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Calcium balance in embryos and larvae of the freshwater-adapted teleost, *Oreochromis mossambicus*

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Abstract

Changes in Ca^{2+} content and flux, and the development of skin chloride cells in embryos and larvae of tilapia, *Oreochromis mossambicus*, were studied. Tilapia embryos hatched within 96h at an ambient temperature of 26–28°C. Total body Ca^{2+} content was maintained at a constant level, about 4–8 nmol per individual, during embryonic development. However, a rapid increase in body Ca^{2+} level was observed after hatching, 12.8 to 575.3 nmol per individual from day 1 to day 10 after hatching. A significant influx and efflux of Ca^{2+} occurred during development, with the average influx rate for Ca^{2+} increasing from 5.9 pmol mg^{-1} h^{-1} at 48h postfertilization to 47.8 pmol mg^{-1} h^{-1} at 1 day posthatching. The skin was proposed as the main site for Ca^{2+} influx before the development of gills, and the increased Ca^{2+} influx may be ascribed to gradual differentiation of skin surface and chloride cells during embryonic development. Ca^{2+} efflux was 16–56 pmol mg^{-1} h^{-1} in 1-day-old larvae. The resulting net influx of Ca^{2+}, 10–12 pmol mg^{-1} h^{-1}, accounted for the increased Ca^{2+} content after hatching. When comparing the measured and estimated ratios of efflux and influx, active transport was suggested to be involved in the uptake of Ca^{2+}. Chloride cells, which may be responsible for the active uptake of Ca^{2+}, started to differentiate in the skin of embryos 48h after fertilization, and the density of chloride cells increased following the development. A possibility of active transport for Ca^{2+} in early developmental stages of tilapia is suggested.

Introduction

The ability of adult teleosts to maintain constant plasma Ca^{2+} levels in diverse calcium environments has been well documented (Pang *et al.* 1980). Freshwater teleosts actively accumulate Ca^{2+} from both food and surrounding medium. The gills, intestine, and kidneys are the most important sites for Ca^{2+} exchange between the external and internal environments. The gills, however, are believed to be the major route for direct absorption of Ca^{2+} from water (Perry and Wood 1985; Flik *et al.* 1985). Physiological studies have demonstrated that chloride cells are the sites responsible for the Ca^{2+} uptake in the gill of freshwater teleosts (Payan *et al.* 1981; Perry and Flik 1988; Marshall *et al.* 1992; McCormick *et al.* 1992; Perry *et al.* 1992).

Embryos and larvae of several teleosts, in which the gills or kidneys are poorly developed, have been found to be able to maintain constancy in the ion concentrations and osmolality of their body fluids

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(Alderdice 1988). The outer membrane of teleosts eggs, the chorion, is permeable to both salts and water but not to large molecules. Thus the vitelline membrane is the only effective barrier between embryos and the external medium (Eddy 1982). Similar to the adult, embryos and larvae have to face the freshwater environment which contains less Ca^{2+} than the body fluid. Brown an Lynam (1981) examined the mortality of brown trout, *Salmo trutta*, eggs incubated in freshwater with different concentrations of Na^+ and Ca^{2+} and suggested that a certain level of Ca^{2+} in the incubation water is of prime importance during its early development. Lee and Hu (1983) found that Ca^{2+} rather than Mg^{2+} is indispensable to the embryonic development of mullet (*Mugil cephalus*). Alderdice (1988) reported that the addition of Ca^{2+} and Na^+ to hatchery water of low ionic activity resulted in a significant reduction in mortality of chinook salmon (*Oncorchynchus tshawytscha*) alevins. Recently, ambient cadmium was found to inhibit Ca^{2+} uptake and result in death in the embryos and larvae of tilapia (*Oreochromis mossambicus*) (Fu and Lock 1990; Hwang *et al.* 1994). However, little is known about the mechanisms of Ca^{2+} balance in embryos or larvae. The purpose of this study was to elucidate the mechanism of Ca^{2+} balance in embryos and larvae of freshwater-adapted tilapia (*Oreochromis mossambicus*). The profile of Ca^{2+} content and fluxes during the embryonic and larval development were clarified, and differentiation of the skin surface and chloride cells were also studied to elucidate the possible mechanism of Ca^{2+} balance.

