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Enhanced salt tolerance of euryhaline tadpoles depends on increased Na^+, K^+-ATPase expression after salinity acclimation

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ABSTRACT

Understanding physiological responses and osmoregulatory mechanisms for dealing with salinity stress is essential to clarify how amphibians living in coastal areas adapt to fluctuating salinity levels. Euryhaline species are rare among reported tadpole species inhabiting saline habitats, and few studies addressed the osmoregulatory mechanisms. We quantified the effects of salinity acclimation on survival, osmolality, water content, ion concentration, and gill Na^+, K^+-ATPase (NKA) expression of euryhaline tadpoles of *Fejervarya cancrivora*, to examine time-course changes of osmoregulatory responses of tadpoles subjected to salinity stress and how osmoregulatory mechanisms were involved in the process. Acclimation to 10 ppt for 24 h increased tadpole survival of *F. cancrivora* in 15 ppt, and it activated osmoregulatory mechanisms such as increase in NKA expression, which enabled them to maintain a stable osmolality below that of the surrounding media, to reach lower sodium and chloride concentrations of body fluid, and to modulate dehydration at higher salinities. The minimum required acclimation period is shorter than that reported previously on this species and non-euryhaline tadpoles. This study highlights that these physiological mechanisms are ecologically relevant and critical for tadpoles living in coastal brackish waters, improving their survival in coastal microhabitats with highly variable salinity levels.

1. Introduction

Understanding physiological responses and the osmoregulatory mechanisms for dealing with osmotic stress may be key to assessing how amphibian populations living in coastal areas adapt to fluctuating salinity levels (Albecker and McCoy, 2017; Kearney et al., 2014; Wu et al., 2014), as their habitats are often impacted by storms and saltwater intrusion caused by rising sea levels as a consequence of global warming (Hopkins and Brodie, 2015; Hopkins et al., 2016; Moreira et al., 2015; Rios-López, 2008; Soares De Oliveira et al., 2016a; Soares De Oliveira et al., 2016b; Wu and Kam, 2009a). Most amphibian larvae are poorly adapted to saline environments because of high ion permeability of skin and gill, and poor osmoregulatory ability, impeding water and ion balance (Balinsky, 1981; Ultsch et al., 1999), and therefore adult amphibians generally avoid breeding in brackish water and therefore their larvae are normally restricted to freshwater (Wells, 2007). The salt tolerance threshold of most amphibian species studied is around 9–12 ppt (Hopkins and Brodie., 2015; Wu and Kam, 2009b), which may represent a general upper limt of tolerance for amphibian species (reviewed by Hopkins and Brodie, 2015). Therefore, amphibians that can tolerate salinities higher than 9–12 ppt may be defined as euryhaline species. Euryhaline species are extremely rare among reported tadpole species inhabiting saline habitats (Hopkins and Brodie, 2015; Wu and Kam, 2009b). A well-known amphibian that has euryhaline tadpoles is *Fejervarya cancrivora* (formerly *Rana cancrivora*), which is the only known species whose tadpoles can tolerate full-strength seawater and regulate osmolality of body fluid below that of the external medium (Balinsky, 1981; Gordon and Tucker, 1965; Shoemaker et al., 1992). There are only a few limited reports on tadpole osmoregulatory mechanisms, which mostly focus on non-euryhaline tadpoles (Alvarado and Moody, 1970; Boonkoom and Alvarado, 1971; Dietz and Alvarado, 1974) rather than truly euryhanline tadpoles (Uchiyama and Yoshizawa, 1992; Wu and Kam, 2009b).

Aquatic amphibians need to maintain the homeostasis of body fluid when their body fluids are not isosmotic with the environmental salinity (Balinsky, 1981; Ultsch et al., 1999; Wu et al., 2014). The imbalance in water and ion homeostasis decreases their survival (Gomez-Mestre et al., 2004; Wu et al., 2014). Gills are considered important

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sites for ion exchange and regulation in tadpoles (Alvarado and Moody, 1970; Boonkoom and Alvarado, 1971; Dietz and Alvarado, 1974; Uchiyama and Yoshizawa, 1992) and their roles in osmoregulatory mechanisms is thought to resemble that of teleosts (Bradley, 2009; Ultsch et al., 1999). The euryhaline teleosts secrete excess salts via gills for maintaining physiological homeostasis (Bradley, 2009; Hwang and Lee, 2007). Mitochondrion-rich cells (MR cells, also formerly called chloride cells) in gills of teleosts are the main sites responsible for the active transport of ions (Hwang and Lee, 2007). Four types of MR cells were reported in the internal gills of *F. cancrivora* tadpoles (Uchiyama and Yoshizawa, 1992). The osmoregulatory mechanism involved by a stepwise acclimation to higher salinity was observed on changes in the proportion of four types of MR cells in gills of tadpoles subjected to different salinities (Uchiyama and Yoshizawa, 1992). When acclimated in 60% seawater (19.2 ppt), the type-4 MR cells appeared (Uchiyama and Yoshizawa, 1992). The type-4 cell structurally resembles the deep-hole MR cell observed in euryhaline teleosts (Uchiyama and Yoshizawa, 1992), which its major function is Cl^- secretion (Chang et al., 2003; Hiroi et al., 2005; Wang et al., 2009). The Na^+-K^+-ATPase (NKA) is mainly located in MR cells of gill epithelia of aquatic animals and responsible for active cellular outflow transport of Na^+ and cellular K^+ inflow for maintaining intracellular homeostasis, providing a driving force for other ion channels and transporters (Bradley, 2009; Hwang and Lee, 2007). The increased NKA expression of fish gills under both hyper- and hyposmotic stress is regarded as an indicator of osmoregulation in euryhaline teleosts (Hwang and Lee, 2007; Hwang et al., 2011). The NKA in gills of tadpoles is also the main enzyme for the active transport of ions (Bernabò et al., 2013; Boonkoom and Alvarado, 1971; Wu et al., 2014). However, elevations in abundance and/or activity of branchial NKA are often required for acclimation in response to osmotic challenge (Hwang and Lee, 2007; Hwang et al., 2011; Wu et al., 2014).

