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# Ontogeny and apoptosis of chloride cells in the gill epithelium of newly hatched rainbow trout

Concepción Rojo and Encina González

Departamento de Anatomía y Anatomía Patológica Comparadas
Facultad de Veterinaria
UCM. Avda./Puerta de Hierro s/n.
ES-28040 Madrid, Spain

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**Abstract**

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We studied the characteristics of chloride cells using transmission and scanning electron microscopy in newly hatched rainbow trout (*Oncorhynchus mykiss*), reared in freshwater. Ultrathin (25 nm) sections from the gills were stained with lead citrate. The first sign of differentiation of chloride cells was the presence of cells with numerous mitochondria in the epithelia of gill filaments and lamellae. In addition to mitochondria, well-developed chloride cells displayed extensive membranous systems and apical membranes of different structure. Not only mature and immature, but also intermediate developmental stages and degenerate chloride cells, were found in the epithelia of the lamellae and interlamellar regions. Our study indicates that variations in the morphology and distribution of chloride cells in rainbow trout alevins, are the result of adaptative responses to different environmental requirements when reared under natural freshwater conditions. These results agree with those reported in other freshwater species, thus suggesting that the morphological variations of chloride cells are partially the representation of different stages during the cell cycle, and partially the consequence of differences in salinity and ionic composition of the water.

Concepción Rojo, Departamento de Anatomía y Anatomía patologica Comparadas, Facultad de Veterinaria, UCM., ES-28040 Madrid, Spain. Tel: 341 3943729; E-mail: RojoSalv@vet.ucm.es

## Introduction

The mitochondria-rich or chloride cells (CCs) of fish gill epithelium play a major role in the osmoregulation of freshwater, euryhaline and seawater teleosts (Goss *et al.* 1995; Pisam *et al.* 1995; Witters *et al.* 1996). Their structural features include large numbers of mitochondria and an extensive tubular network, characteristics found in ion-transporting cells. Contradictory statements have been published concerning the existence of different subtypes of CCs. On the basis of the morphological appearance, stainability and specific location of these cells, Pisam *et al.* (1987) distinguished two types of CCs – $\alpha$ and $\beta$ – in the gills of freshwater guppies. Hootman and Philpott (1980) and Pisam *et al.* (1989) defined a third subtype, the accessory chloride cell. On the other hand, it has been noted that in salmonids there appears to be varying surface morphologies (Perry and Laurent 1989; Perry *et al.* 1992; Goss *et al.* 1995). It has been proposed that these heterogeneities in the morphology of the CCs do not represent two different populations, but instead constitute a uniform population across which an arbitrary division has been placed (Goss *et al.* 1995). Indeed, it has been pointed out that the current classification of the CCs is unsatisfactory (Maina 1990).

Morphological variations in the CCs are known to reflect an adaptive response to particular environmental conditions (Laurent *et al.* 1985; King *et al.* 1989; King and Hossler 1991). Wendelaar Bonga *et al.* (1990) suggest that the varying morphological features of the CCs represent different stages in the cell cycle.

Most of the previous studies on the CCs have been carried

out in adult fish adapted to various environmental conditions. Morphological and physiological studies have focused on the modulation of branchial ionic movements during adaptive responses (Laurent *et al.* 1985; King *et al.* 1989; Pisam *et al.* 1995). However, the ultrastructure of CCs in post-hatched larvae has received little attention. The density and abundance of CCs in teleost larvae appear to be correlated with the different requirements for osmoregulation (Hwang 1988 1989). The change from an 'intracapsular' to a 'free-living' type of life, which is characteristic of fish, has great significance in ontogeny (Yamagami 1988). In fact, post-hatched larvae are susceptible to physiological stress factors occurring during a short period of time, such as the beginning of exogenous nourishment and the beginning of gas exchange through the gill epithelium. Moreover, the extremely variable chemical properties of freshwater environments have important consequences on the internal acid-base and ionic status of freshwater fish (Dejours 1988). One might therefore expect structural differences in the CCs in post-hatched trout alevins.

In the present study, the ultrastructure of the CCs as well as the distribution of these cells in the gill epithelium of newly hatched rainbow trout (*O. mykiss*) reared in natural soft water, were investigated.

## Materials and Methods

### *Specimen acquisition*

Observations were performed on newly hatched alevins (stage 36 according to Vernier 1969) of rainbow trout (*Oncorhynchus mykiss* (Walbaum 1792)), collected from a local privately owned, trout hatchery. The alevins were kept in running fresh water at 100°C ± 1, and under a daily 12 h photoperiod. Concentrations of the main ions (in mM) were: $Na^+$ 4.9; $K^+$ 0.05; $Ca^{2+}$ 0.8; $Mg^{2+}$ 0.2; Cl 4.0; pH, 7.4. After anaesthesia with MS 222, gills were quickly dissected and fixed for 6 h at 40C in a solution containing 1% glutaraldehyde and 4% paraformaldehyde in 0.1 sodium phosphate buffer (pH 7.2).

### *Transmission and scanning electron microscopy preparation*

For transmission electron microscopy, tissue pieces were washed with 0.1 m sodium phosphate buffer, and then post-fixed for 1 h at room temperature in a 1/1 mixture of 2% aqueous osmium tetroxide and 3% aqueous potassium ferrocyanide (Karnovsky 1971). After dehydration in a series of acetone, tissues were embedded in Epon 812. Thin sections (25 nm), parallel to the long axis of the filament, perpendicular to secondary lamellae, were stained with lead citrate, and examined at 80 Kv with a Zeiss EM 902 electron microscope.

For scanning electron microscopy, tissues were post-fixed in 1% $OsO_4$ buffered in sodium phosphate, for 1 h at room temperature. Then they were dehydrated in acetone, critical-point dried over $CO_2$ (Balzers SCD 004), sputter coated with gold for 3 min (Balzers SCD 004), and then studied in a JEOL Model JSM 6400 scanning electron microscope operating at 20 kV.

## Results

### *Distribution of the chloride cells in newly hatched alevins*

In the alevins the gills consist of four arches bearing primary lamellae or filaments, from which radiate secondary lamellae, as can be seen in the scanning electron micrographs (González *et al.* 1996). The epithelium covering the gill filaments (primary epithelium) and the lamellae (secondary epithelium) is formed not only by pavement and undifferentiated cells, but also by chloride and mucous cells.