Materials and methods

Animals

Mature tilapia, *Oreochromis mossambicus*, were obtained from the Tainan Branch of the Taiwan Fisheries Research Institute and kept under natural photoperiod at 27 ± 1 °C in a 180l closed circulating system in which freshwater was controlled to a constant condition. The condition of freshwater was Na^+, 0.23 ± 0.01 mmol l^{-1}; K^+, 0.035 ± 0.015 mmol l^{-1}; Ca^{2+}, 0.17 ± 0.03 mmol l^{-1}; Mg^{2+}, 0.09 ± 0.02 mmol l^{-1}, which applied to all the freshwater used in this study. Fertilized eggs were retrieved from the mouths of females which had just started mouth-breeding (within 12h after fertilization) as described previously (Hwang and Wu 1993). The fertilized eggs were incubated in aerated 1000 ml bottles in a medium identical to that described above.

Table 1. The average time schedule and main developmental feature of *Oreochromis mossambicus* at 26–28°C

Hours after	Developmental feature
0–1	1 cell
1.5	2 cells
2	4 cells
3	8 cells
4	16–32 cells
12	Blastula, appearance of melanophore on yolk sac
24	Gastrula
36	Heart beating
48	Onset of blood circulation
60	Appearance of melanophore on optic lobes
72	Eye stage
96	Hatching

Embryos were dechorionated just before the $45Ca^{2+}$ tracer flux experiment or the digestion treatment. Dechorionation could not succeed until 48h after fertilization and was conducted by tearing off the chorion with watchmaker forceps # 5 under a stereomicroscope. Damage to the embryos from this procedure could be detected by the occurrence of yolk leaking, fading in body or yolk, or the cease of heart beating during the subsequent 8h for flux measurement. Damaged embryos (less than 5% of the total number of the treated embryos) were discarded. The dechorionated embryos survived as well as the intact embryos if the incubation medium was not contaminated. The morphological development of embryos was checked under a stereomicroscope with appropriate lighting to determine the time sequence of different developmental stages (Table 1). Larvae were not fed during the experimental period. Samplings began 12h after fertilization or hatching, and subsequent serial samplings were conducted at various stages of develop-

ment. Twenty to thirty individuals of tilapia were collected as a pooled sample. Only the larvae used for weight measurement were anesthetized with 100 mg l^{-1} MS222.

Ion content in tissue and media

Dechorionated embryos (intact embryos for day 1 after fertilization) were used for measurement of cation content. Pooled samplse of 20–30 embryos or larvae were briefly rinsed in deionized water, the solution on the body surface was wiped off, and the animals were subjected immediately to digestion with 5 ml of 13.1N HNO_3 and 10 ml of H_2O_2 at 400°C for 15 min in a digestion apparatus (23130-20, Hach, USA). Digested solutions were diluted with a 0.2N HCl including 1.3% $LaCl_3$ for subsequent analysis. Water samples from incubation media were diluted with double-deionized water (Milli-RO60, Millipore, USA). Tissue, water samples as well as appropriate blanks were subjected to atomic absorption spectrophotometer (Z-8000, Hitachi, Japan) to measure Ca^{2+}, Na^+, K^+ and Mg^{2+} concentrations.

$^{45}Ca^{2+}$ tracer flux experiment

Ca^{2+} influxes were determined by placing individual dechorionated embryos or larvae in aerated 50 ml of $^{45}Ca^{2+}$-labelled (final concentration: 1 μCi ml^{-1}) medium for 8h to allow the count rate to reach 5–10 times that of background. Animals were removed, washed in nonradioactive fresh water, homogenized and mixed with counting solution. The radio-activity of samples were determined on a liquid scintillation β-counter (Beckman, USA). Based on the assumptions that the specific radio-activity of bathing medium was constant and the backflux over the experimental period was negligible, the influx of Ca^{2+} was determined by the formula (Kirshner 1970):

$$Q_{emb} = J_{in} \cdot X_{out} \cdot t$$

Q_{emb}: radio activity of embryo or larva (cpm per individual) at time t; J_{in}: influx (pmol $individual^{-1}$ h^{-1}); X_{out}: specific activity of the incubation medium (cpm $pmol^{-1}$); t: time (h). J_{in} was calculated from a slope, $J_{in} \cdot X_{out}$, which was derived from a simple linear regression of a plot of Q_{emb} against t.

