiescent "stem" cells. To address this question, we attempted in the present study to localize the cellular target of cortisol in the gill.

The presence of glucocorticoid receptors in the gill tissue of salmonid and non-salmonid fish has been established (Sandor *et al.* 1984; Chakraborti *et al.* 1987). As far as we know, no attempt has been made to localize the corticosteroid in the target cell. By using an antibody against cortisol, we attempted to detect endogenous or exogenous hormone into specific cells. Whether only part of immunostained cortisol was bound to *bone fide* gill receptors cannot be asserted. Nevertheless, the anti-cortisol antibody strongly stained and only stained elongated pear-shaped cells in the gill filament epithelium of trout. In IPW transferred fish, the stained cells were more numerous than in STW fish. In lamellae, the stained cells were rare. It is worth noting that in control (not cortisol treated) fish, immunostained cells were still present, indicating that it was not a

simple cellular uptake of exogenous cortisol which caused a positive immunoreaction, rather cortisol released from interrenal cells. It is of interest to mention that the anti-cortisol antibody stained cells in the head-kidney (Laurent, unpublished observation). By their shape and their situation, these stained gill cells were found to correspond with young incompletely differentiated CCs. They were often, but not always, seen contacting the gill external surface by a narrow aperture. If we reasonably consider this staining as an evidence for the presence of cortisol receptors, then cortisol receptors were undetectable with this method in mature CCs perfectly identifiable with the LM on the basis of their large apical surface contacting the external milieu, particularly after cortisol and IPW transfer. These data suggest, therefore, that young premature CCs were the target of cortisol, a hormone which, presumably, participates in their differentiation. It is interesting to note that cortisol did not label any of the small undifferentiated cells present in the inner layer of the primary epithelium and which comprise the future CCs (see review by Pisam and Rambourg 1991). This might be because the primary trigger of the chloride cell differentiation is not cortisol but another unknown messenger. The localization of cortisol only in immature chloride cells might be explained by the disappearance of the adequate receptors from mature cells.

Growth hormone was used in this study to test the ability of the method to demonstrate an increased rate of cell division in the gill epithelia. The quality of the immunohistochemical preparations and the reproducibility of the results suggest that the method was reliable, permitting individual and group comparisons.

In addition to testing the reliability of the method, there was a second reason for studying GH effect. It has been shown that the action of mammalian or fish GH interests not only growth (see review by Weatherley and Gill 1987) but also osmoregulation and, for instance, SW adaptability in salmonids (Komourdjian *et al.* 1976; Clarke *et al.* 1977; Yao *et al.* 1991). GH treatment increases the gill (Na^+, K^+) ATPase activity (Richman and Zaug 1987; Madsen 1990a,b), an enzyme known to be present on the baso-lateral membrane of the CCs (Hootman and Philpott 1979). It is worth noting that the density of labelled nuclei doubled in both filament and lamella after 2 injections (in 5 days) (Fig. 11). This similarity of action suggests that GH does not affect any particular cell type. The SEM study displayed an effect on individual CCs and fractional surface areas which was relatively small in comparison with the effects of IPW. No effect was observed concerning the numerical density of CCs contacting the environment. The positive immunostaining reactions obtained in the hypophysis with anti-ovine and anti-rat GH antibodies, in contrast to the absence of a comparable reaction in the gill, suggested inadequate specificity/sensitivity of our mammalian GH antibodies seeing that some binding was demonstrated in gill tissue with salmon-GH (Yao *et al.* 1991). Further studies are needed to demonstrate the gill localization of GH with immunohistochemistry.

To conclude, the adaptive response of the gill epithelium to transfer of the salmonid *Oncorhynchus mykiss* to IPW includes an immediate phase of CC differentiation (12h) followed by a delayed phase of proliferation (48h). In the lamellar epithelium, the present results do not support a process of proliferation but rather suggest a process of CC migration from the filament as a result of the overcrowding of this part of the gill epithelia. These processes lead to a dramatic increase of the total apical surface area of chloride cells explaining, at least in part, a large increase of the ability for the fish to take up ions from an extremely diluted environment (Perry and Laurent 1989).