In newly hatched alevins, most of the CCs are observed in the primary epithelium either in the interlamellar region or at the base of the secondary lamellae (Fig. 1A–C). However, a few isolated CCs appear in the secondary epithelium of the lamellae on both sides of the lamellae, thus facing the CCs located in the lamellar epithelium of neighbouring lamellae (Fig. 1D). It is common to find clusters of two or more CCs in the interlamellar region and at the base of the lamellae (Figs 1A, B, 2A, B) whereas in other locations, CCs occur singly.

In the interlamellar region, the CCs locate not only on the superficial layer of the epithelium but also in the intermediate and basal layers. Undifferentiated and degenerating CCs, are observed near the basement membrane of the gill epithelium (Figs 1A, 2A, B, C). The CCs located at the base of the lamellae are usually in contact with the basement membrane of the pillar capillary (Figs 1A, E, 3B).

### *Morphology of the chloride cells in newly hatched alevins*

The density and degree of maturation of the CCs in both the secondary lamellae and the interlamellar region, progress with increasing age. The CCs at these locations show signs of higher activity than those of the filament, e.g. a wide apical exposure, numerous mitochondria and a developed vesicular system. The epithelium of the efferent and afferent sides of the filaments where intercellular spaces are abundant, contains isolated CCs covered by pavement cells (Fig. 2C). Cytoplasmic organelles common to these CCs are a few round or elongated mitochondria, a conspicuous Golgi apparatus, and numerous cisternae of rough endoplasmic reticulum, thus indicating that a significant number of CCs in the filament are immature. Mature CCs are also present in the epithelium of the gill filament, especially in the efferent side (Fig. 2D). These

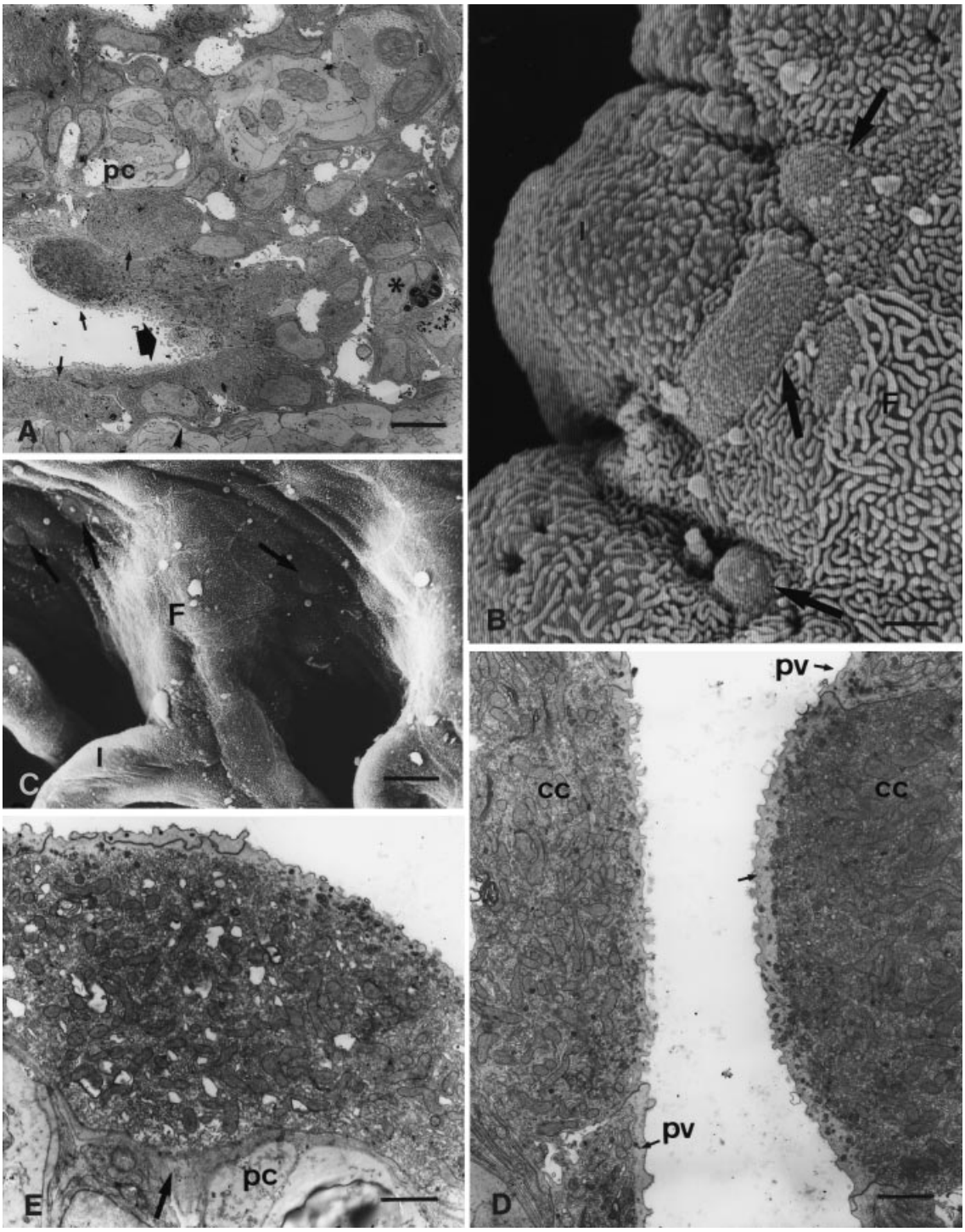
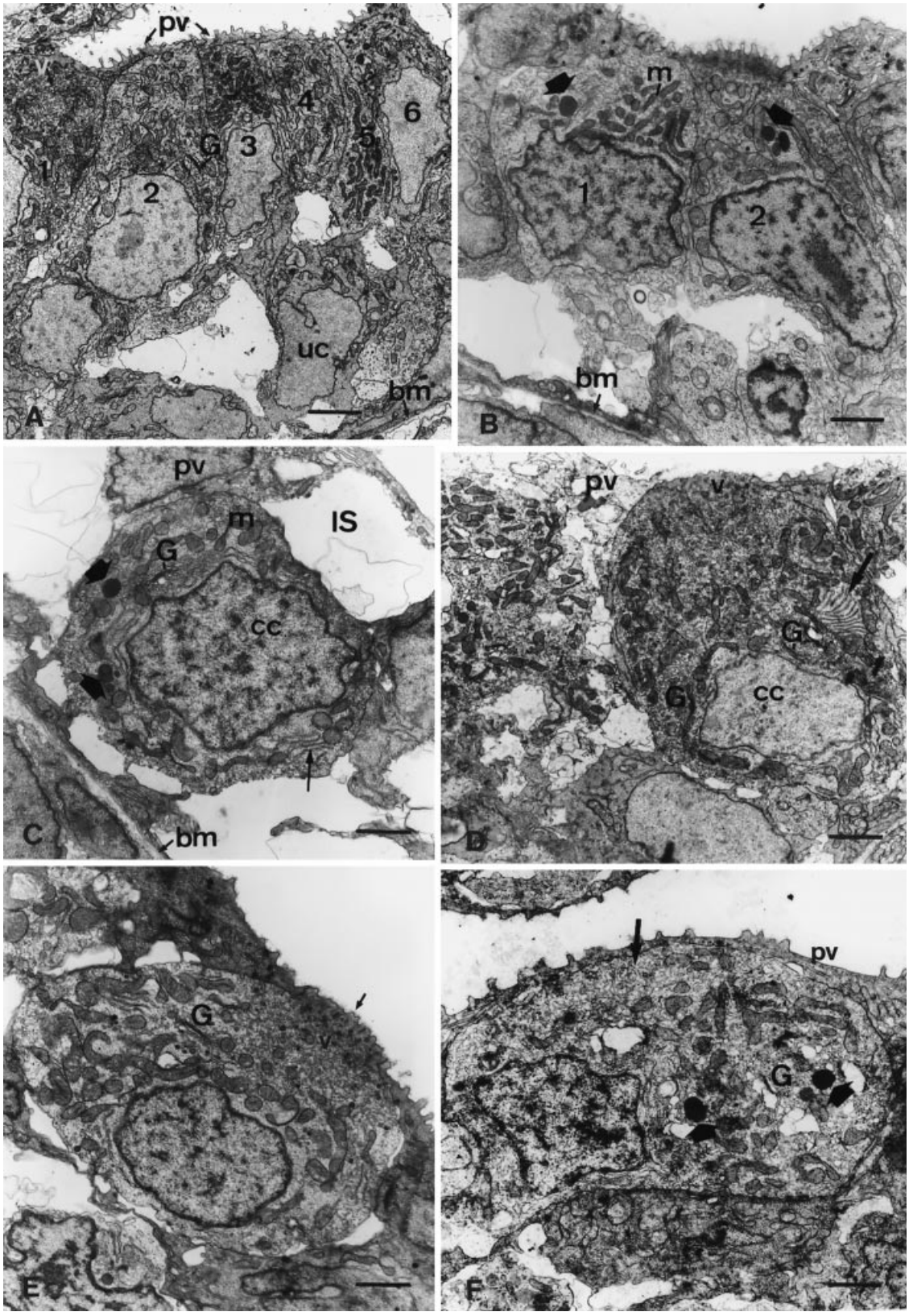