Acclimation to increasing salinity is an important mechanism for enhancing salt tolerance of amphibians, although its effects may not be universal among reported species (reviewed by Hopkins and Brodie, 2015). Even in the euryhaline tadpoles of *F. cancrivora*, previous studies still showed that a stepwise acclimation to higher salinity is necessary for them to build up the appropriate osmoregulatory machinery that will allow them to survive at 16 ppt or above (Hsu et al., 2012; Uchiyama and Yoshizawa, 1992). Sufficient acclimation period is essential for an increase of salt tolerance and for activating osmoregulatory mechanisms in tadpoles (Hsu et al., 2012; Uchiyama and Yoshizawa, 1992; Wu et al., 2014). Previous studies showed that 48 h was the minimum required acclimation period for increasing salinity tolerance in the reported tadpole species (Gordon and Tucker, 1965; Uchiyama et al., 1990; Uchiyama and Yoshizawa, 1992; Wu et al., 2014). However, unlike non-euryhaline tadpoles, the euryhaline tadpoles of *F. cancrivora* naturally inhabit coastal wetlands with widely fluctuating salinity levels (0–35 ppt) (Gordon et al., 1961; Gordon and Tucker, 1965; Hsu et al., 2012; Uchiyama et al., 1990). These tadpoles may experience rapid salinity increases that can even reach harmful levels within days because of high evaporation rates (Gordon et al., 1961). Therefore, in theory, a faster activation of osmoregulatory mechanisms in *F. cancrivora* tadpoles would be vital to cope with unpredictable and short-term dramatic changes in salinity. So far, how the euryhaline tadpoles physiologically adjust to increased salinity, especially their response physiologically to acute hyperosmotic stress and osmoregulatory mechanisms for survival during the stress period, is largely unknown.

This aim of this study is to examine time-course changes of osmoregulatory responses in the euryhaline tadpoles of *F. cancrivora* subjected to salinity stress after acclimation to intermediate salinity and how osmoregulatory mechanisms were involved in the process. We experimentally manipulated larval exposure to salinity and measured survival, osmolality, water content, ion concentration and expression of gill NKA of tadpoles. Understanding the effects of salinity acclimation to salt stress on osmoregulatory responses of tadpoles subjected to hyperosmotic stress is helpful to understand how the euryhaline tadpoles physiologically adapt to increasing salinity of habitats, especially when facing increased salinization of coastal areas.

2. Materials and methods

2.1. Study animals

Fejervarya cancrivora is a medium- to large-sized frog (50–90 mm) distributed throughout East, Southeast, and South Asia (Kurniawan et al., 2010). This species is found in coastal lowland areas of southern Taiwan (Lin-bien and Jia-tung township), where it was first recorded in 2005 (Shang et al., 2009). The preferred habitats of this species at this locality experience increased salinization of its habitats because of seawater intrusion resulting from groundwater overexploitation (Wang et al., 2004), frequent flooding with seawater, and a unique agricultural practice where bell-fruit (*Syzygium samarangense*) growers introduce seawater to orchards as a way to stimulate fruiting (Hsu et al., 2012). The salinity of water bodies used by *F. cancrivora* ranges from 0 to 16 ppt (Hsu et al., 2012). The climate in this area is characterized by high air temperature (monthly air temperature averages 25.2 °C) and abundant annual rainfall (annual rainfall averages 1943 mm) (2000–2007, data from Central Weather Bureau, Taiwan). *Fejervarya cancrivora* typically breeds in temporary water bodies such as roadside ditches and puddles during the breeding season of May to November, and its larval period is < 2 months (Hsu et al., 2012).

Each time we collected four females and twelve males by hand from the field in Jia-tung township (120.53°E, 22.43°N) in 2011 and 2013 for respect experiments. Frogs were kept in four large containers (69 × 48 × 40.7 cm), each with a female and three males. The use of animal in this study was approved by the Institutional Animal Care and Use Committee (IACUC), Tunghai University, Taiwan (Approval No. 95–06).

