RESEARCH ARTICLE

Salinity Acclimation Enhances Salinity Tolerance in Tadpoles Living in Brackish Water Through Increased Na^+, K^+-ATPase Expression

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ABSTRACT

Amphibians are highly susceptible to osmotic stress but, nonetheless, some species can adapt locally to withstand moderately high levels of salinity. Maintaining the homeostasis of body fluids by efficient osmoregulation is thus critical for larval survival in saline environments. We studied the role of acclimation in increased physiological tolerance to elevated water salinity in the Indian rice frog (*Fejervarya limnocharis*) tadpoles exposed to brackish water. We quantified the effects of salinity acclimation on tadpole survival, osmolality, water content, and gill Na^+, K^+-ATPase (NKA) expression. Tadpoles did not survive over 12 hr if directly transferred to 11 ppt (parts per thousand) whereas tadpoles previously acclimated for 48 hr in 7 ppt survived at least 48 hr. We reared tadpoles in 3 ppt and then we transferred them to one of (a) 3 ppt, (b) 11 ppt, and (c) 7 ppt for 48 hr and then 11 ppt. In the first 6 hr after transfer to 11 ppt, tadpole osmolality sharply increased and tadpole water content decreased. Tadpoles pre-acclimated for 48 hr in 7 ppt were able to maintain lower and more stable osmolality within the first 3 hr after transfer. These tadpoles initially lost water content, but over the next 6 hr gradually regained water and stabilized. In addition, they had a higher relative abundance of NKA proteins than tadpoles in other treatments. Pre-acclimation to 7 ppt for 48 hr was hence sufficient to activate NKA expression, resulting in increased survivorship and reduced dehydration upon later transfer to 11 ppt. *J. Exp. Zool. 321A:57–64, 2014.* © 2013 Wiley Periodicals, Inc.

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Maintaining the homeostasis of body fluid by efficient osmoregulatory mechanisms is a challenge for all aquatic animals whose body fluids are not isosmotic with the environmental salinity. Increased salinity affects the physiology of many aquatic organisms with high ion permeability of their skin, gill, or other organs (Balinsky, '81; Hwang and Lee, 2007). This is particularly true for amphibian larvae (Ultsch et al., '99). Most amphibian larvae are poorly adapted to saline environments because their skin is highly permeable and has poor osmoregulatory ability,

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impeding water and ion balance (Balinsky, '81; Duellman and Trueb, '94). Tadpoles are sensitive to salinity, and moderate salinity (≤9 parts per thousand, ppt; equivalent to ≈27% seawater; 9.2 g NaCl L^{-1}; 20,500 μS) often decreases survival, slows development and reduces size at metamorphosis (Christy and Dickman, 2002; Gomez-Mestre and Tejedo, 2003; Chinathamby et al., 2006; Sanzo and Hecnar, 2006; Wu and Kam, 2009). Most amphibians breed in freshwater and avoid breeding in high salinity (Wells, 2007). Nevertheless a small number of species have populations breeding naturally in brackish water bodies (Gordon and Tucker, '65; Balinsky, '81; MØbjerg et al., 2000; Gomez-Mestre et al., 2004; Haramura, 2004; Wu and Kam, 2009; Sillero and Ribeiro, 2010), or in freshwater ponds threatened by increased salinization from either natural causes (e.g., sea levels rise) (Rios-López, 2008), or anthropogenic causes (e.g., road deicing salt run-off) (Sanzo and Hecnar, 2006; Karraker et al., 2008; Collins and Russell, 2009). Given their high sensitivity to osmotic stress, the imbalance in water and ion homeostasis is a serious threat to amphibian survival (Voyles et al., 2007, 2009).