**Fig. 1**.—**A,** Gill epithelium of a newly hatched rainbow trout, showing the abundance of grouped chloride cells at the base of the lamellae (small arrows) and in the interlamellar region of the filament (large arrow); *, phagocytic cell with apoptotic remnants in its cytoplasm; pc, pillar capillary; arrowhead, basement membrane. Scale bar = 7.71 $\mu$m.—**B,C,** Scanning electron micrographs of the gill filaments (F) and lamellae (l) of a newly hatched rainbow trout. The chloride cells (arrows) are mainly distributed in the interlamellar region and at the base of lamellae. Scale bars = 21 $\mu$m and 101 $\mu$m, respectively.—**D,** Chloride cells (cc) in the lamellar epithelium of two neighbouring lamellae. pv, pavement cells; arrow, cytoskeleton elements. Scale bar = 1.51 $\mu$m.—**E,** The chloride cells located at the base of the lamellae are in contact with the basement membrane (arrow) of the pillar capillary (pc). Scale bar = 1.21 $\mu$m.



CCs show numerous electrondense mitochondria, conspicuous Golgi apparatus and elongated cisternae of rough endoplasmic reticulum. The characteristic feature of mature CCs is the presence of a developed vesicular system toward the apical surface. Although the mature CCs of the filament appear in clusters, the cytoplasm of a pavement cell is always present between two neighbouring CCs (Fig. 2D).

A high number of the CCs located on the lamellae are ovoid-shaped. However, the ultrastructural characteristics of these CCs are similar to those of the CCs in the interlamellar region (compare Fig. 2A, B with 2E, F, 3A, B). The mitochondria are uniformly distributed over the cytoplasm, and contain a matrix paler than their counterparts in adjacent CCs (Fig. 2A, B). The Golgi apparatus, which is commonly found in developing CCs, is composed of numerous saccules and small vesicles (Fig. 2A, E, F). The elongated cisternae of rough endoplasmic reticulum are abundant throughout the cytoplasm, especially in the developing CCs (Fig. 2F). However, the mature CCs of the lamellae, show membranous tubules of uniform diameter which spread out in the supranuclear cytoplasm (Fig. 2E). The numerical density of these tubules increases with the maturation stage of the CCs, whereas the cisternae of rough endoplasmic reticulum decrease in number. Ocassionally some pigment granules are observed in the cytoplasm of developing CCs (Fig. 2B, C, F), regardless of their location.

The shape of the CCs varied from ovoid to columnar, but did not depend on the location of the cell nor the stage of maturation. Most of the mature CCs were columnar or cuboidal, extending from the basal lamina of the epithelium to the apical surface (Fig. 3A, B). The mature CCs at both the secondary lamellae and the interlamellar region, displayed few elements of the tubular system intermingled with both mitochondria and cisternae of rough endoplasmic reticulum (Figs 3A, 5A). A large proportion of the vesicles appeared near the apical plasma membrane (Fig. 3A, B). Some of these vesicles were related to the apical microvilli, where exocytotic figures were present (Figs 3A, 4C, 5A).

The interlamellar region of alevins is rich in flattened superficial cells, which contain numerous electrondense vesicles distributed throughout their cytoplasm and few mitochondria. These cells, covering the developing CCs, are pavement cells: their apical surface bears numerous fuzzy coated microridges different from those of the CCs (Fig. 3C).

A few mature CCs showed membranous structures separated from the apical membrane by a cytoplasmic band corresponding to the web of cytoskeleton elements (Figs 1D, 3B, C). This finding occurred in most of the mature CCs, but it was not related to the location of the CCs.

