

## Effects of Environmental $\text{Cl}^-$ Levels on $\text{Cl}^-$ Uptake and Mitochondria-rich Cell Morphology in Gills of the Stenohaline Goldfish, *Carassius auratus*

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**Il-Chi Chang, Tsung-Han Lee, Hong-Chih Wu and Pung-Pung Hwang (2002)** Effects of environmental  $\text{Cl}^-$  levels on  $\text{Cl}^-$  uptake and mitochondria-rich cell morphology in gills of the stenohaline goldfish, *Carassius auratus*. *Zoological Studies* 41(3): 236-243. The objective of the present study was to examine the correlation between morphological changes in gill MR cells and modulations in  $\text{Cl}^-$  uptake in a stenohaline teleost. Goldfish (*Carassius auratus*) were acclimated to high- $\text{Cl}^-$  and low- $\text{Cl}^-$  artificial freshwater for 1 wk, and then the numbers of gill MR cells with different morphologies as well as whole-body  $\text{Cl}^-$  influx were measured. Based on the size and ultrastructural features of the apical surface, gill MR cells in goldfish were classified into types I, II, and III. Type III MR cells display the largest apical dimension (6-9  $\mu\text{m}$ ); type II MR cells are of intermediate size (3-6  $\mu\text{m}$ ); while type I MR cells are the smallest (1-3  $\mu\text{m}$ ). Comparing the morphological and flux data, a positive correlation exists between enhanced  $\text{Cl}^-$  influx and the appearances of type III MR cells in the low- $\text{Cl}^-$  group. In contrast, fish exposed to high- $\text{Cl}^-$  developed type I MR cells (1-3  $\mu\text{m}$ ) in the gills and showed a reduced  $\text{Cl}^-$  influx. These results suggest that MR cells expand their apical surface to enhance their capacity for  $\text{Cl}^-$  uptake. <http://www.sinica.edu.tw/zool/zoolstud/41.3/236.pdf>

**Key words:** Mitochondria-rich cells, Apical surface, Chloride, Uptake.

The gill is the most important extra-renal organ responsible for ion regulation in teleosts. Mitochondria-rich (MR) cells have been suggested to be the major site for ion transport in fish gills (Laurent 1984). In seawater,  $\alpha$  (Pisam et al. 1987 1993) or deep-hole MR cells (Lee et al. 1996c) were described and claimed to be involved in the active secretion of  $\text{Cl}^-$  (Foskett and Scheffey 1982). Plenty of morphological, biochemical, and physiological data (Epstein et al. 1980, Foskett and Scheffey 1982, Zadunaisky 1984, Hwang 1987, Hwang et al. 1988 1989, Laurent and Perry 1991) support the model for  $\text{Cl}^-$  secretion in seawater by gill MR cells. On the other hand, polymorphism of MR cells was demonstrated in gill epithelia of freshwater teleosts (Lee et al. 1996a c, Pisam et al. 1987), and was suggested to be associated with the uptake of diverse ions, i.e.,  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{Ca}^{2+}$ , as well as with acid-base regulation

(Avella et al. 1987, Laurent and Perry 1990, Perry et al. 1992, Bindon et al. 1994). However, the complicated relationships between morphological differences and the uptake of diverse ions in gill MR cells in freshwater fish are still being debated (Perry 1997).

Recent studies focused on the relationships among  $\text{Na}^+$ ,  $\text{Cl}^-$ , and MR cells. In studies with injection of exogenous cortisol (Laurent and Perry 1990, Perry et al. 1992) or with treatment using ion-deficient media (Avella et al. 1987, Perry and Laurent 1989, Greco et al. 1996), adjustments in uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  were found to be synchronized with alternations in the apical area of MR cells in *Salmo gairdneri* and *Oncorhynchus mykiss*. Some other studies demonstrated correlations between MR cells and  $\text{Cl}^-$  uptake in *Ictalurus nebulosus* and *Salmo trutta* (Goss et al. 1992a, Morgan et al. 1994 1995), but they did not