The osmoregulatory mechanism of tadpoles is thought to resemble that of teleosts (Ultsch et al., '99), and their gills are considered important sites for ion exchange and regulation in tadpoles, either living in freshwater (Alvarado and Moody, '70; Boonkoom and Alvarado, '71; Dietz and Alvarado, '74) or in brackish water (Uchiyama and Yoshizawa, '92). However, compared to our knowledge on fish osmoregulatory mechanisms, little is known about tadpole osmoregulation in response to salinity (Ultsch et al., '99). The tadpoles of *Fejervarya cancrivora* (*Rana cancrivora*) can tolerate up to full strength seawater and regulate plasma osmolarity below that of the hyperosmotic media (Gordon and Tucker, '65; Balinsky, '81; Shoemaker et al., '92). However, most tadpoles of *F. cancrivora* did not survive more than one day after direct transfers from 3 to 16 ppt or above, unless they were acclimated to higher salinity stepwise (Uchiyama et al., '90a; Uchiyama and Yoshizawa, '92; Hsu et al., 2012), indicating that their osmoregulatory mechanisms require stepwise acclimation. Mitochondrion-rich cells (MR cells) have been shown to be the main sites responsible for the active transport of ions in gills of teleosts (Hwang and Lee, 2007). MR cells were reported in the internal gills of tadpoles living in freshwater (Brunelli et al., 2004) and those living in brackish water (Uchiyama et al., '90b; Uchiyama and Yoshizawa, '92). Four types of MR cells have been found in the gills of *F. cancrivora* tadpoles after acclimated to progressively higher salinity, and the proportion of MR cells changed in tadpoles subjected to different salinities, suggesting the possible role of MR cells in osmoregulatory functions (Uchiyama and Yoshizawa, '92). The osmoregulatory function of MR cells is mediated by Na^+, K^+-ATPase (NKA), which are large membrane-bound proteins that help maintaining intracellular homeostasis. NKAs are responsible for active cellular outflow transport of Na^+ and cellular K^+ inflow, providing a driving force for other ion channels and transporters (Hwang and Lee, 2007). Variations in abundance and/or activity of branchial NKA are often required for acclimation to osmotic challenge in fishes (Hwang and Lee, 2007; Hwang et al., 2011). Boonkoom and Alvarado ('71) reported that NKA activity was higher in gills than in the skin of larval *Lithobates catesbeianus* (formerly *Rana catesbeiana*) immersed in a hypotonic solution, suggesting that gills played a critical role in ionic regulation. So far, however, there has been little work assessing changes in the branchial NKA expression, especially for that of tadpoles in saline environments.

Tadpoles of the Indian rice frog (*Fejervarya limnocharis*, Dicroglossidae) inhabit the brackish water of coastal rock pools formed by uplifted coral reef on the tropical Green Island of Taiwan (Wu and Kam, 2009). This species provides an excellent model system to study the effect of salinity acclimation because its tadpoles are frequently exposed to widely fluctuating salinity levels (Wu and Kam, 2009; Wu et al., 2012). Wu and Kam (2009) observed that tadpoles of *F. limnocharis* survived at 11 ppt in the field, but most tadpoles could not survive 48 hr after transfer to 11 ppt in the laboratory. Those that did survive 48 hr lived at least for two weeks (Wu and Kam, 2009), suggesting that the 48 hr window after a steep salinity increase is a critical period determining survival of tadpoles. Therefore, we hypothesized that a minimum time (48 hr) is required for tadpoles to activate osmoregulatory mechanisms. Here we studied the effect of salinity acclimation on larval *F. limnocharis* salinity tolerance and osmoregulatory physiology. We experimentally manipulated larval exposure to salinity and recorded tadpole survival, osmolality, water content, and expression of NKA in the tadpoles' gills, to advance our understanding of the mechanisms involved in adaptation to osmotic stress in amphibians.

MATERIALS AND METHODS

Study Animals

F. limnocharis is a medium-sized frog (30–60 mm) widely distributed in Taiwan, ranging from sea level up to elevations of 1,000 m in mainland Taiwan and off-shore islands (Alexander et al., '79; Yang, '98). This species usually breeds in temporary freshwater pools such as rice pools and roadside ditches from February to September, but some populations breed in the brackish water of rock pools above the high tide line of coastal areas (Wu and Kam, 2009). These rock pools are uncommon breeding sites for amphibians because given their small size, seawater spills and strong rainfalls combine during the monsoon season to cause frequent and large salinity fluctuations. Salinity in these pools varies between 0–23 ppt, although no tadpoles have been found in pools with salinity over 12 ppt (Wu and Kam, 2009).

