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Modulation of calcium balance in tilapia larvae (*Oreochromis mossambicus*) acclimated to low-calcium environments

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**Abstract** This study examined how developing fish larvae regulate their Ca$^{2+}$ balance for acclimation to low ambient Ca$^{2+}$. Calcium balance in newly hatched larvae was examined individually. Developing larvae not only increased Ca$^{2+}$ influx but also decreased Ca$^{2+}$ efflux when they were acclimated to low-Ca$^{2+}$ environments. After acclimation for 8 days, the influx and efflux of the low-Ca$^{2+}$ (0.02 mM) group were about 106% and 43%, respectively, compared to those of the high-Ca$^{2+}$ (1.0 mM) group. Sensitivity and response to low-Ca$^{2+}$ environments are age-dependent. Upon acute exposure to low Ca$^{2+}$, newly hatched (H0) larvae increased both Ca$^{2+}$ influx (from 24% to 67% of high-Ca$^{2+}$) and net uptake (from 5% to 69%) within 38 h, while 3-day-posthatching (H3) larvae managed to reach the levels of the control within 38 h. Declining Ca$^{2+}$ efflux in H3 larvae occurred 14 h after exposure, much faster than those in H0 larvae (38 h). It is suggested that modulation of Ca$^{2+}$-balance mechanisms in developing larvae is dependent upon the levels of Ca$^{2+}$ in the larval body.

**Keywords** Fish larvae · Ca$^{2+}$ influx · Ca$^{2+}$ efflux · Ca$^{2+}$ content · Environmental Ca$^{2+}$

**Abbreviations** H0 larvae larvae immediately after hatching · H1 larvae larvae 1 day post-hatching · H3 larvae larvae 3 days post-hatching · H7 larvae larvae 7 days post-hatching · MR mitochondria rich

**Introduction**

Freshwater teleosts maintain their plasma Ca$^{2+}$ levels within a narrow limit (2–4 mM) in a wide range of external Ca$^{2+}$ concentrations. Gill mitochondria-rich (MR) cells (or chloride cells) are the major sites for Ca$^{2+}$ uptake from freshwater environments (Payan et al. 1981; Perry and Wood 1985). Active Ca$^{2+}$ uptake in response to low ambient Ca$^{2+}$ is therefore a prerequisite for Ca$^{2+}$ homeostasis (Perry and Wood 1985; Flik et al. 1986; McCormick et al. 1992; Partrick et al. 1997).

Fish embryos and larvae, whose gills or kidneys are poorly developed, are able to maintain constancy in the ion concentrations and osmolality of their body fluids (Guggino 1980; Hwang and Wu 1993; Lin et al. 1999). Skin chloride cells were suggested to be responsible for the active transport of ions before the complete development of the gills (Hwang and Hirano 1985; Hwang 1990; Hwang et al. 1994, 1999; Van der Heijden et al. 1999). Embryos and larvae have to face freshwater environments with a wide range of external Ca$^{2+}$ concentrations, however, little is known about the mechanism of Ca$^{2+}$ balance in embryos or larvae. Hwang et al. (1994) reported that tilapia increase their Ca$^{2+}$ content rapidly after hatching through an increased uptake of Ca$^{2+}$ from the environment by a mechanism that may involve active transport via skin MR cells. In subsequent studies, developing tilapia larvae were found to be capable of stimulation of their Ca$^{2+}$ uptake mechanism, via enhancement of Ca$^{2+}$ influx, to maintain normal body Ca$^{2+}$ content levels and growth in harsh environments such as those with low levels of Ca$^{2+}$ or with heavy metals like Cd$^{2+}$ present (Hwang et al. 1996; Hwang and Yang 1997; Chang et al. 1997, 1998). Although regulation of Ca$^{2+}$ efflux was also suggested to be involved in this mechanism (Hwang et al. 1996), no empirical data are available.

In this study, calcium balance in newly hatched larvae was examined individually. First, we examined the changes in Ca$^{2+}$ influx and efflux in tilapia (*Oreochromis*...
mossambicus) larvae following development from the newly hatched to 7-day-old larvae. Second, Ca\(^{2+}\) influx and efflux were compared between the larvae acclimated to different ambient Ca\(^{2+}\) levels. Third, modulations of Ca\(^{2+}\) influx and efflux upon acute challenge by low ambient Ca\(^{2+}\) levels were compared between different larval stages.