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discuss which MR cell type is involved in Cl<sup>-</sup> uptake. Recently, we used artificial fresh water containing various ionic compositions (but without acid-base disturbance) for treatment and have identified 3 MR cell types with different apical morphologies in gills of tilapia (*Oreochromis mossambicus*) (Lee et al. 1996c). Moreover, the relative abundance of each cell type was correlated with ionic compositions in artificial media and particular ion uptake in tilapia. The so-called wavy-convex (with the largest apical surface) MR cells were suggested to be responsible for NaCl uptake (Chang et al. 2001). In the skin of tilapia larvae, an increase in the density of wavy-convex MR cell was also found to be associated with enhanced Cl<sup>-</sup> influx (Lin and Hwang 2001).

In addition, these previous studies mostly focused on euryhaline species as described above: tilapia, *O. mossambicus* (Lee et al. 1996c), guppy, *Lebistes reticulatus* (Pisam et al. 1987), or salmonids (Avella et al. 1987, Laurent and Perry 1990, Bindon et al. 1994, Morgan et al. 1994 1995, Greco et al. 1996). It is important to study stenohaline species in order to more clearly elucidate the correlation between the morphologies and functions of gill MR cells, because stenohaline teleosts inhabit and adapt to various hypotonic media (such as soft water, hard water, and acidic water), which exhibit considerably different ionic compositions.

In early morphological or cytochemical studies (Kikuchi 1977, Ishihara and Mugiya 1987), the function of MR cells in stenohaline freshwater teleosts was suggested to be associated with ion uptake. In comparing the relative abundance of different gill MR cell types in several stenohaline teleosts (medaka, *Oryzias latipes*; carp, *Cyprinus carpio*; and goldfish, *Carassius auratus*) acclimated to artificial fresh water, Lee et al. (1996a b) suggested that these different types of gill MR cells may carry out different functions. However, these authors provided no convincing physiological data (such as ion influx) to demonstrate the functions of gill MR cells in stenohaline freshwater

teleosts. Therefore, the goal of the present study was to investigate the physiological functions of gill MR cells in the stenohaline teleost, the goldfish. Based on data from euryhaline teleosts as described above, the present study was designed to examine the relationship between Cl<sup>-</sup> uptake and the relative abundances of different MR cell types in goldfish acclimated to various levels of environmental Cl<sup>-</sup>.

## MATERIALS AND METHODS

### Animals

Goldfish (*Carassius auratus*) weighing 0.3-0.7 g were obtained from laboratory stocks. All individuals were reared in 26-28°C aerated local tap water with a photoperiod of 12L:12D before the acclimation experiments.

### Acclimation experiments

Two kinds of artificial fresh water, low-Cl (L-Cl) and high-Cl (H-Cl), were prepared by adding appropriate amounts of NaCl, Na<sub>2</sub>SO<sub>4</sub>, MgSO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and CaSO<sub>4</sub> to double-deionized water (Milli-RO60, Millipore, Bedford, MA, USA). The ion concentrations of K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> in artificial freshwater were near the ranges found in local fresh water. Na<sup>+</sup> in artificial media was maintained at a high level, 12 mM, because low environmental Na<sup>+</sup> levels would inhibit Cl<sup>-</sup> influx according to our previous study (Chang and Hwang 2001). The ionic compositions (Table 1) of the 2 media were confirmed by measuring the Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> concentrations with an atomic absorption spectrophotometer (Hitachi Z-8000, Tokyo, Japan) and Cl<sup>-</sup> with a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The pH of the media was kept between 6.2 and 6.7, and water temperatures were 26-28°C. Goldfish were acclimated to the H-Cl and L-Cl media for 1 wk, and then were sampled for morphological obser-