2.2. Experimental setup

For each experiment, tadpoles were collected from four egg clutches (ca 3000 eggs each), which were raised in a container (69 × 48 × 40.7 cm) at 3 ppt and room temperature (ca. 26°C) until hatching (ca. three days). The salinity we reared eggs is based on the salinity of water bodies where eggs and tadpoles of *F. cancrivora* were found in the field (Hsu et al., 2018; Hsu et al., 2012). Tadpoles hatching from each clutch were mixed together and then randomly assigned to different treatments. Tadpoles attaining Gosner stages 35—38 were reared in 3 ppt (ca 95 mOsm/kg, ≈9% seawater) before transfer to: a) 3 ppt (a control group); b) 15 ppt (a non-acclimated group, ca 455 mOsm/kg, ≈45% seawater); and c) 10 ppt (an acclimated group, ca 300 mOsm/kg, ≈30% seawater) for 24 h and then 15 ppt. In both experiments (see below), each replicate consisted of an individual tadpole in a plastic container (10.5 × 7.5 × 4.5 cm) holding 100 ml of water. Containers were covered with a transparent, perforated lid to reduce evaporation. We monitored tadpole survival and water salinity daily, keeping salinity constant throughout the experiment. We fed tadpoles boiled Chinese spinach (*Amaranthus inamoenus*) ad libitum and changed all of the water every third day. Tadpoles were kept in incubators at 30 °C under a 12 h:12 h light-dark cycle. The incubator temperature was selected based on previous study (Hsu et al., 2012). We prepared different saline solutions by dissolving Coralife scientific grade marine salt (Energy Savers Unlimited, INC, Carson, CA, USA) in distilled water, and the level of salinity was checked at 30 °C with a handheld digital salinity/temperature/TDS meter (Rixen brand, Model SM-10, Taiwan).

1) Experiment I: Survival to time-course salinity exposure

The number of surviving tadpoles was recorded at 1, 3, 6, 12, 24, and 48 h after transfer to different treatments. Each treatment was replicated 30 times.

2) Experiment II: Osmolality, water content, ion concentration, and NKA Expression in different salinity regimes

We measured osmolality (mOsm/kg), water content (%), ion concentration (sodium and chloride), and gill NKA expression of different batches of tadpoles at 0, 1, 3, 6, 12, 24, and 48 h after transfer to different salinity treatments. We anesthetized tadpoles in a 150 ml beaker holding original water on ice for 5—10 min before conducting measurements. The method of hypothermia followed by cooling for anesthesia and euthanasia has been suggested to use for smaller ectothermic vertebrates and is currently acceptable (Lillywhite et al., 2017).

2.3. Osmolality

The osmolality was determined with a vapor pressure osmometer (Wescor brand, model 5520, US) from whole-body homogenates because of the small size of the tadpoles, as is often the case in amphibians (Gomez-Mestre et al., 2004; Ultsch et al., 1999; Wu et al., 2014). After being euthanized, two tadpoles were pooled and immediately homogenized in 1.5 mlmicro tubes with a hand-operated grinder (Kontes Com., Article No.749540–0000, US) for 3—5 min. The homogenates were centrifuged at 7000*g* for 20 min and the supernatant was taken for osmolality determination.

2.4. Water content (%)

Each individual was placed on a tinfoil dish after euthanasia, dried at 50 °C to constant weight in an oven for 3 h, and weighed to the nearest 0.0001 g (Wu and Kam, 2009a). Percentage of body water content was then calculated.

2.5. Ion concentration

The procedure of collecting body fluid for measuring ion concentration was the same as that for measuring osmolality. The samples were diluted with deionized water before sodium concentration and chloride measurements. We measured sodium concentration using an atomic absorption spectrophotometer (Z-5000, Hitachi, Japan). The dilution rates for samples were 1:5000, except for samples collected from tadpoles subjected to 15 ppt at 48 h after acclimation to intermediate salinity (1:10000). The procedure for measuring chloride concentration followed Franson (1985). The dilution rate for samples assigned to chloride determination was 1:5000. We measured chloride concentration using a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 600 nm.

2.6. Gill NKA expression

1) Sample Preparation

The procedure for measuring gill NKA expression followed Tsai and Lin (2007) and Huang et al. (2011) with modifications. The gill baskets excised from three tadpoles were pooled in 1.5 ml micro tubes, adding 120 µl homogenization medium (5 mM Na_2EDTA, 200 mM sucrose, 0.1% sodium deoxycholate, 100 mM imidazole-HCl buffer, pH 7.6) with proteinase inhibitors (3.31 mM Antipain, 2.16 mM Leupeptin, 63.86 mM Benzamidine in aprotinin saline solution) (5–10 trypsin inhibitor units per ml, Sigma-Aldrich, MO, USA), and then homogenized with an ultrasonic homogenizer (CT 15RE, Hitachi, Japan), always keeping the samples in ice. The resulting gill homogenates were first centrifuged at 6000*g* for 10 min at 4 °C and then were centrifuged at 20000*g* for 20 min at 4 °C (Ultrasonic Processor, SONICS, USA). After centrifugation, the supernatant was taken for determination of protein concentration and immunoblotting.

One µl of supernatant was diluted to 800 µl with deionized water and then mixed with 200 µl of protein dye reagent (Bio-Rad) at room temperature for 5 min. Protein concentrations were determined by a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 595 nm using bovine serum albumin (Pierce) as a standard. The supernatants were stored at −80 °C until immunoblotting.

2) Antibodies

For immunoblotting, the NKA α5 monoclonal antibody (1:2500, from chicken, DSHB, USA) and β-actin monoclonal antibody (1:5000, from mouse, Chemicon, USA) were used as primary antibodies. The horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG were used as a secondary antibody (1:5000, Jackson Immuno Research Laboratories, West Grove, PA, USA).