The apical structures consist of tubules and/or cisternae of rough endoplasmic reticulum, as well as coated vesicles filled with an electrondense material similar to that of the fuzzy coat (Fig. 4A-D). The membranous tubules are located either just beneath the apical vesicles (Fig. 4D) or intermingled with both mitochondria and vesicles (Fig. 4B). These tubules frequently branched and anastomosed, especially in the more mature CCs (Figs 4B–D, 5A).

The high numerical density of the apical vesicles is usually directly related to a more elaborate pattern of microvilli, the presence of exocytotic figures and the wider extent of the exposed apical surface. Figure 4A shows the apical opening of a developing CC between two pavement cells. The CC shows a smooth surface, numerous mitochondria and cisternae of rough endoplasmic reticulum, but vesicles are scarce. However, this is not a general rule since some CCs show a flattened apical membrane with numerous vesicles inside (Fig. 4B), whereas others show an extended apical surface which projects short microplicae, covered by a conspicuous fuzzy coat and related to few vesicles (Fig. 4C).

In the SEM images the heterogeneity in the morphology of the apical surface can be seen (Fig. 4E). Pores representing the apical surface of the CCs, open along the borders of adjacent pavement cells, which display concentrically arranged surface ridges. Most of the openings of the CCs located in the interlamellar region and in the lamellae exhibit protruding surfaces with apical projections. In some cases, the pores resemble rounded-to-oval holes with little or no internal apical structures (Fig. 4E).

The pavement cells are connected with the CCs by

**Fig. 2.**—**A,** Multicellular complex formed by six chloride cells (1–6) in the interlamellar region of rainbow trout alevins. Most of these cells are developing chloride cells, except for number 1, which is a mature cell showing a developed vesicular system (v); G, Golgi apparatus; pv, pavement cells; bm, basement membrane; uc, undifferentiated chloride cells. Scale bar = 3.11 $\mu$m.—**B,** Multicellular complex formed by two chloride cells (1,2) exposed to the outer medium, in the interlamellar region of a newly hatched alevin. arrows, pigment granules; m, mitochondria; bm, basement membrane. Scale bar = 1.31 $\mu$m.—**C,** Isolated immature chloride cell (cc) in the epithelium of the gill filament. large arrow, pigment granules; G, Golgi apparatus; small arrow, rough endoplasmic reticulum; m, mitochondria; bm, basement membrane; IS, intercellular spaces; pv, pavement cell. Scale bar = 1.31 $\mu$m.—**D,** Mature chloride cell (cc) in the epithelium of the gill filament. v, vesicular system; arrow, rough endoplasmic reticulum; G, Golgi apparatus; pv, pavement cell. Scale bar = 2.11 $\mu$m.—**E,** Isolated mature chloride cell in the lamellar epithelium. v, vesicular system in the apical exposed surface; arrow, microvilli; G, Golgi apparatus. Scale bar = 1.31 $\mu$m.—**F,** Developing chloride cell in the lamellar epithelium, showing the abundance of rough endoplasmic reticulum (long arrow) and mitochondria. short arrows, pigment granules; G Golgi apparatus; pv, pavement cells. Scale bar = 1.31 $\mu$m.

