Salinity tolerance and structure of external and internal gills in tadpoles of the crab-eating frog, *Rana cancrivora*

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Summary. Salinity tolerance and histology of gills were studied in *Rana cancrivora* larvae. The tadpoles at the external gill stages (W stages 21–22) were able to survive in media containing up to 40% seawater, but died in water of higher salinity. Their external gills appear to have no critical role in adaptation to seawater. However, advanced tadpoles with internal gills (T-K stages I–XVIII) were able to tolerate 50% or higher seawater. In the internal gills, there are numerous mitochondria-rich cells (MR cells) scattered on the ventral and lateral epithelia of the gill arches and the gill tufts in both freshwater- and seawater-acclimated tadpoles. In freshwater-acclimated tadpoles there are three types of MR cell: (1) microplicated, (2) microvillous, and (3) apically vacuolated. In tadpoles acclimated to dilute seawater, the ratio of type-1 to type-2 cells is lower, although all three types of MR cell are present. In 60%-seawater-acclimated tadpoles there are three types of MR cell: (1) microplicated, (2) microvillous, and (3) apically vacuolated. In tadpoles acclimated to dilute seawater, the ratio of type-1 to type-2 cells is lower, although all three types of MR cell are present. In 60%-seawater-acclimated tadpoles, a few MR cells with a lumen and concave cytoplasm at the apical membrane (type 4) are present. The changes in MR cell morphology under ambient conditions of low or high salinity may reflect alterations in the physiological roles of the gills with regard to transport of ions.

Key words: Osmotic stress – Gills – Mitochondria-rich cells – Polymorphic cell – *Rana cancrivora* (Anura)

*Rana cancrivora* larvae inhabit brackish environments, where salinity corresponds to 19–75% seawater (Alcala 1962; Gordon and Tucker 1965). Under laboratory conditions, *R. cancrivora* tadpoles (T-K stages III–XIX) can tolerate environmental salinities ranging from fresh water to full strength seawater (Gordon and Tucker 1965). Dunson (1977) also reported that the survival ratio of *R. cancrivora* tadpoles was more than 50% when they were acclimated in diluted seawater (up to 80% seawater). However, it is not clear what mechanisms make this possible. According to the hypothesis of Gordon and Tucker (1965), tadpoles of this species are good osmoregulators and drink ambient water, and then excrete salt by extrarenal pathways. We also assumed that they drink seawater and compensate for water loss in hyperosmotic environments based on histological observations of the alimentary tract (unpublished data). The disadvantage of drinking is that it also brings about a great influx of ions. Assuming that the mechanism truly works in *R. cancrivora* tadpoles acclimated to seawater, surplus ions must be excreted on extrarenal salt-excreting organs, as in nonmammalian vertebrates inhabiting the seawater environment. Although we have examined serial sections of complete tadpoles with the light microscope, so far, we have not found any salt excretory glands (unpublished data). Gordon and Tucker (1965) suggested that the osmoregulatory mechanisms used by *R. cancrivora* tadpole may be similar to those used by euryhaline teleosts. In these fishes, it is generally accepted that seawater is swallowed and that this electrolytic entry is balanced by active excretion of monovalent ions through the chloride (Cl) cells of gills and opercular membranes (Keys and Willmer 1932; Copeland 1948; Laurent 1984). The Cl cell is thought to take up small quantities of ions in fresh water and secrete large amounts of salts in seawater (Foskett and Scheffey 1982; Karmak et al. 1984; Gardaire et al. 1985). In several teleosts, it is known that osmoregulatory mechanisms develop in early larvae and that the Cl cells, which are present in the skin and branchial epithelium of the embryos, larvae and juveniles seem to play a critical role in their salinity adaptation (e.g. O’Connell 1981; Hwang and Hirano 1985). In contrast, it is reported that early embryonic development of *R. cancrivora* tadpoles is only advanced in a low-salinity environment, less than 20% seawater (Gordon and Tucker 1965). We also observed that eggs from *R. cancrivora* frogs kept in 50% seawater remained undeveloped and that development of tadpoles kept in high salinities (40–100% seawater) was delayed and they did not metamorphose (Uchiyama et al. 1990a). These results suggest that early embryos and
metamorphic climax tadpoles cannot adapt well to high salinities because of the lack of gill development in the embryonic stages and the loss of gills during metamorphosis.

In anuran tadpoles it is reported that internal gills are important for absorption of ions in fresh water (Dietz and Alvarado 1974), and it has been suggested based on ultrastructural observations that mitochondria-rich (MR) cells may participate in ion exchange and osmoregulation of anuran larvae (Hourdy 1974; Uchiyama et al. 1990b). However, few details are known about the seawater-adaptation mechanism of \textit{R. cancrivora} larvae. A hypothesis about the adaptation mechanism proposed by Gordon and Tucker (1965) is based on their limited experiments and speculation. It is therefore interesting to investigate when the high ability of salinity tolerance is obtained by tadpoles and what mechanisms are involved in their osmoregulation in seawater. The present paper reports salinity tolerance experiments and morphological observations of the external and internal gills in developing tadpoles of \textit{R. cancrivora} showing tolerance to salinity.