3) Immunoblotting

We mixed supernatants of homogenized gills (50 µg / lane) with sample-loading buffer (20 mM Tris-Hcl, pH 6.8, 8% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 40% glycerol, and 0.4% Bromophenol Blue). Samples were denatured at 37 °C for 30 min. All protein samples were separated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 5% and 10% polyacrylamide gels and then the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, NEN Life Science, Boston, MA, USA). To minimize nonspecific binding, blots were pre-incubated in PBST buffer (136.9 mM NaCl, 2.68 mM KCl, 6.39 mM $Na_2HPO_4{\cdot}2H_2O$, 1.76 mM KH_2PO_4, and 0.5% Tween 20, pH 7.4) with 5% (*w/v*) nonfat dried milk at room temperature for one hour and washed in PBST four times. The blots with the primary antibodies were incubated at room temperature (ca. 26 °C) for 1 h. After washed in PBST three times, the blots with the secondary antibodies were incubated at room temperature for 1 h and then washed in PBST five times. We used western blot chemiluminescence reagent plus system (NEL105, NEN Life Science) to detect the proteins and then captured and photographed the signals by an Intelligent Dark Box II with Fujifilm LAS-1000 digital camera. We analyzed the relative protein abundance of immunoreactive with Image Gauge 4.0 (Fujifilm). The relative protein abundance of immunoreactive bands was obtained from the value of samples divided by that of β-actin (control) and then multiplied by 100.

2.7. Data analyses

The Kaplan-Meier survival analysis was used to compare the curves of tadpole survivorship within 48 h in different treatments. Because of small size of the tadpoles, we must sacrifice tadpoles and then used whole-body animals for measurements of osmolality, water content and ion concentration, and a pooled gills for measurements of the NKA expression at each time point (at 0, 1, 3, 6, 12, 24, and 48 h after transfer to different salinity treatments). Therefore, measurements of different variables for each time point were taken from different individuals or experimental unit. To understand the trend of time-course variation in osmolality, water content (%), ion concentration, and NKA expression, we tested differences among different test time-points within each treatment in these variables by using ANOVA. We also used ANOVA to test for differences among treatments at the same test time-point in osmolality, water content, ion concentration, and NKA expression, in order to further understand whether there are differences in time-course variations of these variables among treatments groups. We checked the parametric assumptions for all variables before analyzing with ANOVA. We used Fisher LSD test for multitiple comparisons. Data analysis was conducted with SPSS 17.0 (SPSS, Chicago, IL, USA). All

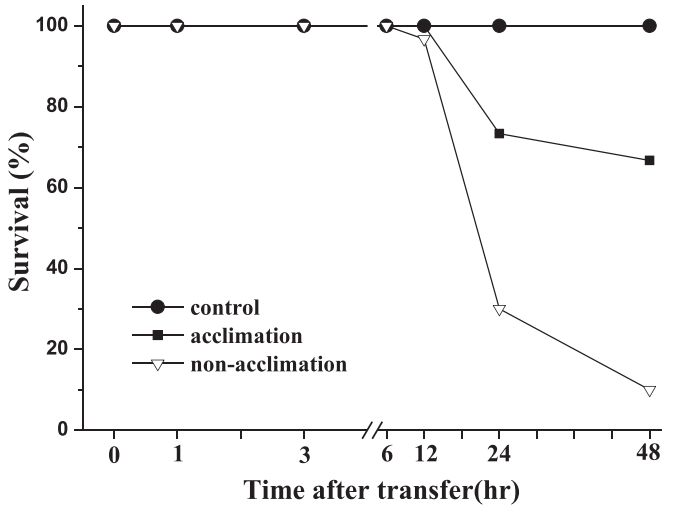

Fig. 1. A 48-h survival (%) of *F. cancrivora* tadpoles (n = 30) reared in different salinity regimes (control: transferred to 3 ppt; non-acclimation: transferred directly to 15 ppt; acclimation: acclimated to 10 ppt for 24 h before transferred to 15 ppt).

data were expressed as means ± SE.

3. Results

3.1. Survivorship

Tadpole survival differed statistically among treatments (log rank test, X^2 = 64.37, P < .001, df = 2; Fig. 1). Most tadpoles did not survive for > 48 h if directly transferred to 15 ppt, but 67% of tadpoles pre-acclimated for 24 h in 10 ppt survived 48 h (Fig. 1). Survival in tadpoles pre-acclimated in 10 ppt for 24 h and then transferred to 15 ppt was significantly higher than in tadpoles directly exposed to 15 ppt (log rank test, X^2 = 29.92, P < .001, df = 1).

3.2. Osmolality

The osmolality of tadpoles after being directly transferred to 15 ppt significantly increased within the first 3 h and attained the maximum at 24 h (480.5 ± 12.4 mOsm/kg), which was on average 84% higher than that of tadpoles kept in the control group (254–267 mOsm/kg) (Fig. 2A). For tadpoles that first acclimated to 10 ppt for 24 h before transfer to 15 ppt, the osmolality gradually increased within the first 3 h and attained the maximum at 12 h (434 ± 5.07 mOsm/kg), and then managed to maintain a lower level that ranged 376—380 mOsm/kg after 24 h (Fig. 2A). At 24 h after transfer, the osmolality of tadpoles pre-acclimated to 10 ppt for 24 h was lower than that of individuals directly transferred to 15 ppt (Fig. 2A). No significant changes in osmolality over time were found in the control group (Fig. 2A).

3.3. Water content

The water content over time was maintained on average at 88% in the control group (ANOVA, $F_{6,63}$ = 0.52, P = .789) (Fig. 2B). When directly transferred to 15 ppt, tadpoles gradually dehydrated over the first 6 h and then sharply dropped to 81.5% at 24 h (Fig. 2B). However, tadpoles that were pre-acclimated in 10 ppt slowly dehydrated during the first 12 h but then stabilized on average at 84% between 12 and 48 h (Fig. 2B).