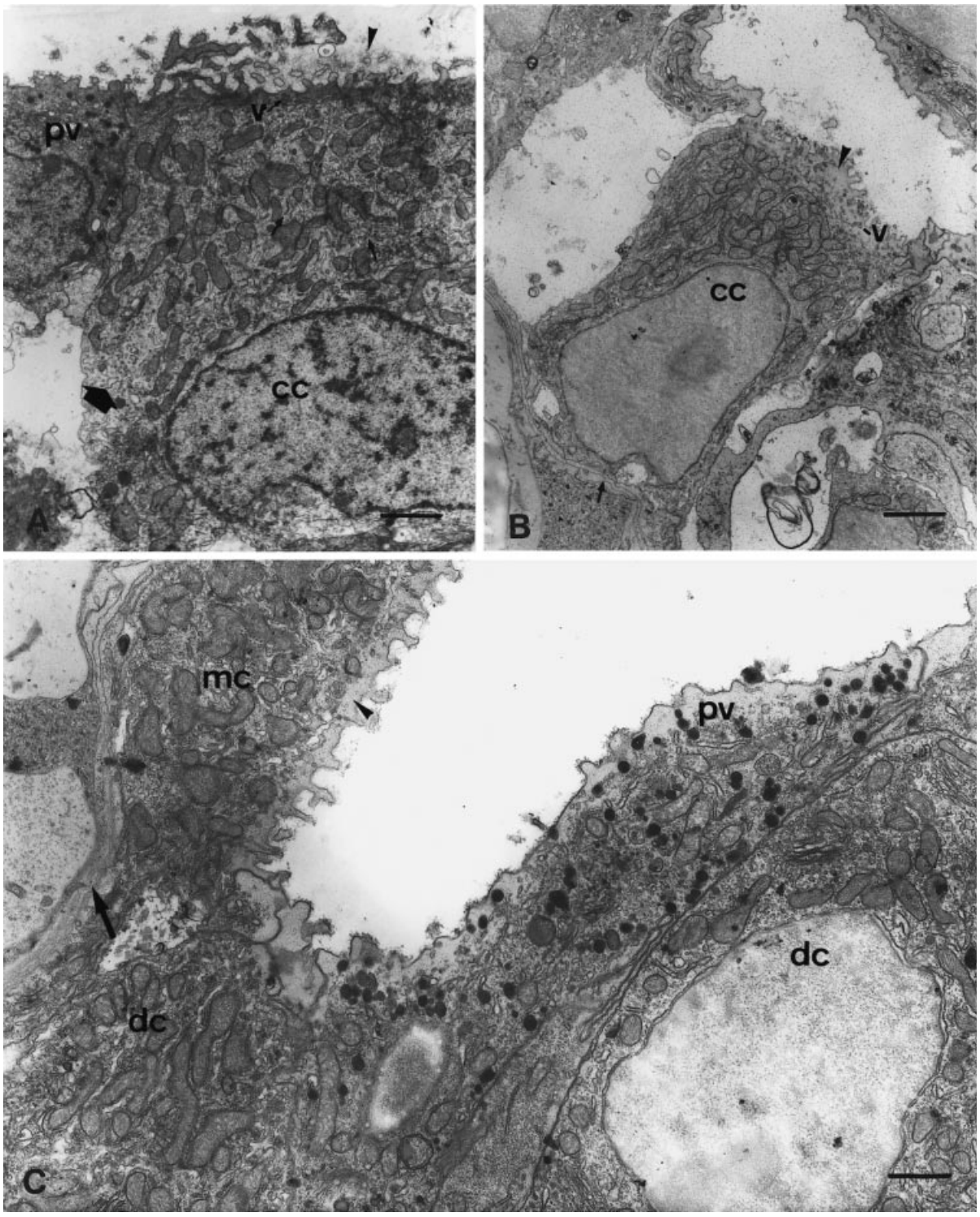



**Fig. 3**.—**A,** Mature chloride cell (cc) in the lamellar epithelium. small arrows, tubular system; large arrow, cisternae of rough endoplasmic reticulum; v, vesicular system; arrowhead, exocytotic vesicles; pv, pavement cell. Scale bar = 1.31 $\mu$m.—**B,** Mature chloride cell (cc) at the base of the lamellae. arrow, basement membrane; v, vesicles; arrowhead, cytoplasmic band of cytoskeleton elements. Scale bar = 1.31 $\mu$m.—**C,** Interlamellar region. dc, developing chloride cells; pv, pavement cells; mc, mature chloride cell; arrowhead web of cytoskeleton elements; arrow, basement membrane. Scale bar = 0.71 $\mu$m.

desmosomes and tight junctions (Fig. 4B). Attachments between neighbouring CCs are different from those between CCs and pavement cells. The lateral membranes of two adjacent CCs of the interlamellar region, show neither interdigitations nor desmosomes (Fig. 5A). This kind of junctional appositions resemble leaky pathways.

Degenerating CCs were also observed in the gill epithelium of newly hatched stages. These cells display ultrastructural signs corresponding with apoptosis (physiologically programmed cell death). The major occurrence of apoptotic CCs took place in the interlamellar epithelium as can be inferred from the images of Figs 1A and 5C. We have detected advanced stages of apoptosis charaterized by the formation of apoptotic bodies which include cytoplasmic (Fig. 5C–E) and/or nuclear fragments. These apoptotic bodies appeared engulfed in the cytoplasm of a phagocytic cell (Fig. 5B–D) which was usually located in the proximities of blood vessels.

## Discussion

### *Distribution of chloride cells*

The results of this study clearly show that the CCs of the gill epithelium of newly hatched rainbow trout are not only found in the filament and at the base of the lamellae (Laurent 1984; Witters *et al.* 1996), but also in the epithelium of the lamellae. This confirms the reports of Laurent *et al.* (1985) on adult salmonids reared in natural soft-water streams or hatcheries, and those of Hwang (1988) on freshwater teleost larvae. Although Dunel and Laurent (1980) reported the presence of CCs only on the afferent side of the filament, we found that in trout, as has been shown in other species (Wendelaar Bonga and van der Meij 1989; Pisam *et al.* 1995), the epithelium of the afferent as well as the efferent side of the filament, display chloride cells. As in brown trout (Rojo 1997), in rainbow trout alevins, the CCs are more numerous on the efferent side of the filament.

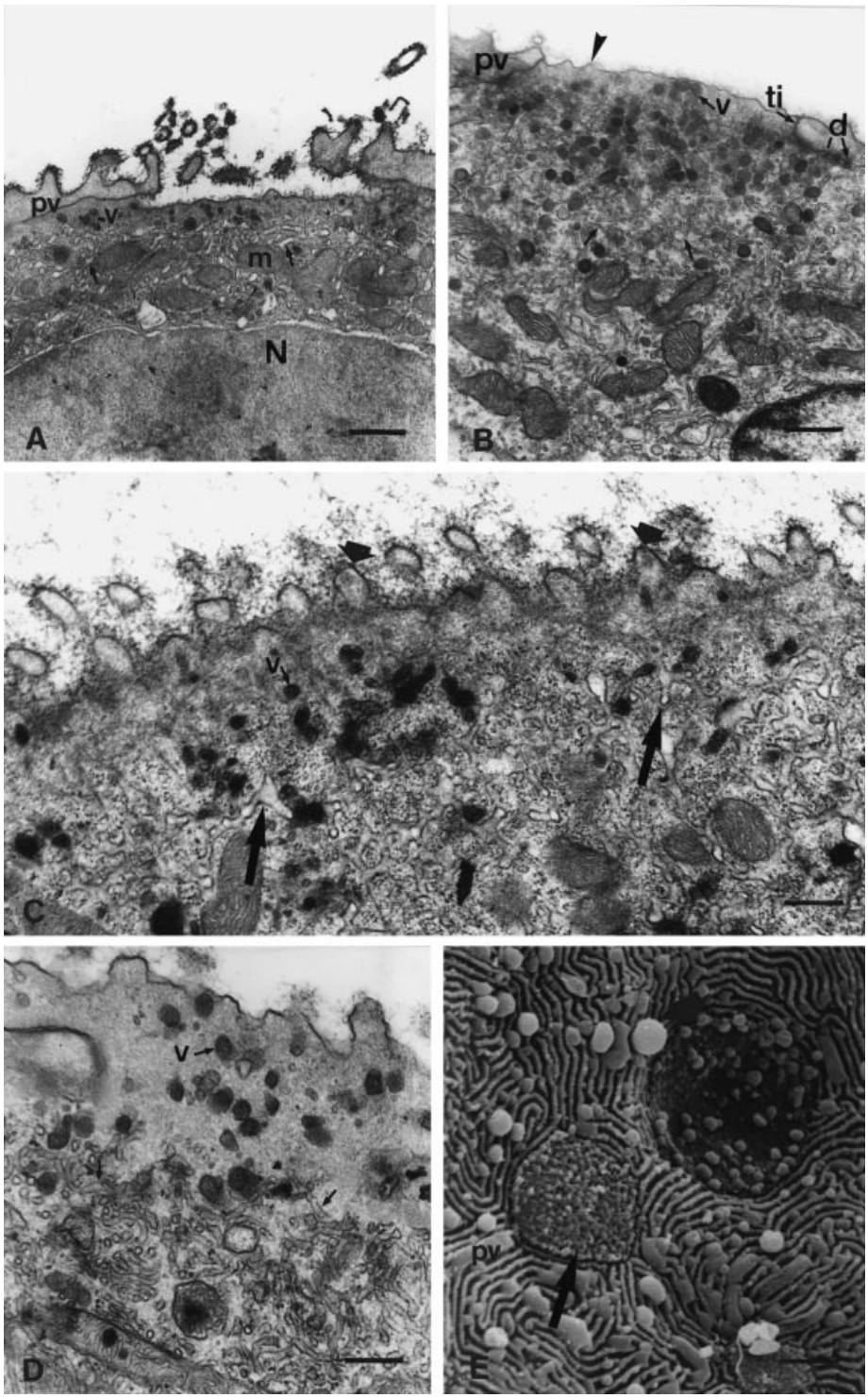
Osmoregulation in fish embryos and larvae has been investigated by a few authors who related the osmoregulatory function to the presence of CCs (Shen and Leatherland 1978; Guggino 1980; O'Connell 1981). The numerical density and the distribution of CCs in teleost larvae seem to vary depending on both the species and the different requirements for osmoregulation (Hwang 1989). This author has shown that CCs are not present in the gill epithelium of newly hatched alevins of flounder and ayu. However, in both rainbow trout and brown trout, the CCs appeared before hatching, when the filaments began to develop from the gill arches, and then the number and maturation of these cells quickly increased in post-hatching stages (González *et al.* 1996; Rojo 1997).