Experimental Setup

For each experiment, we collected tadpoles of *F. limnocharis* at Gosner stages 26–28 (Gosner, '60) from five pools with salinities below 3 ppt on Green Island (121.28°E, 22.35°N). Tadpoles

collected in May–August 2007 were used to measure survival, osmolality, and water content. Tadpoles collected in April–August 2008 were used to measure NKA expression in the gills. In both experiments, tadpoles from all pools were mixed together and randomly assigned to plastic containers (28.5 cm × 18.5 cm × 12 cm) holding 3,000 mL of water. Each container had 38 tadpoles reared at 3 ppt for at least 2 weeks. We obtained the different saline solutions by dissolving TAAM Real Ocean salt (TAAM, Inc., Camarillo, CA, USA) in distilled water, and the level of salinity was checked at 26°C with a handheld digital salinity/temperature/TDS meter (Rixen brand, Model SM-10, Seoul, South Korea). We fed tadpoles boiled Chinese spinach (*Amaranthus inamoenus*) ad libitum and changed the water every third day. Tadpoles were kept in incubators at 26°C under a 12 hr:12 hr light–dark cycle. We monitored tadpole survival and water salinity daily, adjusting salinity when necessary.

Experiment I: Survival to Time-Course Salinity Exposure

Tadpoles attaining Gosner stages 34–37 (body mass: 356.98 ± 11.16 mg, $n = 30$) were reared in 3 ppt (ca 95 mOsm/kg) (≈9% seawater; 3.1 g NaCl L^{-1}; 6800 μS) before transfer to: (a) 11 ppt (*ca*. 335 mOsm/kg) (≈33% seawater; 11.1 g NaCl L^{-1}; 25,000 μS); (b) 7 ppt (*ca*. 230 mOsm/kg) (≈20% seawater; 7.1 g NaCl L^{-1}; 16000 μS) for 24 hr and then 11 ppt; and (c) 7 ppt for 48 hr and then 11 ppt. Each treatment was replicated 10 times, each replicate consisting of an individual tadpole in a plastic container (10.5 cm × 7.5 cm × 4.5 cm) holding 100 mL of water. Containers were kept covered with a transparent perforated lid to reduce evaporation. We recorded the number of surviving tadpoles at 1, 3, 6, 12, 24, and 48 hr after transfer to different treatments, adjusting salinity if necessary. Salinity fluctuations were <0.5 ppt within each treatment throughout the experiment.

Experiment II: Osmolality, Water Content, and NKA Expression in Different Salinity Regimes

Tadpoles at Gosner stages 35–37 had been reared in 3 ppt for at least two weeks before transfer to one of (a) 3 ppt (a control group); (b) 7 ppt for 48 hr and then 11 ppt (a pre-acclimated group); and (c) 11 ppt (a non-acclimated group). Each tadpole after transfer to different treatments was raised individually in plastic containers (10.5 cm × 7.5 cm × 4.5 cm). We measured osmolality (mOsm/kg), water content (%), and NKA expression of different batches of tadpoles at 0, 1, 3, 6, 12, 24, and 48 hr after transfer to different salinity treatments. We placed tadpoles in a 150 mL beaker holding original water on ice for 5–10 min for anesthesia before conducting measurements.

Osmolality

We determined osmolality with a vapor pressure osmometer (Wescor brand, model 5520, Logan, UT, US) from whole-body homogenates due to the small size of the tadpoles, as is often the case in amphibians (Ultsch et al., '99; Gomez-Mestre et al., 2004). After being euthanized, tadpoles were immediately homogenized in 1.5 mL microtubes with a hand-operated grinder (Kontes Com., Article No.749540-0000, Vineland, NJ, US) for 3–5 min. The homogenates were centrifuged at 7,000*g* for 20 min and the supernatant was taken for osmolality determination. Each test point consisted of five tadpoles.