3.4. Ion concentration

The sodium concentration over time was maintained on average at

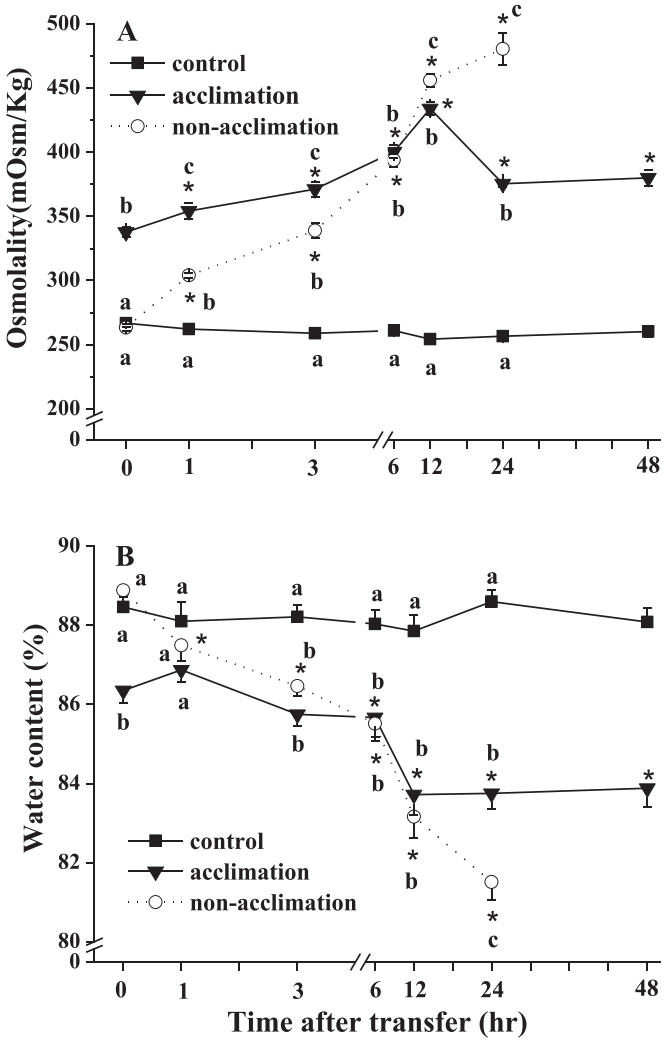

Fig. 2. Changes in osmolality (mOsm/kg) (A) and water content (%) (B) of tadpoles reared in different salinity regimes (control: transferred to 3 ppt; non-acclimation: transferred directly to 15 ppt; acclimation: acclimated to 10 ppt for 24 h before transferred to 15 ppt). Sample sizes were 10 per test point. Each replicate consisted of two individuals in the measuremens of osmolality but each replicate represents one individual in the measurement of water content. Asterisks (*) indicate significant differences between the starting point (0 h) and test points. Identical letters indicate no significant difference between treatments at the same test time-point. Bars represent ± SE.

60 mM in the control group (ANOVA, $F_{6,63}$ = 1.44, P = .215) (Fig. 3A). When directly transferred to 15 ppt, Na^+concentration of tadpoles significantly increased after 1 h (ANOVA, $F_{5,54}$ = 138.5, P < .001) and then attained the maximum at 12 h (122.4 ± 1.7 mM), which was on average 200% higher than that at 0 h (60 ± 1.3 mM) (Fig. 3A). However, Na^+concentration of individuals that were pre-acclimated in 10 ppt for 24 h before being transferred to 15 ppt significantly increased within the first 12 h and then decreased (Fig. 3A). Six hours after the transfer Na^+concentration of individuals in the acclimation treatment was higher than that in the control treatment, but lower than that in the non-acclimation treatment (Fig. 3A).

The chloride concentration over time was maintained on average at 74 mM in the control group (ANOVA, $F_{6,63}$ = 0.56, P = .761) (Fig. 3B). When directly transferred to 15 ppt, Cl^-concentration of tadpoles significantly increased after 1 h (ANOVA, $F_{5,54}$ = 43.04, P < .001) and then attained the maximum at 24 h (160.8 ± 7.4 mM), which was on average 230% higher than that at 0 h (73.3 ± 3.6 mM) (Fig. 3B). The Cl^-concentration of individuals in the acclimation treatment