**Table 1.** Ionic compositions (mM) in artificial fresh water

Medium	[Na <sup>+</sup> ]	[Cl <sup>-</sup> ]	[Ca <sup>2+</sup> ]	[K <sup>+</sup> ]	[Mg <sup>2+</sup> ]
L-Cl	12.4 ± 2.2	< 0.001	0.18 ± 0.01	0.39 ± 0.04	0.17 ± 0.03
H-Cl	12.74 ± 1.7	7.8 ± 0.3	0.18 ± 0.01	0.40 ± 0.05	0.22 ± 0.04

Mean ± SE (n = 7).

vations (4-6 individuals for each test) and ion influx measurements (4-6 individuals for each test). In order to maintain the water quality, the acclimation media were aerated with a filtered air pump and changed every 2 d. Fish were fed on commercial pellets 1 h immediately prior to the water change, but feeding was stopped 3 d before sampling.

### Morphology of gill MR cells

After anesthetization with MS222, 4-6 fish from each medium were sacrificed to excise their gills. The 1st gill arch from each side was fixed at 4°C in fixative consisting of 5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.2) for 12 h. After rinsing with 0.1 M PB, specimens were post-fixed with 1% osmium tetroxide in 0.2 M PB for another 1 h. After rinsing with PB and dehydration with ethanol and acetone, specimens were critical-point-dried using liquid CO<sub>2</sub> in a critical-point drier (Hitachi HCP-2, Tokyo, Japan), and sputter-coated for 3 min with a gold-palladium complex in a vacuum evaporator (Eiko 1B-2, Tokyo, Japan). Coated specimens were examined in a scanning electron microscope (Hitachi S-2500, Tokyo, Japan) at an accelerating voltage of 15 kV.

It was previously found that MR cells are concentrated on the afferent and interlamellar regions of the filament, and only scattered MR cells are found on the efferent filamental surface and lamellae in goldfish gills (Lee et al. 1996a). Densities of different types of MR cells were measured according to Lee et al. (1996a c). Areas on the afferent side of the filament near the lamellae were chosen at random for counting at 1250 × magnification to measure the densities of different types of MR cells. One area (4250 μm<sup>2</sup> each) was counted on each of 2 branchial filaments from a fish. An average for each morphological parameter of an MR cell in these 2 areas was obtained. Six individuals per group were examined.

### Ion influxes

Whole-body Cl<sup>-</sup> influx was measured following Wood (1992) with some modifications. Tracer media were prepared by adding appropriate amounts of <sup>36</sup>Cl<sup>-</sup> (Amersham, Piscataway, NJ, USA) to artificial freshwater ([Ca<sup>2+</sup>] = 0.148 mM, [Na<sup>+</sup>] = 0.547 mM, and [Cl<sup>-</sup>] = 0.308 mM) to give a final working specific activity of <sup>36</sup>Cl<sup>-</sup>, (2.1-2.6) × 10<sup>5</sup>/cpm/μmole. After rinsing briefly with deionized water, fish from each acclimation medium were

transferred to plastic flux chambers with 20 ml of tracer media for 2.5 h. The tracer media in flux chambers were gently aerated, and the water qualities were confirmed to show no significant change during the period of incubation. The plot of accumulated radioisotope against time was linear within the first 6 h (*r* = 0.974; *n* = 6; sampling times: 1, 2, 3, 4, 5, and 6 h), and the calculated influxes at each period during the first 6 h were constant. Moreover, preliminary experiments with fish over-anesthetized on ice indicated that radioisotope adhering to the surfaces of flux chamber and fish accounted for less than 2% of the total radioisotope in the chamber, and the amount becoming adhered reached saturation within the first 10 min of incubation (*n* = 5; sampling times: 0, 10, 20, 30, 60, and 150 min). Therefore, water samples (100 μl) were collected at 0.5 and 2.5 h after incubation in order to exclude the effects of adhering radioisotopes. Counting solution (Fluoran-safe Scintran, BDH, Poole, UK) was added to water samples from the <sup>36</sup>Cl<sup>-</sup> media, and then radioactivities were counted with a beta counter (LS6500, Beckman, Fullerton, CA, USA). Volumes of the tracer media were measured after 0.5 and 2.5 h of incubation. The ion influxes in fish from different acclimation media were measured at the same levels of ions in order to compare the ion uptake capacities.