Water Content (%)

After euthanasia, each individual was placed on a tinfoil dish, dried at 50°C to constant weight in an oven (for ∼3 hr), and weighed to the nearest 0.0001 g. A percent body water content was then calculated (Wu and Kam, 2009). Each test point consisted of five tadpoles.

NKA Expression

Sample Preparation. Immediately after euthanasia we excised the gill baskets from each tadpole, pooling together gills from four individuals in 2 mL microtubes, adding 150 μL homogenization medium (5 mM Na_2EDTA, 200 Mm sucrose, 0.1% sodium deoxycholate, 100 Mm imidazole–HCl buffer, pH 7.6) and 3 μL proteinase inhibitors (10 mg antipain, 5 mg leupeptin, 1 g benzamidine in 5 mL aprotinin), and then homogenized with a homogenizer (Polytron PT1200E, Lucerne, Switzerland) at maximum speed for 20 sec, always keeping the samples in ice. The resulting gill homogenates were centrifuged at 13,000*g* for 20 min at 4°C and the supernatant taken for determination of protein concentration and immunoblotting. Protein concentrations were determined by the BCA Protein Assay Kit reagents (Pierce, Hercules, CA, USA) using bovine serum albumin (Pierce) as a standard. The supernatants were stored at −80°C until immunoblotting.

Antibodies. A mouse monoclonal antibody (α5)(Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of the avian NKA and the alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA) for immunoblotting were used as primary and secondary antibodies, respectively. The dilution rates of the antibodies were 1:500 for the primary antibody and 1:2,500 for the secondary one.

Immunoblotting. The procedure for immunoblotting followed Tang et al. (2008) with minor modifications. We mixed supernatants of homogenized gills (100 μg/lane) with loading dye, adding the supernatant of milkfish (*Chanos chanos*) gill homogenate as a positive control (50 μg/lane). Samples were heated at 37°C for 30 min. Protein molecular weight standard was purchased from Fermentas (SM0671, Hanover, MD, USA). All protein samples were separated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 7.5% polyacrylamide gels and then the separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA). To minimize non-specific binding, blots

were pre-incubated in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% Tween 20, pH 7.4) with 5% (w/v) non-fat dried milk at 4°C overnight. The blots were incubated at room temperature for 2 hr, with the primary antibodies diluted in PBST with 1%BSA (w/v) and 0.05% sodium azide, and then washed in PBST. The blots were incubated at room temperature for 1 hr with the secondary antibodies diluted in PBST. The blots were washed and then incubated at room temperature for 5 min in the reaction buffer (5 mM $MgCl_2$, 100 mM Tris-Base, 100 mM NaCl, pH 9.5) and then developed with the BCIP/NBT Kit (Zymed, South San Francisco, CA, USA). Immunoblots were scanned to produce TIF files. We obtained converted numerical values by analyzing immunoreactive bands with MCID software version 7.0 (Imaging Research, Inc., Ontario, Canada). The relative protein abundance of immunoreactive bands was obtained from the value of samples divided by that of positive control and then multiplied by 100.

Data Analyses

We used the Kaplan–Meier survival analysis to compare the curves of tadpole survivorship within 48 hr in different treatments. We tested for differences of the NKA abundance at the starting point (0 hr) among treatments and differences between 0 hr and test points (1, 3, 6, 12, 24, and 48 hr) in osmolality, water content (%) and NKA expression within each treatment by using ANOVA. We ranked variables before conducting an ANOVA because data did not meet parametric assumptions, and used Tukey HSD test for multiple comparisons. Data analysis was conducted with SPSS 11.0 (SPSS, Chicago, IL, USA). All data were expressed as means ± SE.