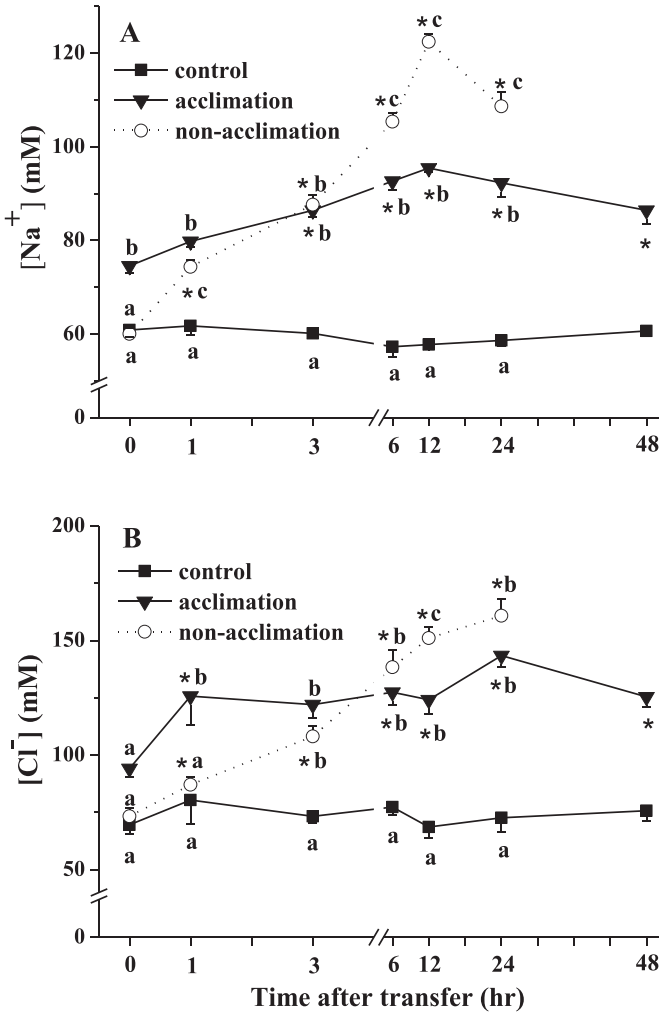

Fig. 3. Changes in sodium concentration (mM) (A) and chloride concentration (mM) (B) of tadpoles reared in different salinity regimes (control: transferred to 3 ppt; non-acclimation: transferred directly to 15 ppt; acclimation: acclimated to 10 ppt for 24 h before transferred to 15 ppt). Sample sizes were 10 per test point. Each replicate consisted of two individuals. Asterisks (*) indicate significant differences between the starting point (0 h) and test points. Identical letters indicate no significant difference between treatments at the same test time-point. Bars represent ± SE.

significantly increased over time (ANOVA, $F_{6,63} = 4.76$, $P < .001$) but stabilized on average at 130 mM between 1 and 48 h (Fig. 3B). Six hours after the transfer Cl^- concentration of individuals in the acclimation treatment was higher than that in the control treatment but lower than that in the non-acclimation treatment (Fig. 3B).

3.5. NKA expression

Single immunoreactive bands of NKA at approximately 100 kDa were detected in immunoblots of gills in the acclimation group (Fig. 4A), the non-acclimation group (Fig. 4B) and the control group (Fig. 4C). Relative abundance of branchial NKA proteins in both control and non-acclimated tadpoles remained unchanged over time ($F_{6,63} = 0.20$, $P = .98$; $F_{5,54} = 0.35$, $P = .88$, respectively) (Fig. 4D). However, the relative protein abundance of NKA in tadpoles in the acclimation treatment significantly changed over time (ANOVA, $F_{6,63} = 3.02$, $P = .01$)(Fig. 4D). During the 6–24 h period, the relative protein abundance of branchial NKA of tadpoles in the acclimation treatment was higher than that in the other two treatments (Fig. 4D).