Cl<sup>-</sup> influx was calculated by the following formula:

$$J_i = (Q_i \times V_i - Q_f \times V_f) \div (1/2 \times (SA_i + SA_f) \times t \times W);$$

where *Q<sub>i</sub>* and *Q<sub>f</sub>* (cpm · mL<sup>-1</sup>) refer to the initial (0.5 h) and final (2.5 h) radioactivities in the tracer media, *V<sub>i</sub>* and *V<sub>f</sub>* (mL) refer to initial and final volumes of the tracer media; *SA<sub>i</sub>* and *SA<sub>f</sub>* are initial and final specific activities (cpm/μmole), *t* (2 h) is incubation time, and *W* (g) is body weight of the fish. Decay of the radioisotope during influx experiments was corrected for. Since all water samples were in the same condition, the effects of quenching on data were excluded through calculation.

### Statistical methods

All values are presented as the mean ± SE (*n*). The significance of difference between treatments was assessed by Student's *t*-test, and the significance of intra-treatments was assessed by one-way ANOVA (using Tukey's pairwise comparison).

## RESULTS

### Gill MR cells in different artificial freshwater media

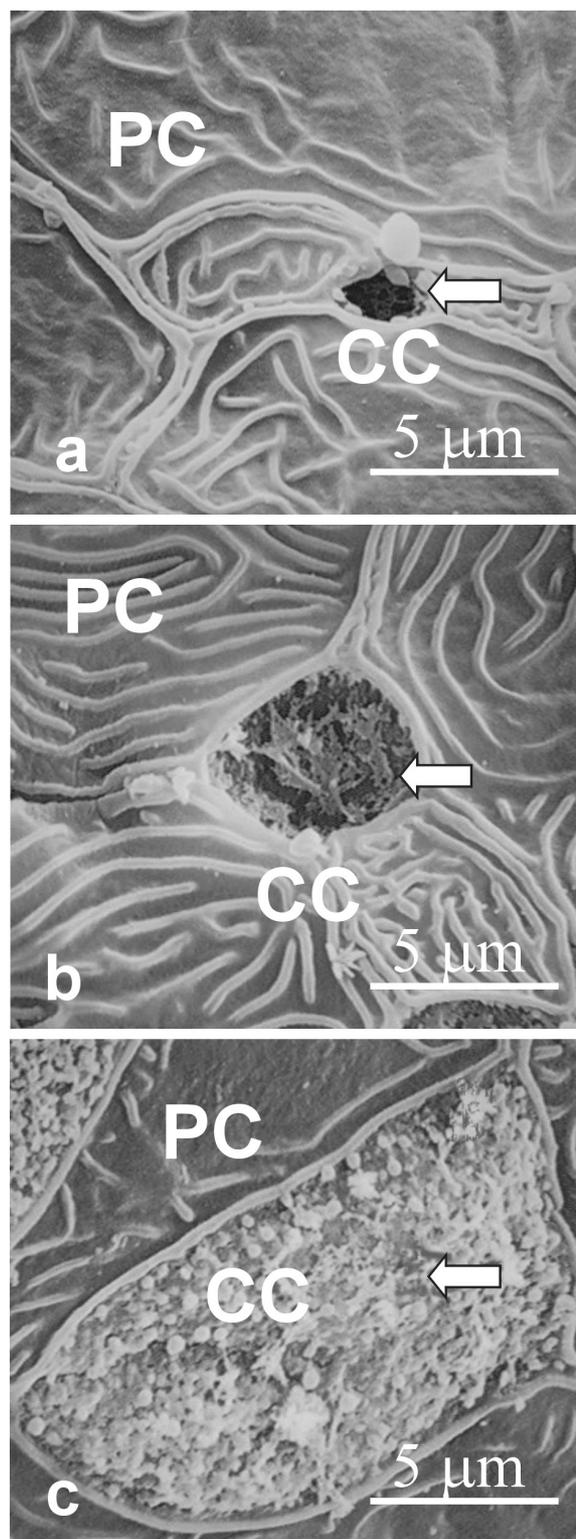
Based on the longest diameter and ultrastructural features of the apical surface, 3 MR cell types were differentiated in goldfish gill filament. Type I MR cells display deep, narrow apical pits with a diameter of 1-3  $\mu\text{m}$  (Fig. 1a). Broad and shallow apical crypts with apical dimensions of 3-6  $\mu\text{m}$  appear in type II MR cells (Fig. 1b). Type III MR cells, with the maximum dimension of 6-9  $\mu\text{m}$ , display a flattened or convex apical surface (Fig. 1c). There was no significant difference in total MR cell density between the H-Cl and L-Cl groups (Table 2). However, 7-d acclimation caused a significant difference in relative abundances of types I and III MR cells. H-Cl treatment resulted in type I MR cells dominating (100%) in gills of the H-Cl group (Fig. 2a, Table 2). On the contrary, L-Cl treatment resulted in an increase in type III MR cells, which reached 95% of total MR cells (Fig. 2b, Table 2). This indicates that declining environmental Cl<sup>-</sup> concentrations stimulate the appearance of type III MR cells. Type II MR cells showed no significant difference in their relative abundance between the 2 treatments.