RESULTS

Survivorship

Tadpole survival differed statistically among treatments (log rank test, $\chi^2 = 27.92$, $P < 0.0001$, df = 2; Fig. 1). Tadpoles did not survive for more than 12 hr if directly transferred to 11 ppt, but most tadpoles pre-acclimated for 24 hr in 7 ppt survived ≥12 hr (Fig. 1). Survival in tadpoles pre-acclimated in 7 ppt for 24 hr and then transferred to 11 ppt was significantly higher than that directly exposed to 11 ppt (log rank test, $\chi^2 = 8.17$, $P = 0.0042$, df = 1). However, if acclimated to 7 ppt for 48 hr, 90% tadpoles could survive 11 ppt for 48 hr (Fig. 1). Upon transfer to 11 ppt, survivorship in tadpoles pre-acclimated in 7 ppt for 48 hr was significantly higher than that of those acclimated for 24 hr ($\chi^2 = 15.17$, $P < 0.0001$, df = 1).

Osmolality, Water Content, and NKA Expression

The osmolality of tadpoles after being directly transferred to 11 ppt sharply increased within the first 6 hr and attained the maximum at 6 hr (400.6 ± 6.78 mOsm/kg), which was on average 67% higher than that of tadpoles kept at 3 ppt (Fig. 2A).

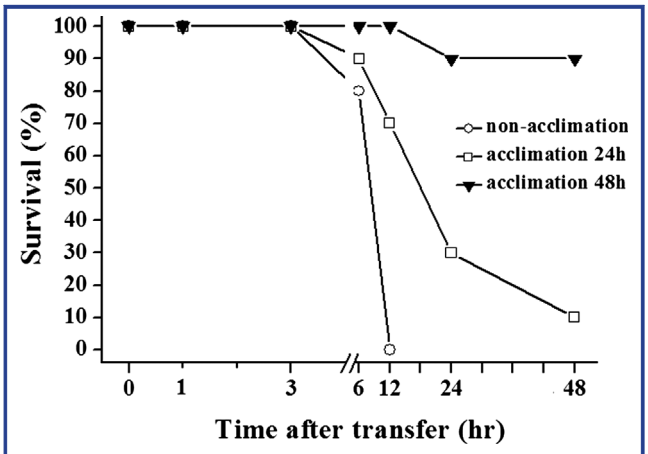

Figure 1. A 48-hr survival (%) of *F. limnocharis* tadpoles ($n = 10$) reared in different salinity regimes (non-acclimation: transferred directly to 11 ppt; acclimation 24 hr: acclimated to 7 ppt for 24 hr before transferred to 11 ppt; acclimation 48 hr: acclimated to 7 ppt for 48 hr before transferred to 11 ppt).

If first acclimated to 7 ppt for 48 hr before transfer to 11 ppt, osmolality of tadpoles sharply increased within the first 3 hr and then managed to maintain a lower level, ranging 370–390 mOsm/kg after transfer for 3 hr (Fig. 2A). The osmolality of tadpoles pre-acclimated to 7 ppt for 48 hr was lower at 6 hr than that of individuals directly transferred to 11 ppt (Fig. 2A). No significant changes in osmolality over time in the control group were found (Fig. 2A).

The water content was maintained on average at 89% in the control group (Fig. 2B). When directly transferred to 11 ppt, tadpoles gradually dehydrated over the first 6 hr (Fig. 2B), and the water content dropped to 84% at 6 hr (Fig. 2B). If tadpoles were pre-acclimated in 7 ppt for 48 hr before being transferred to 11 ppt, they immediately reduced their water content during the first 6 hr, but regained water after 6 hr and then stabilized on average at 87% between 12 and 48 hr (Fig. 2B).

The relative protein abundance of branchial NKA of tadpoles before being transferred to 11 ppt (i.e., at 0 hr) significantly differed among treatments (ANOVA, $F_{2,\ 13} = 10.00$, $P = 0.002$). Post hoc comparison showed that tadpoles pre-acclimated to 7 ppt for 48 hr had significantly higher NKA abundance than the control and the non-acclimation treatments (Fig. 3). The relative protein abundance of NKA did not statistically increase after direct transfer to 11 ppt for 6 hr (Fig. 3). If tadpoles were pre-acclimated 7 ppt for 48 hr and then transferred to 11 ppt, they managed to maintain high relative protein abundance after transfer, compared to the other treatments (Fig. 3). No significant changes of the NKA expression over time in the control group were found (Fig. 3).