For the measurement of Ca^{2+} efflux, newly-hatched larvae were incubated in slightly-aerated 50 ml of $^{45}Ca^{2+}$ freshwater (final concentration: 1 μCi ml^{-1}) for 24h. The larvae were removed from the medium, washed with nonradioactive freshwater for 5 min, and then transferred into a beaker with 10 ml nonradioactive freshwater. The medium in the beaker was pumped out and replenished with freshwater into the beaker at the same speed (about 2 ml min^{-1}) to maintain the radioactivity in the medium low enough to eliminate a significant backflux of tracer into the animals. Water samples in the beaker were collected at certain periods. At the end of the experiment, the animals were sampled and treated as described in the influx experiment. The effluxes were determined as follows (Kirshner 1970):

$$\ln Q_{emb} = (J_{out}/Q_{in}) \cdot t + C$$

J_{out}: efflux (pmol $individual^{-1}$ h^{-1}); Q_{in}: total Ca^{2+} in the larva; C: constant; Q_{emb} and t: the same as described before. As described above, J_{out} was obtained by calculating J_{out}/Q_{in}, which is the slope of a simple linear regression analysis of ln Q_{emb} against t.

Preliminary experiments have been conducted to confirm that 1 μCi ml^{-1} $^{45}Ca^{2+}$ did not show significant effects on the development of embryos. Difference in quenching effect between water and tissue has been calibrated before calculting the data as described by Perry and Wood (1985).

Scanning electron microscopy

Embryos or larvae of different stages were immersion-fixed with 4% glutaraldehyde in phophate buffer (pH 7.4) at 4°C for 12h, dehydrated through a graded acetone series followed by drying with liquid CO_2 on a critical point dryer (HCP-2, Hitachi, Japan). Dried samples were

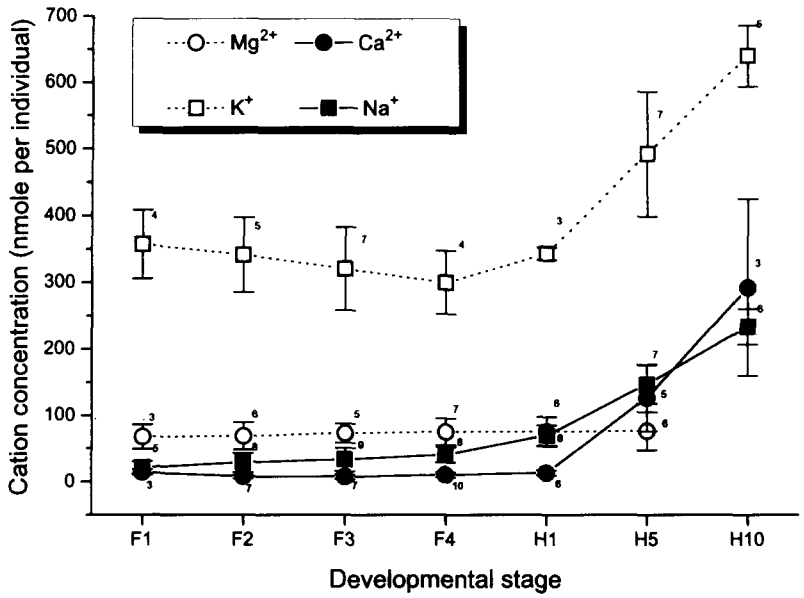

Fig. 1. Changes of whole-body cation contents with developmental stages. Mean, SEM, and N (small number) were indicated. F1–4, 1–4 days (*i.e.*, 24–96 h after fertilization as indicated in Table 1) after fertilization; H1–10, 1–10 days after hatching.

mounted on an aluminum stub and coated with gold/palladium using an ion coater unit (IB-2, Giko, Japan). The specimens were observed on an Hitachi S-2500 scanning electron microscope.