### Cl<sup>-</sup> influx in different artificial freshwater media

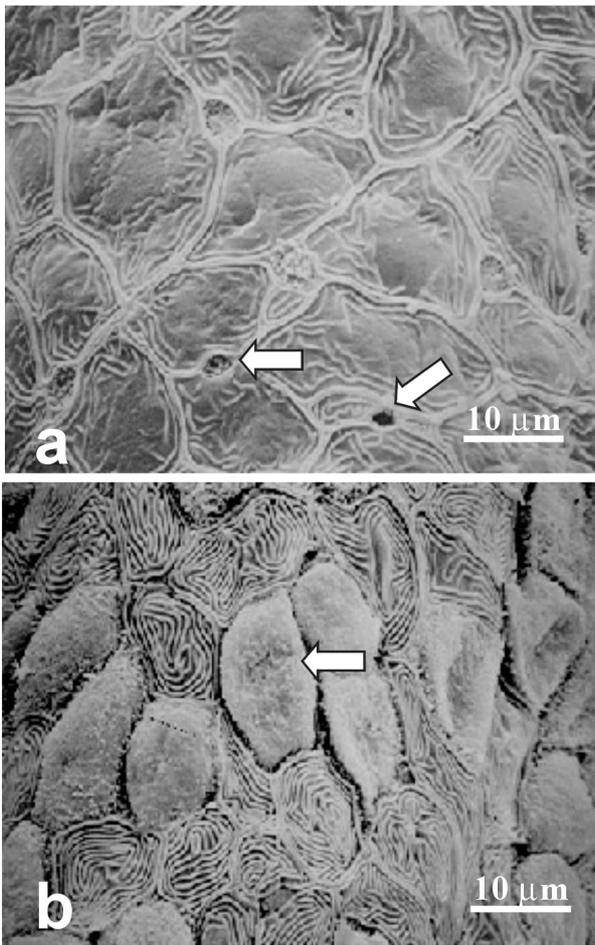
Cl<sup>-</sup> influx in L-Cl tilapia was about 3.9 times higher than that in H-Cl tilapia (Table 2). This result indicates that declining environmental Cl<sup>-</sup> levels stimulate Cl<sup>-</sup> influx in goldfish.