In addition to immature or developing stages of the CCs, we found mature and apoptotic CCs. Immature, mature and apoptotic CCs, were mainly distributed in the interlamellar region and the lamellar epithelium. This common finding in the gill epithelium of rainbow trout alevins reared under natural freshwater conditions, reveals an increased cell turn-over in the lamellar and filament epithelium.

Proliferation of branchial CCs, is the normal response during exposure of fish to dilute media such as freshwater (Laurent *et al.* 1985; Avellá *et al.* 1987; Perry and Laurent 1989). According to the hypothesis suggested by Wendelaar Bonga *et al.* (1990), the diversity of ultrastructural features for the CCs may be the result of a complex synergy between external (water ion content, acid-base disturbances, feed) and internal (endocrine, branchial gas and ion exchange) factors. The extremely variable ionic concentration of freshwater media (Laurent *et al.* 1985) is thought to be the cause of adaptive responses of the CCs, including compensatory physiological (Avellá *et al.* 1987; Perry and Laurent 1989) and morphological adjustments. With regard to the latter, we detected a number of qualitative modifications in the distribution and morphology of the CCs. Thus, most of the CCs located both in the interlamellar region and at the base of the lamellae, formed multicellular complexes. Such multicellular complexes have been shown to contain leaky junctions in larvae in seawater (Hwang 1988; King *et al.* 1989; King and Hossler 1991). These leaky junctions are known to provide paracellular pathways for the ionic exchange mechanisms in seawater (Karnaky 1986) as well as in freshwater fish (Hwang 1988; King and Hossler 1991). Since salinity tolerance depends upon the ability to modify junctional structures and intercellular organization, multicellular complexes have been demonstrated to reflect the ability of fish to adapt to different salinities (Hwang and Hirano 1985; King and Hossler 1991). Clusters of CCs have been mainly described in seawater fish (Laurent 1984) although groups of these cells have been reported in freshwater species (Laurent and Dunel 1980).

The present results show that clusters of CCs are common in rainbow trout alevins in the same locations as Hwang (1988) reported for other species of freshwater teleosts. In the epithelium of the afferent and efferent sides of the filament, the CCs did not form multicellular complexes since the cytoplasm of a pavement cell was always present between two neighbouring CCs. It seems reasonable to think that the presence of multicellular complexes in the gill epithelium of newly hatched rainbow trout reared under natural freshwater conditions, may point to physiological stress, but it is also possible that these complexes are typical for cells developing in the high-salinity environment of the embryos (the fluid in the egg capsules).