Results

Cation content of embryos and larvae

Figure 1 shows the changes in the tissue content of four cations following the early development of tilapia. Tissue contents of Na^+, K^+ and Ca^{2+} remained at a constant level during the embryonic stages and increased rapidly after hatching. Tissue Na^+ content did not show a significant change until 1 day after hatching, while significant changes did not appear in either K^+ or Ca^{2+} until 5 days after hatching (oneway ANOVA, Tukey's pairwise comparisons). Ten-day-old (*i.e.*, 10 days after hatching) tilapia larvae had higher levels of tissue Na^+, K^+ and Ca^{2+}, about 8, 2, and 60 times that of the embryo, respectively. However, the level of tissue Mg^{2+} remained constant during the embryonic and larval stages (oneway ANOVA, Tukey's pairwise comparisons).

Ca^{2+} influx and efflux

As shown in Table 2, Ca^{2+} influx (J_{in}) increased following the development. There was less than 3-fold increase in J_{in} (in pmol individual^{-1} h^{-1}) before hatching, while about 12 times the J_{in} of 48h-postfertilization embryos was measured in the larvae 1 days after hatching.

Efflux of Ca^{2+} (J_{out}) was only measured in 1-day-old larvae (120h after fertilization). The level of J_{out} was lower than that of J_{in} in the same brood of larvae, resulting in a significant net flux of Ca^{2+} (Table 3).

Development of skin and chloride cell

The skin of the 36h-postfertilization embryos was covered by mosaic pavement cells which were distinguished by their notable cell boundary. The surface of these pavement cells did not show any ridge structure or folding. Forty-eight hours after fertilization the surface of pavement cells showed some faint and irregular folding of the cell membranes (Fig. 2). This folding of the cell membranes increased rapidly following embryonic development.

Table 2. Ca^{2+} influx of tilapia embryos and larvae

Hours after fertilization	J_{in} (pmol individual^{-1} h^{-1}	J_{in}** (pmol mg^{-1} h^{-1}	Wet*** Weight (mg)
48	23.4± 1.0 (42)	5.9±0.3	3.97
72	39.0± 1.0 (42)	9.8±0.3	3.98
96*	58.8± 1.4 (63)	11.8±0.3	4.98
120	286.8±20.0 (63)	47.8±3.3	6.00

Mean ± SEM (N) values are given; * Hatching; ** Calculated by dividing the J_{in} (in pmol individual^{-1} h^{-1}) by the wet weight of embryos or larvae; *** Average of 10–15 individuals

Table 3. Comparison of Ca^{2+} influx and efflux rates in 1-day-old (120 hours after fertilization) larvae of tilapia

	Measured data				
Brood no.	J_{in}*	J_{out}*	Net influx* (J_{in}-J_{out})	J_{out}/J_{in}	Estimated J_{out}/J_{in}**
I	67.3±2.5	56.5±1.7	10.8	0.84	13.2–15.8
II	28.3±0.8	16.6±4.2	11.7	0.59	

* J_{in}, J_{out}, and net influx were expressed in pmol mg^{-1} h^{-1}; ** Estimated flux ratio is based on diffusive movements of Ca^{2+} according to the formula:

$$J_{out}Ca^{2+}/J_{in}Ca^{2+} = [Ca^{2+}]_i/[Ca^{2+}]_0 \exp(zFE/RT)$$

where $[Ca^{2+}]_i$ and $[Ca^{+2}]_0$, Ca^{2+} concentration in plasma and external medium; z, valence; F, Faraday constant; E, transepithelial potential; R, gas constant: T, absolute temperature; Data of $[Ca^{2+}]_i$ and E cited from Pratap *et al.* (1989), Young *et al.* (1988), and McCormick *et al.* (1992).

In 72h-postfertilization embryos, the ridge structures (*i.e.*, folding of cell membrane) were ramifying like labyrinth on the surface of pavement cells but still poorly developed near the center of the cell surface (Fig. 3). Ninety-six hours after fertilization the ramifying of ridges was more intensified and showed more regularity compared with that of the previous stage (Fig. 4, 5).