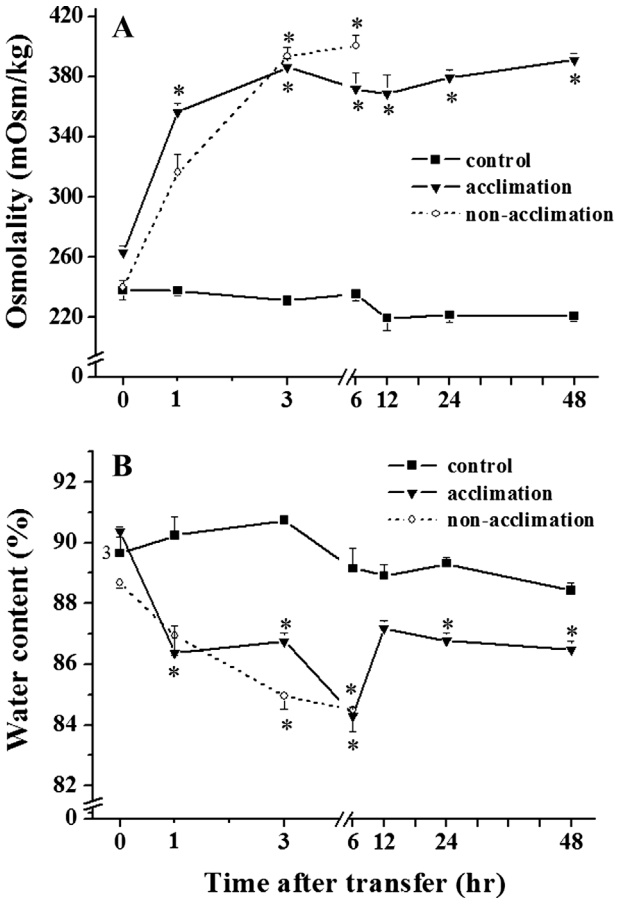

Figure 2. Changes in osmolality (mOsm/kg) (A) and water content (%) (B) of tadpoles reared in different salinity regimes (control: transferred to 3 ppt; acclimation: acclimated to 7 ppt for 48 hr before transferred to 11 ppt; non-acclimation: transferred directly to 11 ppt). Sample sizes were 5 per test point. Asterisk (*) indicates significant differences between the starting point (0 hr) and test points. Bars represent ± SE.