### Materials and methods

#### Fish and acclimation conditions

Mature adult tilapia, *O. mossambicus*, from the Taiwan branch of the Taiwan Fisheries Research Institute were reared in 182-l glass aquaria with plastic chips as gravel. Each tank was supplied with dechlorinated, circulated, and aerated local tap water at 26 ± 1 °C under a photoperiod of 14 h:10 h light:dark. Fish were fed with commercial fish food pellets (Fu-Sow, Taiwan). Fertilized eggs were collected from the mouth of a breeding female 48 h after fertilization (generally taking 96 h to hatch) and incubated in a well-aerated 1250-ml container until the acclimation experiments. Complete absorption of yolk was generally on day 10 post-hatching, and mixed endogenous and exogenous feeding was between 8 days and 10 days post-hatching. During the acclimation experiment (see below, up to 7 days post-hatching) the larvae were not fed.

Artificial fresh water for acclimation experiments was prepared by adding appropriate amounts of MgSO\(_4\), NaCl, K\(_2\)HPO\(_4\), KH\(_2\)PO\(_4\), and CaSO\(_4\) (Merck, Germany) to double-deionized water (Milli-RO60, Millipore, USA). The concentrations of Na\(^+\), K\(^+\) and Mg\(^{2+}\) in the artificial media were near the ranges of local fresh water (Table 1), while that of Ca\(^{2+}\) was 0.02, 0.2, or 1.0 mM depending upon the experiments (Table 1). The 0.02 mM Ca\(^{2+}\) was about 1/10 the concentration of our local tap water and was lower than the Ca\(^{2+}\) concentrations in natural soft water reported as 0.04–0.27 mM by Laurent et al. (1985); the 0.2–1.0 mM was considered as a normal range according to Perry and Wood (1985), Hwang et al. (1996) and our measurement (Table 1). Preliminary experiments indicated a similar survival and growth of tilapia larvae maintained in 0.02–1.0 mM Ca\(^{2+}\). Eggs or larvae were incubated in 1000-ml containers. These containers were well aerated in the same conditions as those of adults in order to diminish any effect from the change of temperature or photoperiod. The water in the test containers was changed daily to maintain water quality (Table 1; dissolved oxygen 7.5 ± 0.5 mg l\(^{-1}\); pH 6.9 ± 0.3).

#### Ion content in tissue and media

After measurements of body weight and total length, tilapia larvae were killed with an overdose of anesthetic (MS 222, 0.38 mM) and then digested overnight with 13.1 N HNO\(_3\) at room temperature. Digested solutions as well as the water samples from incubation media were diluted with double-deionized water and were subjected to atomic absorption spectrophotometry (Z-8000, Hitachi, Japan) for Na\(^+\), K\(^+\), Mg\(^{2+}\) (water samples only), and Ca\(^{2+}\) concentrations. Ion standard solutions (from Merck) were used as standard curves for the measurements. Matrix effects were calibrated by standard addition.

### Table 1

<table>
<thead>
<tr>
<th>Medium</th>
<th>[Ca(^{2+})]</th>
<th>[Na(^+)]</th>
<th>[K(^+)]</th>
<th>[Mg(^{2+})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local freshwater</td>
<td>0.180–0.240</td>
<td>0.480–0.520</td>
<td>0.029–0.031</td>
<td>0.150–0.170</td>
</tr>
<tr>
<td>0.02 mM Ca(^{2+})</td>
<td>0.021–0.034</td>
<td>0.533–0.559</td>
<td>0.032–0.042</td>
<td>0.130–0.190</td>
</tr>
<tr>
<td>0.20 mM Ca(^{2+})</td>
<td>0.195–0.225</td>
<td>0.529–0.594</td>
<td>0.032–0.042</td>
<td>0.120–0.175</td>
</tr>
<tr>
<td>1.00 mM Ca(^{2+})</td>
<td>0.958–1.055</td>
<td>0.600–0.734</td>
<td>0.030–0.045</td>
<td>0.144–0.180</td>
</tr>
</tbody>
</table>