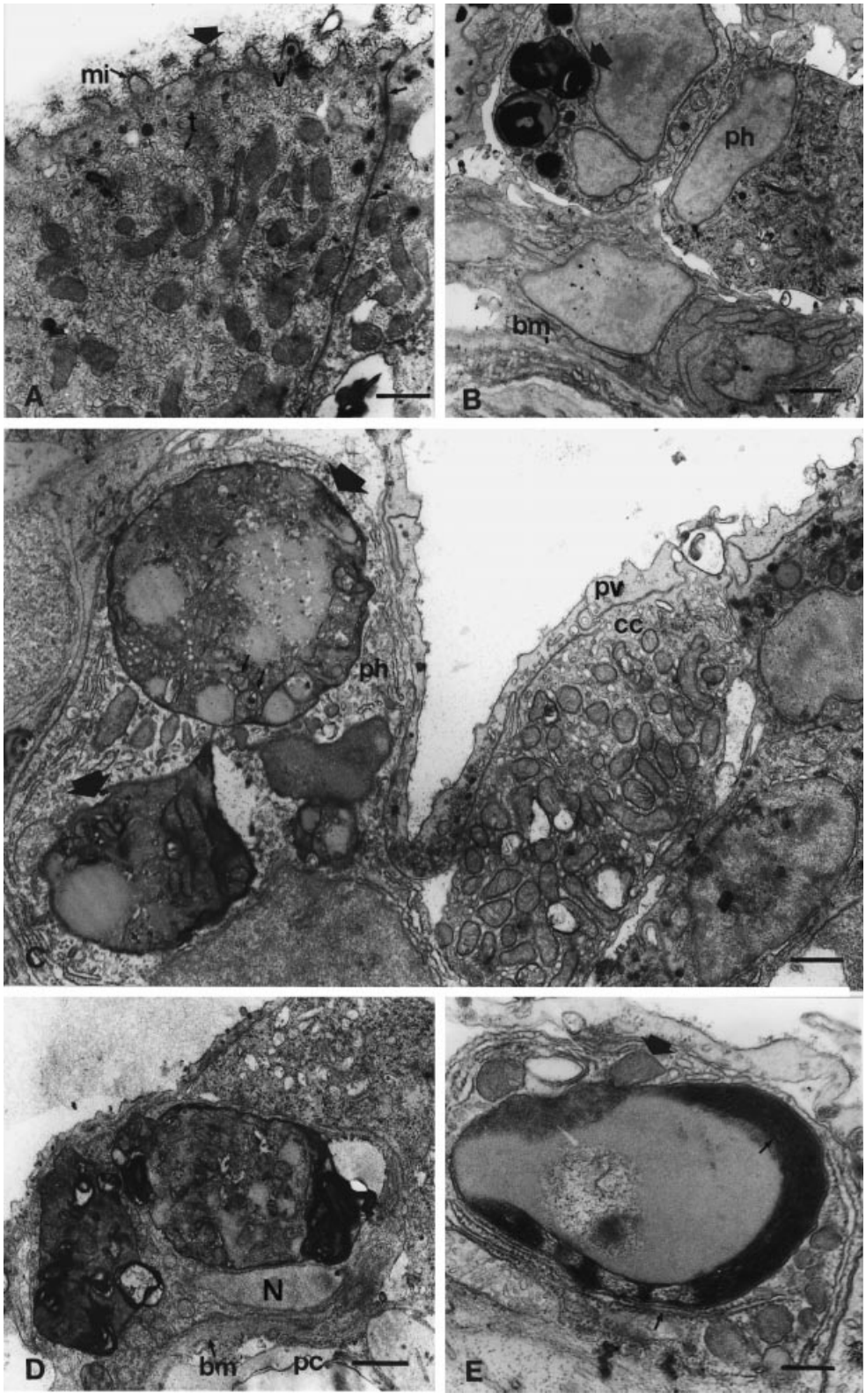
*Morphology of chloride cells*

Multiple sizes and shapes were shown by the CCs in the gill epithelium of rainbow trout alevins, as well as variations in the apical region of the cells and in the cytoplasmic content. This heterogeneity in the morphology and ultrastructure of the CCs occurs regardless of the location of the cells, and seems to represent successive stages of the chloride cell cycle, as Wendelaar Bonga *et al.* (1990) stated for freshwater tilapia. A few investigators have noted the ultrastructural variation of the CCs population in the gill epithelium of euryhaline freshwater teleosts (Pisam *et al.* 1987; 1989; King *et al.* 1989; Franklin 1990) and stenohaline (Shen and Leatherland 1978; Avellá *et al.* 1987; Perry and Laurent 1989). Pisam *et al.* (1987) distinguised two types of CCs-$\alpha$ and $\beta$-on the basis of their location, shape and cytoplasmic features. In addition, accessory CCs have been shown to be either a specific chloride cell type of seawater fish (Sardet *et al.* 1979; Laurent and Dunel 1980; Chretien and Pisam 1986) or a developing chloride cell (Hootman and Philpott 1980).

In this study we have found CCs which share features of both the so-called $\alpha$ and $\beta$ CCs (Pisam *et al.* 1987). The so-called $\alpha$ CCs are lightly stained columnar cells located at the base of the lamella, with a poorly developed vesicular system but with a tubular system distributed throughout the cytoplasm except for a narrow apical zone. The $\beta$ CCs are more intensely stained ovoid cells located in the interlamellar region, having a conspicuous rough endoplasmic reticulum and a well developed vesicular system. The developing CCs described show some resemblance to the $\alpha$ CCs, and our mature cell with the $\beta$ CCs defined by Pisam *et al.* (1987). However, there were also differences. The columnar CCs of our rainbow trout alevins were frequent both singly or in groups in the interlamellar region as well as in the lamellae. On the other hand, ovoid CCs appearing in both the filament and the lamellar epithelium, showed either a well-developed or a poorly developed vesicular system, regardless of the location of the CCs. Light or dark staining of the CCs did not relate to the presence of a developed tubular system or any other specific ultrastructural feature. Moreover, a few CCs shared ultrastructural features with the accessory cells (showed an extensive endoplasmic reticulum and poor development of the tubular system) which have also been reported in freshwater fish (Chretien and Pisam 1986; Pisam *et al.* 1989; Wendelaar Bonga *et al.* 1990; King and Hossler 1991). There were no clear differences between the cells resembling accessory cells and those resembling young CCs, so that our results are in agreement with those of Wendelaar Bonga *et al.* (1990) who proposed naming the accessory cells 'replacement cells'. The present findings corroborate those of a recent study carried out on brown trout (*Salmo trutta*) by Rojo *et al.* (1997). From both studies we can conclude that there are no clearly defined morphological characteristics to distinguish different subtypes of CCs. There exists only a few distinct morphological and ultrastructural features to distinguish mature from inmature CCs (Fig. 6). The former are characterized by frequently display elongated or columnar shapes, a variable number of membranous tubules and a well-developed vesicular system. The apically located vesicular system appears to be related to a wide exposed apical area bearing microvilli or microplicae covered by a specific fuzzy coat.

The immature CCs display the following features: smaller size, ovoid, rounded or irregular shapes; a tubular system which shows different developmental degrees depending on the degree of maturation of the cell, whereas the vesicular system is absent or poorly developed. On the contrary, the cytoplasm of these immature CCs is rich in rough endoplasmic reticulum cisternae, and one or more Golgi apparatus is commonly found in the different stages of young chloride cells, although it is also present in mature CCs. Therefore, the presence of Golgi apparatus is not a characteristic feature of immature CCs, as Wendelaar Bonga *et al.* (1990) reported in freshwater tilapia. Our study shows that CCs contain either anastomosed or unfenestrated tubules. Despite this fact, both cell types can be considered as mature CCs. The stainability of the CCs does not seem to be related to the development of the tubular system, so that it can not be taken as a distinct feature to classify the CCs in different subtypes. In fact, an alternate explanation to the varying staining characteristics of the CCs has been put forward by Wendelaar Bonga and van der Meij (1989). They contend that the darker-staining CCs may represent cells that are undergoing degeneration rather than different subtypes.

**Fig. 4.**—**A,** Apical structures in a developing chloride cell of the lamellae. v, electrondense vesicles; N, nucleus; m, mitochondria; arrows, cisternae of rough endoplasmic reticulum; pv, pavement cell. Scale bar = 0.81 $\mu$m.—**B,** Apical surface of a mature chloride cell in the lamellar epithelium. The apical exposed surface displays short microvilli (arrowhead). v, vesicular system; arrows, membranous tubules; pv, pavement cells; d, desmosomes; ti, tight junction. Scale bar = 0.81 $\mu$m.—**C,** Apical structures of a mature chloride cell in the filament. v, electrondense vesicles; short arrow, microplicae covered by a fuzzy coat. The tubular elements anastomose and branch (long arrows). Scale bar = 0.421 $\mu$m.—**D,** Apical surface of a mature chloride cell in the lamellae. The apical vesicles (v) distribute in the apical web of cytoskeleton elements, whereas the membranous tubules (arrows) locate beneath them. Scale bar = 0.351 $\mu$m.—**E,** Scanning electron micrograph of the interlamellar region, showing the different morphologies of the apical surfaces of chloride cells: protruding surface projections (long arrow) and smooth oval holes (short arrow). pv, apical microridges of the pavement cells. Scale bar = 51 $\mu$m.