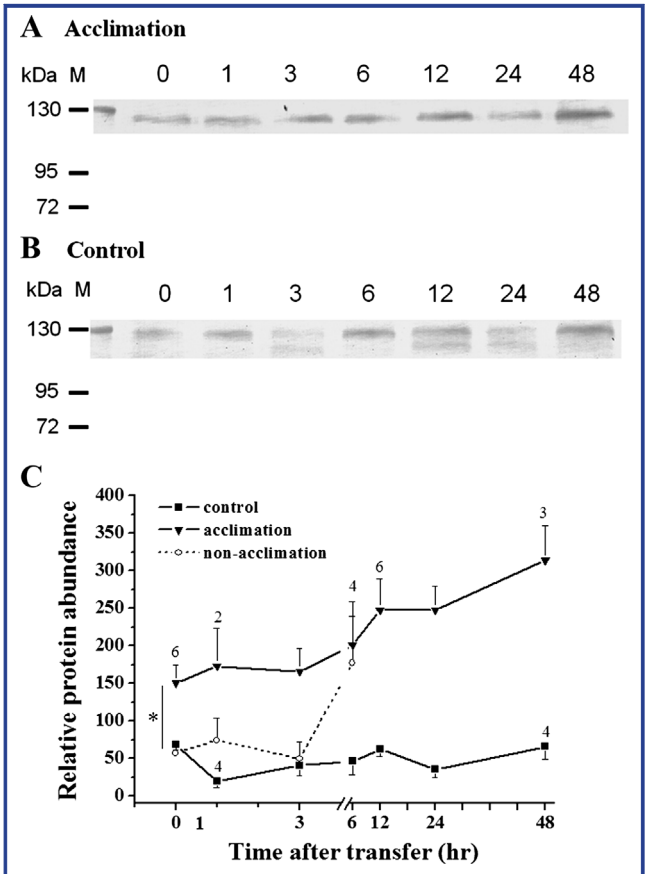

Figure 3. Changes in NKA protein expression in tadpoles reared in different salinity regimes. Representative immunoblots of branchial NKA protein expression in the acclimation group (A) and the control group, kept at 3 ppt (B). The molecular mass of immunoreactive bands is at 114 kDa. (C) Tadpoles acclimated to 7 ppt for 48 hr managed to maintain high relative protein abundance after transfer to 11 ppt, compared with the other treatments. Sample sizes were 5 per test point, except for the point labeled with numbers. Asterisk (*) indicates the NKA protein expression at the starting point (0 hr) of the acclimation treatment was significantly higher than that of the other treatments. Bars represent ± SE. M = marker

DISCUSSION

Acclimation to Intermediate Salinity Enhanced Survival in High Salinity by Modulating Dehydration and Increased NKA Expression

To date, there has been no work explaining the time-course changes of physiological parameters in tadpoles subjected to salinity stress after acclimation to intermediate salinity and which osmoregulatory mechanisms were involved in the process. We found that acclimation at an intermediate salinity (48 hr at 7 ppt prior to transfer to 11 ppt) greatly increased tadpole salinity tolerance and survival under osmotic stress, and it modulated dehydration and activated osmoregulatory mechanisms by increasing NKA expression.

Forty-eight hours at an intermediate salinity seemed to be a sufficiently long pre-acclimation period for activating osmoregulatory mechanisms granting increased tadpole survival under osmotic stress. Similarly, 48 hr was the minimum required acclimation period for increasing salinity tolerance in the euryhaline *F. cancrivora* tadpoles (Gordon and Tucker, '65; Uchiyama et al., '90a; Uchiyama and Yoshizawa, '92). Tadpoles of *F. cancrivora* that were progressively acclimated to increased salinity at a rate of 2 ppt every 2–3 days could survive up to 32 ppt

for 48 hr (Gordon and Tucker, '65). Hsu et al. (2012) further reported a stepwise salinity acclimation at a rate of 2 ppt every 3 days enhanced survival to metamorphosis of *F. cancrivora* tadpoles in high salinity, and the highest salinity threshold (21 ppt) allowing tadpoles to reach metamorphosis was higher than that reported previously (Gordon and Tucker, '65; Uchiyama et al., '90a).

Branchial NKA expression of tadpoles pre-acclimated to 7 ppt for 48 hr was significantly higher than that of non-acclimated tadpoles upon transfer to 11 ppt, suggesting that increased tolerance after acclimation was associated with elevated NKA expression in the gills. The increased NKA expression under different kinds of osmotic stress is regarded as a mechanism for maintaining intracellular homeostasis in animals, which provides a driving force for meeting the functional demands of secondary transport pathways (Hwang and Lee, 2007; Hwang et al., 2011). In euryhaline teleosts, salinity-induced branchial NKA responses include increased NKA in both hyper- and hyposmotic media (Hwang and Lee, 2007; Hwang et al., 2011). In amphibians, the branchial NKA was also the main enzyme for the active transport of ions in tadpoles (Boonkoom and Alvarado, '71). A recent study suggests that higher NKA activity in tadpoles with higher salt tolerance could explain why it could survive in brackish environments (Bernabò et al., 2013). Therefore, we suggest that increased NKA expression in tadpoles' gills was induced to regulate ionic balance in response to increased salinity. Before transfer to 11 ppt (at 0 hr), the NKA expression of tadpoles acclimated in 7 ppt for 48 hr was higher than that of tadpoles kept in 3 ppt, suggesting that increased NKA expression in the beginning was probably critical for tadpole survival when they were acutely dehydrated in 11 ppt within the first 6 hr.