As reported in the previous study (Hwang 1989), most of the chloride cells are concentrated in the skin posterior to the pectoral fins. Therefore, observations were focused on this area of the tilapia embryos or larvae. No apical opening of chloride cell was found in the skin of the seven 36h-postfertilization embryos that have been studied. The first chloride cells were found in the skin of a 48h-postfertilization embryo after examining fifteen individuals. The number of chloride cell increased, and differentiation of cell structure progressed with the development of the skin (Fig. 3, 4). Typical microvilli-like structure of a chloride cell under SEM was evident in the apical opening of the cell (Fig. 3–5). About 2–3 chloride cells with surface area of 80X132 μm^2 were observed in the skin near pectoral fins of newly-hatched larvae (96h after fertilization).

Discussion

Changes of tissue cation contents in the early developmental stages have been determined in several teleosts. Hayes *et al.* (1946) reported that developing salmon gained Na^+ and Ca^{2+} from the en-

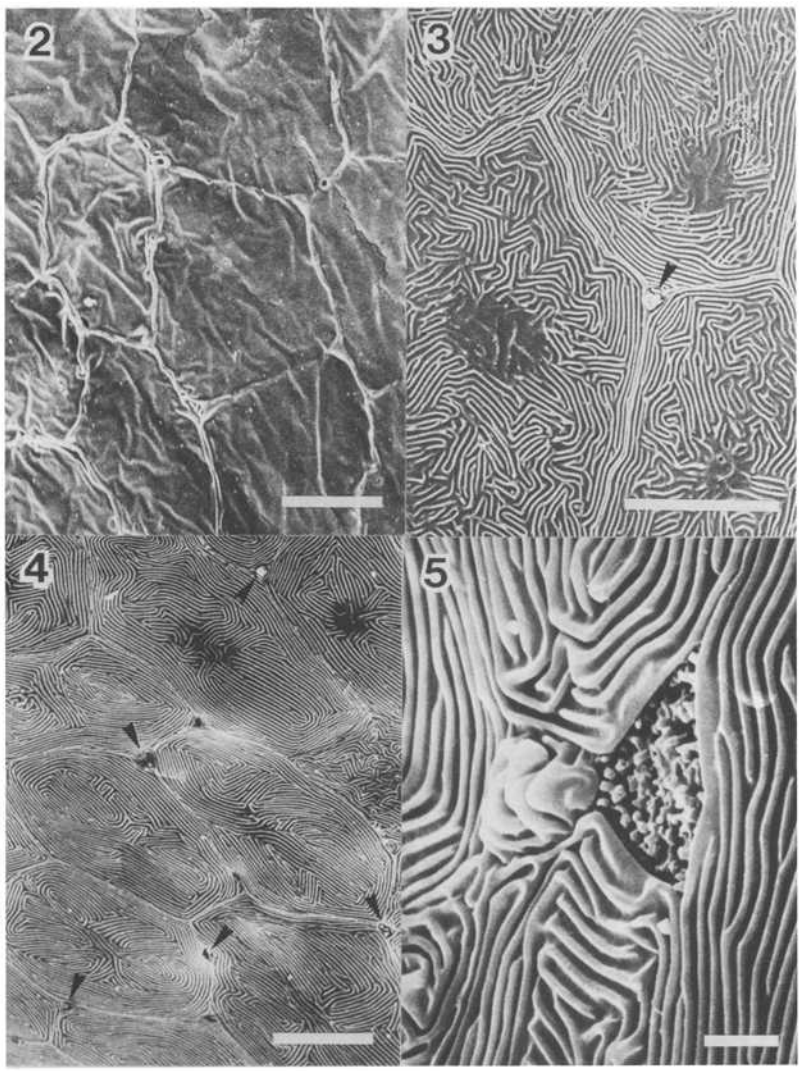

Fig. 2–5. Micrographs of scanning electron microscope observations on the skin of tilapia embryos with different ages.
Fig. 2. 48 h after fertilization. Skin surface looked like mosaic pavement due to notable cell boundary of pavement cells and showed some faint folding of cell membrane. (Scale = 10 μm, × 1,900).
Fig. 3. 72 h after fertilization. Ridges (*i.e.*, folding of cell membrane) were ramifying as labyrinth on the surface of pavement cells but still poorly developed near the center of cell surface. A chloride cell (arrow head) whose apical opening had numerous microvillus appeared at the boundary of three pavement cells. (Scale = 10 μm, × 2,900).
Fig. 4. 96 h after fertilization. Ramifying of ridge was intensified and showed more regularity comparing with that of the previous stage. More chloride cells (arrow heads) were observed. (Scale = 10 μm, × 1,900).
Fig. 5. 96 h after fertilization. Structure of well-developed ridge of pavement cells and microvilli in the apical opening of a chloride cell were noted. (Scale = 1 μm, × 13,400).