Apart from mature and immature CCs and cells with intermediate ultrastructural features, we described the presence of apoptotic chloride cells. Such cells were mainly found at the base of the lamellae and/or in the interlamellar region, where few developing CCs were present. We suggest, together with Laurent *et al.* (1985), that the lamellar epithelium is capable of sustaining CC renewal in the same way it occurs in the filament epithelium (Conte and Lin 1967). However, it is likely that the differentiation of CCs takes place from migrating cells of the filament epithelium (Mackinnon and Enesco 1980; Rojo 1997) rather than from stem cells (Laurent and Dunel 1980). According to Laurent *et al.* (1985) it seems that the proliferation of CCs is not simply a transient modification but a permanent adaptive response to dilute freshwater.

*Apoptotic chloride cells*

Apoptosis of CCs has been reported under pathogenic (Daoust *et al.* 1984; Mallat 1985) and physiological (Wendelaar Bonga and Van der Meij 1989; Wendelaar Bonga *et al.* 1990) conditions in adult fish. More recently, Rojo *et al.* (1997) showed that apoptosis of CCs took place in the gill epithelium of embryos and alevins of brown trout. In both species, brown and rainbow trout, the apoptotic CCs were especially abundant in the gill epithelium, during the hatching and post-hatching stages.

The final stage of apoptosis is the formation of apoptotic bodies which include the cytoplasmic and/or nuclear remnants of the CCs. In rainbow trout alevins, these apoptotic bodies are engulfed by phagocytic cells (a macrophage-like cell) to be removed from the epithelium. This finding seems to be in contrast with those that we have previously shown in brown trout embryos and alevins (Rojo *et al.* 1997). In brown trout we found no evidence of the final formation of apoptotic bodies and their phagocytosis, only initial and intermediate stages of the apoptotic process. However, in alevins of rainbow trout, the evidence of the final stages of apoptosis lead us to confirm the existence of phagocytosis of the apoptotic bodies from CCs, as Wendelaar Bonga and Van der Meij (1989) reported in adult tilapia. Nevertheless, macrophage-like

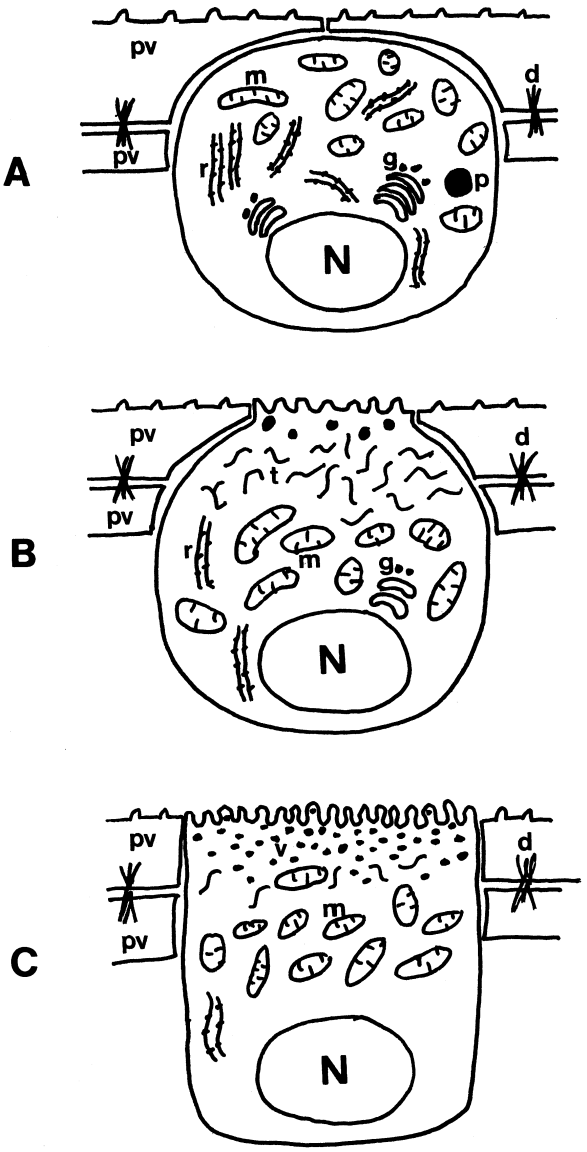



**Fig. 6.** Diagram of the different chloride cell stages, from immature (**A**) to mature (**C**) chloride cell. (**B**) represents a intermediate maturation stage. N, nucleus; m, mitochondria; r, rough endoplasmic reticulum; p, pigment granules; g, Golgi apparatus; t, tubular system; v, vesicular system; pv, pavement cells; d, desmosomes.

**Fig. 5.**—**A,** Mature chloride cells in the interlamellar region, showing apposition of their lateral plasma membranes (small arrows); v, electrondense vesicles; t, anastomosed elements of the tubular system; large arrow, exocytotic figure; mi, microvilli. Scale bar = 1.11 $\mu$m.—**B,** Apoptotic bodies (arrow) engulfed in the cytoplasm of a phagocytic cell in the interlamellar region. The neighbouring cell shows ultrastructural features resembling a phagocytic cell (ph). bm, basement membrane. Scale bar = 1.51 $\mu$m.—**C,** The interlamellar epithelium shows both phagocytic (ph) and developing chloride (cc) cells. The cytoplasm of the phagocytic cell engulf large lysosome-like bodies (big arrows) containing cytoplasmic remnants probably representing apoptotic bodies of chloride cells. small arrows, mitochondria remnants; pv, pavement cell. Scale bar = 0.681 $\mu$m.—**D,** Apoptotic bodies probably derived from chloride cells, engulfed in a phagocytic cell located at the base of the lamellae. N, nucleus of the phagocytic cell; bm, basement membrane; pc, pillar capillary. Scale bar = 1.51 $\mu$m.—**E,** Part of a phagocytic cell showing an apoptotic body containing remnants of both rough endoplasmic reticulum (small arrows) and probably mitochondria (big arrow). Scale bar = 0.351 $\mu$m.

cells or structurally similar cells described as leucocytes, have been observed in the intercellular or lymphoid spaces of several species (Laurent 1984) including the rainbow trout (Morgan and Tovell 1973) and the brown trout (Rojo *et al*. 1997). In brown trout, we ruled out the possibility that both leucocytes and intercellular spaces may play a role in the removal of the apoptotic bodies.

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