## DISCUSSION

Using transmission electron microscopy, Kikuchi (1977) was the first to find the existence of MR cells in gills of stenohaline freshwater teleosts such as goldfish (*C. auratus*), carp (*C. carpio*), and loach (*Misgurnus anguillicaudatus*). Pisam et al. (1990) reported that at least 2 types of MR cell types appear in the stenohaline freshwater gudgeons (*Gobio gobio*) as in euryhaline teleosts (Pisam et al. 1987). Ishihara and Mugiya (1987) localized Ca<sup>2+</sup> with an oxalate method and suggested the function of Ca<sup>2+</sup> uptake by gill MR cells in goldfish (*C. auratus*). These previous studies, however, provided no convincing physiological data, such as ion influx, to support their inference of the functions of gill MR cells in stenohaline



**Fig. 1.** Scanning electron microscopic images of branchial MR cells with different apical morphologies in freshwater goldfish. Arrows indicate MR cells with different apical surfaces. a, type I MR cell; b, type II MR cell; c, type III MR cell; CC, MR cell; PC, pavement cell.



**Fig. 2.** Scanning electron microscopic images of branchial MR cells in goldfish acclimated to H-Cl (a) and L-Cl (b) media. Arrows indicate MR cells with different apical surfaces.

teleosts. The present study demonstrates for the 1st time that  $\text{Cl}^-$  uptake is associated with MR cells, which display expansion of their apical surfaces in goldfish, a stenohaline freshwater teleost.

Morgan et al. (1994 1995) used x-ray microanalysis to demonstrate the  $\text{Cl}^-$  uptake function of gill MR cells in *S. trutta*, and Goss et al. (1992a b) also correlated the  $\text{Cl}^-$  uptake capacity with the apical fractional area of MR cells in *Ictalurus nebulosus* that was acclimated to acidic or alkaline conditions. However, these authors did not discuss which MR cell type is involved in  $\text{Cl}^-$  uptake. Moreover, acid-base disturbances can induce changes in the functions of MR cells as indicated in frog skin (Haverly 1992); it is unknown whether similar impacts of acid-base disturbances occur in fish gills. The present result that type III MR cells are associated with  $\text{Cl}^-$  uptake in goldfish gills has physiological significance, because goldfish were treated with artificial media with various ionic compositions at a constant pH to prevent impacts from acid-base disturbances.

In the present study, goldfish gill MR cells were classified into types I, II, and III (Fig. 1). Types I and III dominated respectively in particular hypotonic medium. In L-Cl medium, the dominant cell type was type III, whose apical morphology is similar to that of cells in the gills of cortisol-treated or  $\text{NaHCO}_3$ -infused rainbow trout (Perry and Goss 1994) and to the so-called wavy-convex MR cells in tilapia gills (Lee et al 1996c, Chang et al. 2001). Taken all together, stimulation of the capacity for  $\text{Cl}^-$  uptake appears to be associated with an

**Table 2.** Relation between whole-body  $\text{Cl}^-$  influx and the relative abundance of gill MR cells in goldfish acclimated to H-Cl and L-Cl media

Medium ion (mM)	Artificial freshwater medium	
	L-Cl	H-Cl
$\text{Na}^+$	$12.4 \pm 2.2$	$12.74 \pm 1.7$
$\text{Cl}^-$	$< 0.001$	$7.8 \pm 0.3$
MR cell type (longest apical diameter, $\mu\text{m}$ )	Cell number/unit area (% of total number)	
Total number	$8.4 \pm 1.1$ (100)	$6.1 \pm 1.3$ (100)
Type I (1-3)	$0.2 \pm 0.1$ (2.4) <sup>a</sup>	$6.1 \pm 1.3$ (100) <sup>a*</sup>
Type II (3-6)	$0.2 \pm 0.1$ (2.3) <sup>a</sup>	$0.0 \pm 0.0$ (0) <sup>b</sup>
Type III (6-9)	$8.0 \pm 0.9$ (95) <sup>b</sup>	$0.0 \pm 0.0$ (0) <sup>b*</sup>
$\text{Cl}^-$ influx (nmole/g/h)	$1272.7 \pm 117.6$	$323.2 \pm 28.5^*$

Mean  $\pm$  SD (%);  $n = 4$  for cell density and 4 for  $\text{Cl}^-$  influx. One-way ANOVA was conducted among different types of MR cells for each medium and different letters indicate a significant difference by Tukey's pairwise comparison. Student's  $t$ -test was conducted in different media for each type of MR cell, and an asterisk indicates a significant difference from the L-Cl group.

increase in gill MR cells with enlarged apical surfaces.