vironment after hatching but lost K^+ and Mg^{2+} to the outside. Seawater-adapted killifish (*Fundulus heteroclitus*) were found to have a gradual decrease in K^+ and a 2–2.5 fold increase in Na^+ from 4 days postfertilization till hatching (Guggino 1980b). Rombough and Garside (1984) also reported that Atlantic salmon (*Salmo salar*) showed a rapid increase in tissue Na^+, K^+ and Ca^{2+} content following the development of alevins. In this study, tilapia revealed a constant level in tissue Na^+, K^+

and Ca^{2+} during the embryonic stages and a rapid increase after hatching, and a constant Mg^{2+} level throughout the embryonic and larval development. This observation was consistent, although not completely so, with that of Fu and Lock (1990) for the same species. The differences among the data of these studies may be ascribed to the difference in species or experimental design. However, this implies that there must be some mechanisms in the embryos to maintain the constancy of most cations including Ca^{2+}.

The measured influx rate of 6–48 pmol Ca^{2+} mg^{-1} h^{-1} for tilapia embryos was near the range (27–146 pmol Ca^{2+} mg^{-1} h^{-1}) of those reported for several species of adult fish including tilapia (Fleming *et al.* 1973; Pang *et al.* 1980; Ichii and Mugiya 1983; Flik *et al.* 1985; Perry and Flik 1988). The Ca^{2+} influx rate in tilapia increased following the embryonic development, and the rate in the 1-day-old larvae turned out much higher than that in adult tilapia (27.9 pmol mg^{-1} h^{-1}). A similar phenomenon has been found in the flux rates of Na^+ and Cl^- in seawater-adapted killifish (Guggibo 1980b) and in the drinking rates of herring (*Clupea harengus*), plaice (*Pleuronectes platessa*) and cod (*Gadus morhua*) (Tytler and Blaxter 1988; Mangor-Jensen and Adoff 1987), which has been ascribed to the larger surface-to-volume ratio of hatched larva than that of adults. This seems to also hold in tilapia, although the exact value of ratio in embryos or larvae of tilapia is unknown.

The chorion may contributed to some extent to the ion balance in freshwater fish embryos by concentrating cations in the perivitelline fluid and developing a potential difference across the chorion (Eddy *et al.* 1990; Peterson and Martin-Robichaud 1986). Guggino (1980a) found that water turnover rates were higher in the dechorionated killifish embryos than in the intact ones, however the rates of both groups showed a similar increase pattern following the development of embryos. Since dechorionated embryos were used in the present study, the Ca^{2+} influx rate of intact tilapia embryos is unknown; however, it is presumable to show a similar increase pattern as that of the dechorionated embryos.

The increase in Ca^{2+} influx with the embryonic and larval stages indicated that the mechanism of Ca^{2+} uptake is not fully developed at hatching but continues to progress during larval development. This increase may be the result of two possible factors: (1) increase of surface area of transporting epithelium, (2) increase of Ca^{2+} permeability. Flik *et al.* (1985) measured the whole-body Ca^{2+} influx rate in tilapia adult and concluded that gills, representing 70–80% of the total body surface, are the main site for more than 97.4% of extraintestinal Ca^{2+} entry in fish. Drinking in embryos or larvae, being in the range of adults as described above, may result in only a negligible fraction of the whole-body Ca^{2+} influx as that observed in tilapia adult (Flik *et al.* 1985). Since tilapia start to develop gills at hatching, the skin is probably the main surface for Ca^{2+} entry in tilapia embryos before the complete development of gills. Besides the elongation of embryos, the differentiation of skin surface and the development of chloride cells during embryonic and larval stages also provides morphological evidence for the increase of surface area for Ca^{2+} transport. It has been suggested that ridges create local water turbulence and increase surface area for respiration (Olson and Fromm 1973). At hatching, development of gills, including filaments and lamellae, greatly increase the surface area of the whole fish. Consequently, a 7-fold increase of Ca^{2+} influx (in pmol $individual^{-1}$ h^{-1}) occurred around hatching in tilapia. Whether changes in Ca^{2+} permeability with embryonic and larval development in tilapia contribute to the increase of Ca^{2+} influx remains to be verified. However, Guggino (1980a) suggested that the increase in water turnover around hatching may be attributed more to an increase in the surface-to-volume ratio than to a change in the water permeability of the epithelia.