On the other hand, the present results support the role played by cortisol in promoting the differentiation of immature CCs without changing or even decreasing the rate of cell division, leading finally to the same functional adaptation as IPW transfer (Laurent and Perry 1990). Finally, GH probably has no direct effect on the ability of the fish to adapt to IPW since, when injected, it does not greatly change the total apical surface of CCs. However, it might have an indirect effect by significantly increasing the rate of gill cell division including the CCs. Thus the hormonal control of the CCs during environmental adaptation to an ion poor environment is probably multifactorial.

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References cited

Avella, M., Masoni, A., Bornancin, M. and Mayer-Gostan, N. 1987. Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. J. Exp. Zool. 242: 159–169.

Barton, B.A., Peter, R.E. and Paulencu, C.R. 1980. Plasma cortisol levels and fingerling rainbow trout (*Salmo gairdneri*) at rest and subjected to handling, confinement, transport, and stocking. Can. J. Fish. Aqu. Sci. 37: 805–811.

Barton, B.A., Schreck, C. and Barton, L.D. 1987. Effects of chronic cortisol administration and daily acute stress on growth, physiological condition, and stress responses in juvenile rainbow trout. Dis. Aquat. Org. 2: 173–185.

Chakraborti, P.K., Weisbart, M. and Chakraborti, A. 1987. The presence of corticosteroid receptor activity in the gills of the brook trout, *Salvelinus fontinalis*. Gen. Comp. Endocrinol. 66: 323–332.

Chrétien, M. and Pisam, M. 1986. Cell renewal and differentiation in the gill epithelium of fresh- or salt-water-adapted euryhaline fish as revealed by [^{3}H]thymidine autoradiography. Biol. Cell. 56: 137–150.

Clarke, W.C., Farmer, S.W. and Hartwell, K.M. 1977. Effect of pituitary growth hormone on growth of *Tilapia mossambica* and on growth and sea water adaptation of sockeye salmon (*Oncorhynchus nerka*). Gen. Comp. Endocrinol. 33: 174–178.

Dunel, S. 1975. Contribution à l'étude structurale et ultrastructurale de la pseudobranchie et de son innervation chez les Téléostéens. Ph. D. Thesis. University of Strasbourg.

Ellwart, J. and Dörmer, P. 1985. Effect of 5-fluoro-2′-deoxyridine (FdUrd) on 5-bromo-2′-deoxyuridine (BrdUrd) incorporation into DNA measured with a monoclonal BrdUrd antibody and BrdUrd/Hoechst quenching effect. Cytometry 6: 513–520.

Gratzner, H., Leif, R.C., Ingram, D.J. and Castro, A. 1975. The use of antibody specific for bromodeoxyuridine for the fluorescent determination of DNA replication in single cell and chromosome. Exp. Cell. Res. 95: 88.

Gratzner, H. 1982. Monoclonal antibody against 5-bromo- and 5-iodo-deoxyuridine: a new reagent for detection of DNA replication. Science 218: 474–475.

Hootman, S.R. and Philpott, C.W. 1979. Ultracytochemical localization of Na^+, K^+-activated ATPase in chloride cells from the gills of euryhaline teleost. Anat. Rec. 193: 99–130.

Kikuyama, S., Kubota, T., Watanabe, M., Ishibiki, K. and Abe, O. 1988. Cell kinetic study of human carcinomas using bromodeoxyuridine. Cell Tiss. Kinet. 21: 15.

Komourdijan, M.P., Saunders, R.L. and Fenwick, J.C. 1976. The effect of porcine somatotropin on growth and survival of Atlantic salmon (*Salmo salar*). Can. J. Zool. 54: 534–535.

Laurent, P. 1984. Gill internal morphology. *In* Fish Physiology. Vol. 10A, pp. 73–183. Edited by W.S. Hoar and D.J. Randall. Academic Press, New York.

Laurent, P. and Dunel, S. 1978. Relations anatomiques des ionocytes (cellules à chlorure) avec le compartiment veineux branchial: Définition de deux types d'épithélium de la branchie des poissons. C.R. Hebd. Séances Acad. Sci., Ser. D286: 1447–1450.

Laurent, P. and Dunel, S. 1980. Morphology of gill epithelia in fish. Am. J. Physiol. 238 (Regulatory Integrative Comp. Physiol. 7V: R147–R159.