In the present study, enhanced Cl<sup>-</sup> uptake was correlated with type III MR cells with an expanded apical surface but not with type I MR cells with small apical pits. This implies that the apical surface area of MR cells plays a crucial role in the function of ion uptake. Goss et al. (1992a b) suggested that a Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger is involved in Cl<sup>-</sup> uptake, and Wilson et al. (2000) recently localized a Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger in the apical membranes of gill MR cells in tilapia. Schwartz et al. (1985) proposed that the number of transporter sites is under the control of rapid membrane recycling, which is accompanied by morphological modifications. Based on this evidence, we suggested that type III MR cells expand their apical membranes to express additional Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers, and consequently enhance their capacity for Cl<sup>-</sup> uptake.

Based on SEM observations, the present study shows that the 3 MR cell types with different apical morphologies exist in goldfish gills, which is consistent with the polymorphism of gill MR cells reported in euryhaline teleosts including tilapia (Pisam et al. 1987; Lee et al. 1996). It is an interesting question to relate the underlying relationships among these MR cell subtypes (Perry 1997). The present study cannot answer this question; however several previous studies provided some clues for it. Some studies (Shikano and Fujio 1998, Tsai and Hwang 1998, Hiroi et al. 1999) indicated that direct morphological transitions between different MR cell types do occur to deal with environmental challenges such as ion levels or salinity. Recently, Lin and Hwang (2001) pointed out that the remodeling of MR cells (especially in wavy-convex and deep-hole types) in tilapia larvae skin without a change in the total number of MR cells was required in response to low ambient Cl<sup>-</sup> medium. Our unpublished data also confirm that morphological conversions between wavy-convex and deep-hole gill MR cells are reversible in adult tilapia during acclimation to various levels of Cl<sup>-</sup>. In the rabbit collecting duct with acid perfusion, Schwartz et al. (1985) demonstrated that intercalated cells (i.e., MR cells in the mammalian kidney) switch function by direct conversion between different cell types. All these data suggest that MR cells regulate their functions by modifying of their morphology to deal with environmental challenges.

In summary, the present study provides convincing physiological data to support the idea that

modifications of the apical surface of MR cells are associated with regulation of the capacity for ion transport, and this appears to occur in both stenohaline and euryhaline teleosts.

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## 環境氯離子對窄鹽性金魚 (*Carassius auratus*) 的氯離子吸收 與鰓絲上MR細胞型態的影響

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本實驗以窄鹽性的金魚 (*Carassius auratus*) 為實驗材料，探討其鰓絲上MR細胞 (mitochondria-rich cell) 型態的改變與氯離子吸收間的關連性。將金魚分別馴養在高氯與低氯的人工水中7天，然後計算不同型態的MR細胞在這兩種處理下密度及魚體的氯吸收。根據MR細胞頂部的口徑大小與型態，金魚的MR細胞可分成3型：type 3 MR細胞具有最大的口徑 (6-9 μm)、type 2 MR細胞的口徑居次 (3-6 μm)，而type 1 MR細胞具有最小的口徑 (1-3 μm)。低氯處理組的MR細胞以type 3 MR細胞為主 (95%)；而高氯處理組則以type 1 MR細胞 (100%) 為主。至於氯離子吸收上，則是低氯處理組顯著高於高氯處理組。從上述結果可知，MR細胞可擴大其頂端面積，使氯離子吸收能力增加。

**關鍵詞：**富含粒線體細胞，頂部表面，氯離子，吸收。

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