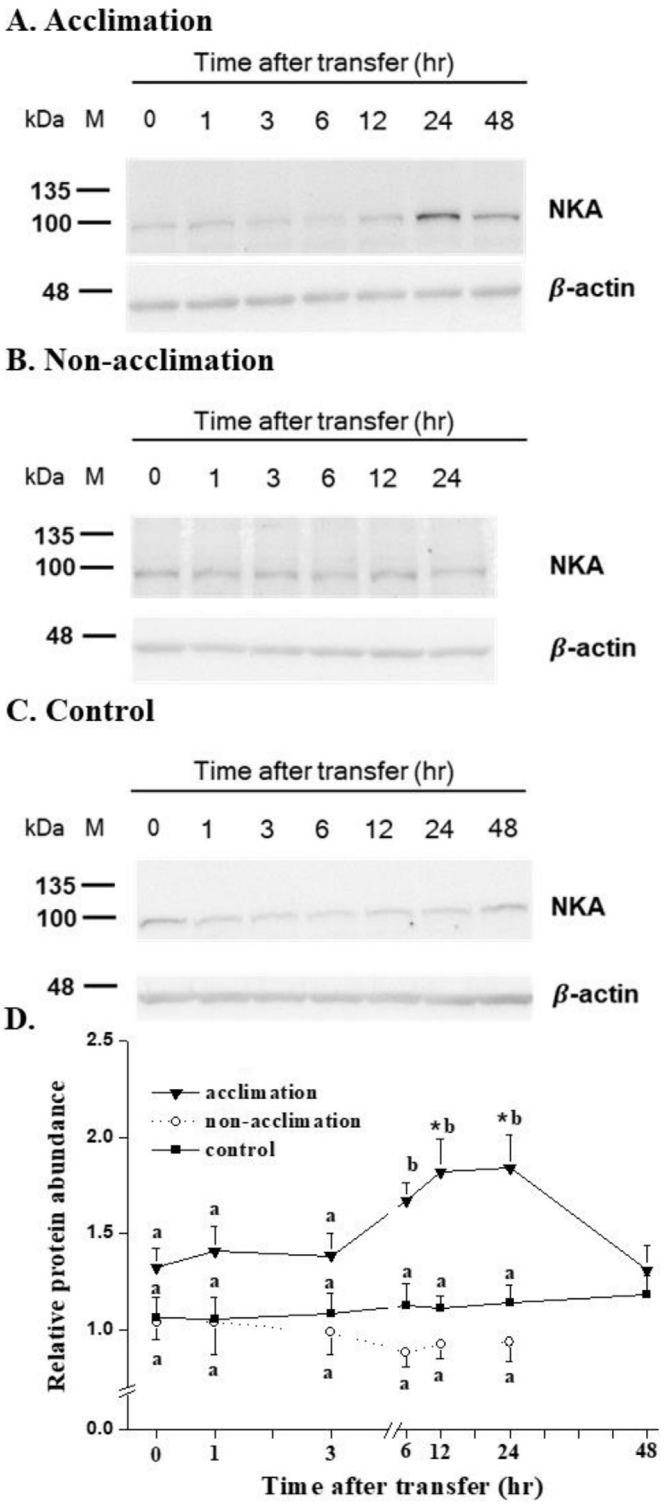

Fig. 4. Representative immunoblots of branchial NKA protein expression in the acclimation group (A), the non-acclimation group (B) and the control group (C). The molecular mass of immunoreactive bands of NKA is at 100 kDa. (D) Changes in NKA protein expression in tadpoles reared in different salinity regimes. Sample sizes were 10 per test point. Each replicate consisted of gills from three individuals. Asterisks (*) indicate significant differences between the starting point (0 h) and test points. Identical letters indicate no significant difference between treatments at the same test time-point. Bars represent ± SE. M = marker.

4. Discussion

4.1. A short-term acclimation to intermediate salinity is sufficient for activating osmoregulatory mechanisms in euryhaline tadpoles

As expected, acclimation at an intermediate salinity activated osmoregulatory mechanisms and increased salinity tolerance of *F. cancrivora* tadpoles. Additionally, twenty-four hours at an intermediate salinity was a sufficiently long pre-acclimation period for activating osmoregulatory mechanisms, suggesting euryhaline tadpoles have the capacity to activate such mechanisms after a relatively short-term acclimation period. The minimum required acclimation period for increasing salinity tolerance in *F. cancrivora* tadpoles is shorter than that reported previously on this species (48 h) (Gordon and Tucker, 1965; Hsu et al., 2012; Uchiyama et al., 1990; Uchiyama and Yoshizawa, 1992) and non-euryhaline tadpoles (*F. limnocharis*) (48 h) (Wu et al., 2014). Gordon and Tucker (1965) reported a stepwise salinity acclimation at a rate of 2 ppt every 2—3 days enhanced survival of *F. cancrivora* tadpoles (up to 32 ppt for 48 h). However, tadpoles of *F. cancrivora* that were progressively acclimated to increased salinity at a rate of 2 ppt every 3 days could survive to metamorphosis in 21 ppt (Hsu et al., 2012). The osmoregulatory mechanism of tadpoles of *F. cancrivora* is thought to resemble that of euryhaline teleosts (Gordon and Tucker, 1965; Uchiyama and Yoshizawa, 1992). Similarly, the appropriate acclimation period for increasing salinity tolerance was also 24 h in the euryhaline teleosts, such as the tilapia (*Oreochromis mossambicus*) (Hwang, 1987; Hwang et al., 1989), Japanese medaka (*Oryzias latipes*) (Inoue and Takei, 2003), and the sailfin molly (*Poecilia latipinna*) (Yang et al., 2011). For example, acclimation to 20 ppt for 24 h resulted in higher survival of the tilapia after transfer to 30 ppt than non-acclimated animals (Hwang, 1987; Hwang et al., 1989). The survival rate in the sailfin molly pre-acclimated for 24 h in 15 pp. was 100% but only half of the fish survived if directly transferred from freshwater to 35 ppt (Yang et al., 2011). In the field, tadpoles of *F. cancrivora* may face ambient water salinity ranging from 0 to 35 ppt (Gordon et al., 1961; Gordon and Tucker, 1965; Hsu et al., 2012) and experience harmful salinities ($\geq$ 19 ppt) within days as a result of high evaporation rates (Gordon et al., 1961). However, the salinity of temporary pools with tadpoles increased about 0.5–2 ppt/day because of evaporation (Hsu et al., 2012), indicating that conditions of habitats allow them to acclimatize since salinity increases gradually by evaporation. Therefore, our results highlight that the mechanisms are especially necessary for tadpoles to physiologically overcome osmotic stress when they face unpredictable and dramatic salinity increases in coastal microhabitats, benefiting their survival.

Branchial NKA expression of tadpoles pre-acclimated to 10 ppt for 24 h was significantly higher than that of non-acclimated tadpoles upon transfer to 15 ppt after 3 h. Also, $Na^+$and Cl^-concentrations of pre-acclimated tadpoles remained lower than in non-acclimated tadpoles after transfer for 6 h (Fig. 3) when NKA expression increased over time (Fig. 4). These results suggest that discharging excess salts for maintaining physiological homeostasis after acclimation was associated with elevated NKA expression in the gills. In contrast to a similar study in *F. limnocharis*, Wu et al. (2014) found that pre-acclimation to 7 ppt for 48 h activated NKA expression of tadpoles living in brackish rock pools of costal areas, resulting in increased survivorship and reduced dehydration upon later transfer to 11 ppt. However, they did not provide a clear evidence on the association between salt excretion and physiological role of elevated NKA expression in gills, since no $Na^+$and Cl^-concentration measurements were available (Wu et al., 2014). The trend of time-course variation in branchial NKA expression of tadpoles in *F. cancrivora* is different from that in non-euryhaline species as *F. limnocharis*. The NKA expression of *F. limnocharis* tadpoles in acclimation treatment before transfer to 11 ppt (at 0 h) was higher than that of tadpoles kept in 3 ppt (Wu et al., 2014). However, the expression of *F. cancrivora* tadpoles in acclimation treatment after transfer to 15 ppt was similar to that kept in 3 ppt within the first 3 h but gradually increased and became higher than that in 3 ppt during 3—24 h.