Laurent, P. and Hebibi, N. 1989. Gill morphometry and fish osmoregulation. Can. J. Zool. 67: 3055–3063.

Laurent, P., Höbe, H. and Dunel-Erb, S. 1985. The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. Cell Tiss. Res. 240: 675–692.

Laurent, P. and Perry, S.F. 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell Tiss. Res. 259: 429–442.

Laurent, P. and Perry, S.F. 1991. Environmental effects on fish gill morphology. Physiol. Zool. 64: 4–25.

Leino, R.L., McCormick, J.H. and Jensen, K.M. 1987. Changes in gill histology of fathead minnows and yellow perch transferred to soft water or acidified soft water with particular reference to chloride cells. Cell Tiss. Res. 250: 389–399.

MacCormick, J.H. 1990. Cortisol directly stimulates differentiation of chloride cells in *Tilapia* opercular membrane. Am. J. Physiol. 159: R857–R863.

Madsen, S.S. 1990a. Cortisol treatment improves the development of hypoosmoregulatory mechanisms in the euryhaline rainbow trout, *Salmo gairdneri*. Fish Physiol. Biochem. 8: 45–52.

Madsen, S.S. 1990b. Enhanced hypoosmoregulatory response to growth hormone after cortisol treatment in immature *Salmo gairdneri*. Fish Physiol. Biochem. 8: 271–279.

Madsen, S.S. 1990c. The role of cortisol and growth hormone in SW adaptation and development of hypoosmoregulatory mechanism in seawater trout parr. Gen. Comp. Endocrinol. 79: 1–11.

Morgan, M. 1974a. The development of gill arches and gill blood vessels of the rainbow trout, *Salmo gairdneri*. J. Morphol. 142: 351–364.

Morgan, M. 1974b. Development of the secondary lamellae of the gills of the trout, *Salmo gairdneri*. Cell Tiss. Res. 151: 509–523.

Perry, S.F., Goss, G.G. and Laurent, P. 1992. The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts: the effects of cortisol. Can. J. Zool. 70: 1765–1786.

Perry, S.F. and Laurent, P. 1989. Adaptational responses of rainbow trout to lowered external NaCl Concentration: Contribution of the branchial chloride cell. J. Exp. Biol. 147: 147–168.

Perry, S.F. and Laurent, P. 1993. Environmental effects on fish gill structure and function. *In* Fish Ecophysiology. pp. 231–263. Chapman and Hall, London.

Pickering, A.D., Pottinger, T.G., Sumpter, J.P., Carragher, J.F. and Le Bail, P.Y. 1991. Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 83: 86–93.

Pisam, M. and Rambourg, A. 1991. Mitochondria-rich cells in the gill epithelium of teleost fishes: an ultrastructural approach. Int. Rev. Cytol. 130: 191–232.

Rahim, S.M., Delaunoy, J.-P. and Laurent, P. 1988. Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. Histochemistry 89: 451–459.

Ricardi, A., Danova, M., Wilson, G., Ucci, G., Dormer, P., Mazzini, G., Brugnatelli, S., Girino, M., McNally, M.J. and Ascari, E. 1988. Cell kinetics in human malignancies studied with *in vivo* administration of bromodeoxyuridine and flow cytometry. Cancer Res. 48: 6238.

Richman, N.H., III, and Zaugg, W.S. 1987. Effects of cortisol and growth hormone on osmoregulation in pre- and desmoltified coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 65: 189–198.

Sandor, T., DiBatista, J.A. and Medhi, A.Z. 1984. Glucocorticoid receptors in the gill tissue of fish. Gen. Comp. Endocrinol. 53: 353–364.

Silvestrini, R., Costa, A., Veroni, S., Del Bino, G. and Persici, P. 1988. Comparative analysis of different approaches to investigate cell kinetics. Cell Tiss. Kinet. 21: 123.

Weatherley, A.P. and Gill, H.S. 1987. The Biology of Fish Growth. Academic Press, New York.

Yao, K., Niu, P., Le Gac, F. and Le Bail, P.Y. 1991. Presence of specific growth hormone binding sites in rainbow trout (*Oncorhynchus mykiss*) tissues: Characterization of the hepatic receptor. Gen. Comp. Endocrinol. 81: 72–82.