### Calcium fluxes

Measurement of calcium influx followed our previous method (Chang et al. 1997, 1998) with some modifications. Briefly, the media for the tracer experiment were prepared by adding an appropriate amount of tracer to artificial fresh water as described in Table 1. Tilapia larvae were rinsed briefly for 3 min with 250 ml solutions similar to the tracer media but without the 45Ca\(^{2+}\) tracer, and then were incubated in 45Ca\(^{2+}\)-labeled tracer media for 1 h. After tracer incubation, larvae were collected, washed in non-radioactive fresh water (three times for a total of about 5 min). After a 5-min rinse, the radioactivity of the subsequent rinse was checked and was not significantly different from the background. The larvae were anesthetized with MS222 and finally treated individually with tissue solubilizer (Soluene, Packard, USA) at 50 °C for 8 h. The digested solutions were mixed with counting solution (Ultima Gold, Packard, USA) and measured with a liquid scintillation β-counter (LS 6500, Beckman, USA). 45Ca\(^{2+}\)-labelled CaCl\(_2\) (889 mCi mmol\(^{-1}\), Amersham, England) was used as tracer at 1–3.5 mCi mmol\(^{-1}\). The calcium influx was determined individually by the formula:

\[
J_{in} = \frac{Q_{lava}}{T_{out} \times T} \div W
\]

where \(J_{in}\) is the influx (nmol mg\(^{-1}\) h\(^{-1}\)), \(Q_{lava}\) is the radioactivity of larva (cpm larva\(^{-1}\)) at the end of incubation, \(X_{in}\) is the specific activity of the incubation medium (cpm mmol\(^{-1}\)), \(T_{in}\) is the incubation time (h), and \(W\) is the body weight (mg). Preliminary experiments showed that the plot of radioactivity in larva against incubation time was linear \((R = 0.999)\) within 8 h, and the specific activity of the tracer medium did not show significant change during the same period. Therefore, 6 h was chosen for the incubation time in the tracer medium. The difference in quenching effect between water and tissue was calibrated before calculating the data as described previously (Hwang et al. 1994).

Measurement of Ca\(^{2+}\) efflux followed our previous method (Hwang et al. 1994) with some modifications. Briefly, larvae were incubated in slightly aerated 45Ca\(^{2+}\)-tracer media (3.5 mCi mmol\(^{-1}\)) for 15–20 h. At the end of incubation, the larvae were washed with 250 ml artificial fresh water without tracer for 5 min to wash out the most adhering tracer. The larvae were then transferred to a 5-ml tube with 2 ml non-radioactive fresh water for another 6 h. Preliminary experiments confirmed that the amounts of 45Ca\(^{2+}\) released from larva were linear during 6 h \((R = 0.998)\), and the calculated efflux rates were constant during the 6 h. After being mixed with the counter solution, radioactivity in the tubes was measured as described above. The efflux was determined individually as follows:

\[
J_{out} = \frac{Q_{out}}{X_{lava}} \div \left( X_{lava} \times T \right) \div W
\]

where \(J_{out}\) is the efflux (nmol mg\(^{-1}\) h\(^{-1}\)), \(Q_{out}\) is the radioactivity released from larva (cpm larva\(^{-1}\)) at the end of incubation, \(X_{lava}\) is the specific activity in larva (cpm mmol\(^{-1}\)), \(T_{in}\) is the incubation time (h), and \(W\) is the body weight (mg). The difference in quenching effect between water and tissue was calibrated before calculating the data as described previously (Hwang et al. 1994). 45Ca\(^{2+}\)-specific activities in larval bodies are necessary for the measurement of Ca\(^{2+}\) efflux. The larvae were too small in size to collect a sufficient amount of body fluids for the measurement of 45Ca\(^{2+}\)-specific activities. Following Guggino (1980) and Hwang et al. (1994) with some modification, 6–10 larvae were anesthetized with MS222, washed with double-deionized water for 5 min, homogenized, and then centrifuged at 19,000 g for 20 min. The