The first 6 hr after a large salinity fluctuation therefore seem critical for tadpole survival in *F. limnocharis*. This effect is not exclusive of amphibians, and in tilapia (*Oreochromis mossambicus*) acclimation to 20 ppt for 24 hr resulted in higher survival after transfer to 30 ppt than non-acclimated animals (Hwang, '87), showing that the degree of dehydration during the first 6 hr was a critical period for tilapia survival (Hwang et al., '89).

Limited Osmoregulatory Ability to Overcome Osmotic Stress

In our experiment, direct transfer from 3 to 11 ppt caused a sharp increase in osmolality and a steep decrease in water content (Fig. 2). Pre-acclimation to 7 ppt for 48 hr did not prevent tadpoles from increasing their osmolality and decreasing their water content to a similar extent upon transfer to 11 ppt for the first 6 hr. However, pre-acclimation allowed tadpoles to survive the transfer and partially bounce back to attain lower osmolality (*ca.* 380 mOsm/kg) and higher water content (*ca.* 87%) than if directly transferred to 11 ppt (Fig. 2). Despite pre-acclimation, tadpoles in 11 ppt experienced high internal osmolality, even above the surrounding media (380 mOsm/kg tadpole osmolality vs. 335 mOsm/kg 11 ppt water), and well above the control group (240 mOsm/kg) (Fig. 2A). This indicates that *F. limnocharis* tadpoles have limited osmoregulatory ability in hyperosmotic environments. Tadpoles can often osmoregulate and hence maintain their internal osmolality constant within a low range of salinity concentrations, but if salinity is further increased then tadpoles can no longer osmoregulate and their osmolality begins to increase, retarding development and decreasing survival (Gomez-Mestre et al., 2004). The rise of osmolality in tadpoles exposed to saline water was mainly attributed to the increase of sodium and chloride (Gordon and Tucker, '65; Gomez-Mestre et al., 2004). Therefore, observed increased osmolality in our experiment is probably due to salt excess that tadpoles failed to excrete. In contrast, *F. cancrivora* tadpoles can regulate their internal osmolality below that of the medium even at salinity $\geq$12.8 ppt (40% sea water; Gordon and Tucker, '65; Shoemaker et al., '92). Maintaining a body fluid slightly hyperosmotic to the environment probably mitigated the dehydration effects on *F. limnocharis* tadpoles in high salinity.

Ecological Implications

In conclusion, our results show that acclimation to 7 ppt for 48 hr allowed tadpoles to withstand acute osmotic stress (11 ppt) by recovering from sharp decreases in water content and increased internal osmolality. The enhanced tolerance attained during acclimation seems to be at least partially mediated by expression of NKA proteins, as these transmembrane proteins are responsible for maintaining intracellular homeostasis and driving other transporter pathways. This mechanism may have been key in allowing *F. limnocharis* populations living in coastal rock pools to adapt to fluctuating salinity levels. Those pools used by tadpoles vary in size and depth, where salinity increases gradually due to high evaporation in temporary ponds, possibly reaching harmful levels within a day (Wu and Kam, 2009). When salinity of the pools gradually rises due to evaporation, tadpoles with sufficient acclimation period could activate the osmoregulatory mechanisms to mitigate the immediate effects of osmotic stress and increase tadpole survival. The activation of osmoregulatory mechanisms is particularly important for early-developing tadpoles because the osmotic stress experienced may have long-lasting or irreversible effects on their life history traits (Wu et al., 2012). In addition, tadpoles progressively acclimated increased survival and metamorphosed at higher salinity levels, hence showing a flexible salinity threshold of metamorphosis that would no longer depend on the timing of raining to facilitate metamorphosis (Gordon and Tucker, '65; Uchiyama et al., '90a; Uchiyama and Yoshizawa, '92; Hsu et al., 2012).

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