Materials and methods

Adult males and females of the crab-eating frog, \textit{Rana cancrivora}, were collected around prawn culture-ponds (salinity 33%) located in a mangrove swamp at Ang Sila near Bangkok, Thailand. They were shipped by air to the laboratory in Niigata, Japan. Larvae obtained from them were used in the present study. Fertilization of the eggs and development of the tadpoles have been described in detail by Uchiyama et al. (1990a). Tadpoles were raised in 10% seawater at 24.5–26°C. The embryonic stages were judged according to the developmental stages for \textit{R. pipiens} by Witschi (1956) and the subsequent larval stages as described by Taylor and Kollros (1946). Ion concentrations in aged tap-water in Niigata city used as fresh water in the present study were (in mM): Na+, 0.65; Ca++, 0.25; Cl−, less than 1. The ion concentrations in full strength seawater (in mM) used in the present study were: Na+, 460; K+, 11; Ca++, 10.6; Cl−, 490. Osmolality and salinity of seawater used were 980 mOsm and 32%, respectively.

Salinity tolerance

Three series of experiments were undertaken. In the first experiment, tadpoles with external gills (W stages 21–22) were acclimated in 10% seawater and transferred directly to various dilutions of seawater (salinity 0–32%). In the second experiment, tadpoles with lids on the external gills (W stages 24–25) were acclimated in 10% seawater or 40% seawater for at least 3 days, and then transferred directly to various dilutions of seawater (salinity 13–32%). In the third experiment, tadpoles (T-K stages III–XVIII) were acclimated in steps with 10–20% changes of salinity every 2–7 days. Death of tadpoles after transfer to diluted seawater was judged by observation of heart beat using a dissecting microscope.

Histology of external gills

Four tadpoles (W stages 21–23) kept in 10% seawater were anesthetized with MS 222 (Sankyo) and then used in this experiment. For light microscopy (LM) two tadpoles were immersed in Bouin’s solution, embedded by the routine paraffin method, and sectioned serially at 8 μm. They were then stained with Mayer’s hematoxylin and eosin. The external gills of two tadpoles were used for observation of ultrastructure. For transmission electron microscopy (TEM), the external gills of two tadpoles were rapidly excised and immediately bathed in a solution of 2.5% glutaraldehyde, 4% para-formaldehyde in 0.1 M sodium cacodylate buffer, and then cut into small pieces and placed in fresh fixative for 6 h. Tissue pieces were washed twice with 0.1 M sodium cacodylate buffer, and post-fixed with 1% osmium tetroxide for 1 h. Then the materials were washed with distilled water, dehydrated in ethanol and embedded in Epon. Thin sections were stained with methanolic uranyl acetate and alkaline lead citrate, and examined with a JEOL JEM-100B electron microscope. Some semithin (1 μm) sections, made by the methods described for TEM, were stained with toluidine blue and observed by LM.

Histology of internal gills

The internal gills of anesthetized tadpoles acclimated to fresh water or diluted seawater (20%, 40%, 60%, 100% SW) for 3 days were dissected quickly and treated as follows. Two to 5 tadpoles (T-K stages VIII–XVII) in each group were used. For LM, the gills of tadpoles were immersed in Bouin’s solution, dehydrated, and embedded in paraffin. They were then sectioned serially at 6 or 8 μm by the routine paraffin method. The stains used were; 1) Mayer’s hematoxylin and eosin or 2) periodic acid-Schiff reaction (PAS) and hematoxylin. The methods used for observation of ultrastructure were the same as those for the external gills.

Results

Salinity tolerance

Tadpoles of the external gill stages (W stages 21–22) were able to survive in media up to 40% seawater, but died within 8 h after being placed in 50% seawater or higher salinity. Tadpoles at the point of death lay on the bottom of the container and the blood circulation of their external gills stopped following severe hyperemia. Tadpoles with lids on the external gills (W stages 24–25) were also well acclimated to 40% seawater, but not to 50% seawater or higher, when they were transferred from 10% seawater to the diluted seawater. However, only a few tadpoles (W stages 24–25) were acclimated well in 60% seawater when they were acclimated to 40% seawater for 3 days before transfer. On the other hand, tadpoles (T-K stages I–XVIII) were acclimated in higher salinities when environmental salinity was increased stepwise. However, tadpoles were dehydrated and died within a few hours when transferred directly from fresh water to 100% seawater. In younger larvae (W stages 21–25), stepwise adaptation to diluted seawater was not done because the tadpoles developed rapidly and changed their stages. These results are shown in Table 1.

External gills

The finger-like external gills become visible at W stage 21 (27 h old). They then elongate and reach a maximum size at W stage 23 (51 h old), gradually becoming smaller thereafter and are covered by the operculum at W stage 25 (96 h old). By LM observation, the external gills appear as protrusions of the epidermis, with connective tissue, blood vessels and muscle inside (Fig. 1A). The