Similar to the Ca^{2+} influx rate described above, the Ca^{2+} efflux rate in 1-day-old larvae was higher than that reported for the adults of tilapia, 11.5 pmol mg^{-1} h^{-1}, (Flik *et al.* 1985) and eel (*Anguilla anguilla*), 7.0 pmol mg^{-1} h^{-1}, (Milet *et al.* 1979). This may also be ascribed to the larger surface-to-volume ratio in hatched larvae. The Ca^{2+} influx rate was about 1.2–1.7 times the value of Ca^{2+} efflux rate in 1-day-old tilapia larvae, resulting in a significant net uptake of Ca^{2+} as reported in the

adult (Flik *et al.* 1985). This net uptake could account for the rapid increase in tissue Ca^{2+} content after hatching, reflecting a physiology demand of Ca^{2+} for bone formation or other metabolism during the larval development.

According to the Ussing flux ratio equation (Ussing 1949), if the estimated ratio for Ca^{2+} passive movement is higher than the measured ratio, then the movement of Ca^{2+} is active. $[Ca^{2+}]_i$ and E (Table 3) in tilapia hatched larvae were unavailable in the present study and were assumed to be close to the plasma Ca^{2+} concentration, 2.5 mM, (Pratap *et al.* 1989) and the transepithelial potential of whole animal or isolated opercular membrane, 3–13 mV, in tilapia adults (Young *et al.* 1988; McCormick *et al.* 1992). $[Ca^{2+}]_0$ and T of the present study were 0.2 mM and 299°K, respectively. Thus, the estimated ratio was much higher than those measured (Table 3). The measured ratios derived from the present study were similar to that reported in the freshwater trout, 0.54, although the estimated ratio for the trout was lower, 2.19, due to the difference in $[Ca^{2+}]_i$ and E (Perry and Flik 1988). It is reasonable to state that active transport is involved in the uptake of Ca^{2+} in embryos and larvae of tilapia as demonstrated in the adult trout (Perry and Flik 1988), since the $[Ca^{2+}]_i$ and E have to be lower than 0.13 mM and −154 mV, respectively, to exclude the possibility of active transport.

Chloride cells have been well documented to be responsible for the active transport of Ca^{2+} in adult fish (Payan *et al.* 1981; Perry and Wood 1985; Perry and Flik 1988; Marschall *et al.* 1992; McCormick *et al.* 1992; Perry *et al.* 1992). Embryos or larvae of teleosts have been found to develop chloride cells in the skin until their gills are fully formed and functioning (Hwang and Hirano 1985; review of Alderdice 1988; Hwang 1989, 1990). The structure and function of these cells have been demonstrated to be similar to that of the gill chloride cells in adults, *i.e.*, the excretion of ions (Hwang and Hirano 1985; Hwang 1990). As described above, the main surface responsible for Ca^{2+} flux before the development of gills is the skin, in which only chloride cells showed a typical structure for active transport, *i.e.*, numerous mitochondria, well-branched tubular system and an apical opening exposed to ambient medium (Hwang and Hirano 1985; Hwang 1989, 1990). It is reasonable to assume that skin chloride cells are the site which performs the active uptake of Ca^{2+} in embryos and larvae.

In summary, tilapia maintained a constant level of Ca^{2+} content during the embryonic stages and had a rapid increase of Ca^{2+} content after hatching through an increasing uptake of Ca^{2+} from the environment, and the active transport *via* skin chloride cells is probably involved in this mechanism.

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