The degree of initial dehydration after transfer to osmotic stresses is relevant to pre-acclimation in an intermediate salinity (Hwang et al., 1989; Wu et al., 2014), which may influence survival of teleosts (Hwang et al., 1989; Wang et al., 2009) and tadpoles (Hopkins and Brodie, 2015; Wu et al., 2014). Euryhaline teleosts subjected to osmotic stress experience a crisis period and a regulatory period (Hwang, 1987; Lin and Lee, 2016; Wang et al., 2009). The early dehydration phase is referred to as a crisis period because of a rapid increase of gill ion exchange and plasma ions (Hwang et al., 1989; Wang et al., 2009). Before experiencing a regulatory period, the teleosts must overcome dehydration during the crisis period (Hwang, 1987; Lin and Lee, 2016; Wang et al., 2009). During the regulatory period, osmoregulatory mechanisms were activated, with an involvement of activity and expression of NKA, as well as a proliferation and/or development of functional MR cells, resulting in net Na^+ and Cl^- efflux increases and maintaining water balance (Hwang, 1987; Hwang et al., 1989; Lin and Lee, 2016; Wang et al., 2009). Our results showed that branchial NKA expression of *F. cancrivora* tadpoles in the acclimation treatment was higher than that in the other two treatments after 6 h, suggesting they began experiencing a regulatory period after 6 h (Fig. 4). In contrast to the study on the NKA activity of rough-skinned newts (*Taricha granulosa*) inhabiting a coastal stream, the result showed a significant increase in osmolality but not NKA activity after only 6 h of exposure to high salinity (Hopkins et al., 2016). It is possible that the exposure period was too short to activate the NKA expression for the newts. Our results show that a pre-acclimation in an intermediate salinity was necessary for *F. cancrivora* tadpoles to overcome acute osmotic challenge during the crisis period and then to exhibit NKA expression during a regulatory period.

4.2. Efficient osmoregulatory ability to overcome osmotic stress

Direct transfer from 3 ppt to 15 ppt caused a sharp increase in osmolality and a steep decrease in water content (Fig. 2). However, pre-acclimation to 10 ppt for 24 h prevented tadpoles from increasing their internal osmolality upon transfer to 15 ppt after 24 h and their osmolality (376—380 mOsm/kg) was below that of the surrounding media (ca 455 mOsm/kg), suggesting they are efficient osmoregulators. Previous studies showed that when non-euryhaline tadpoles are exposed to hyperosmotic environment, internal osmolality is higher than that of surrounding media in *Epidalea calamita* (formerly *Bufo calamita*) (Gomez-Mestre et al., 2004) and *F. limnocharis* (Wu et al., 2014). The rise of osmolality in tadpoles exposed to saline water was mainly due to increased sodium and chloride (Gomez-Mestre et al., 2004; Gordon and Tucker, 1965). The time-course study on *F. limnocharis* tadpoles showed that, despite pre-acclimation in 7 ppt, tadpoles in 11 ppt experienced high internal osmolality, even above that of the surrounding media, indicating that *F. limnocharis* tadpoles have limited osmoregulatory ability under hyperosmotic environments (Wu et al., 2014). Wu et al. (2014) suggest that maintaining a body fluid slightly hyperosmotic to the environment may mitigate the dehydration effects observed in *F. limnocharis* tadpoles at high salinity. By contrast, *F. cancrivora* tadpoles under hyperosmotic environment can regulate internal osmolality below that of the medium by excreting excess salts. However, despite being able to maintain internal osmolality constant and below that of the medium, the osmolality (376—380 mOsm/kg) is still above the control group (254–267 mOsm/kg)(Fig. 2B). This suggests that the osmolality level may be caused by dehydration because of their highly permeable skins or even be contributed by organic osmolytes, as in some marine species (Kinne, 1993).

Our results also found that euryhaline tadpoles display a better dehydration tolerance than reported non-euryhaline tadpoles. When directly transferred to hyperosmotic media, the water content of *F. cancrivora* tadpoles dropped to 81.5% (ca 7.5% water loss) at 24 h (Fig. 2B) and most of them did not survive for > 48 h, but *F. limnocharis*

tadpoles dropped to 84% (ca 5% water loss) at 6 h and most of them did not survive for > 12 h (Wu et al., 2014). A possible explanation for the inability of tadpoles to survive after direct transfer to osmotic stress is gill damage. Bernabò et al. (2013) demonstrated acute effects of salinity on morphology of internal gills of two tadpole species, showing that morphological alterations include severe dehydration of both gill filters and gill tufts, increased mucous secretion, detachment of external layer, alternation of epithelial surface, degeneration phenomena, and appearance of residual bodies. They suggested these alternations were harmful for normal branchial functions of respiration, osmoregulation, and acid-base regulation and hence increased mortality of tadpoles (Bernabò et al., 2013).

4.3. Conclusion

In conclusion, we demonstrate that twenty-four hours at an intermediate salinity was a sufficiently pre-acclimation period for activating osmoregulatory mechanisms, allowing *F. cancrivora* tadpoles to withstand acute osmotic stress by recovering from sharp decreases in water content and increased ionic influx and hence to increase survival under high salinity. As predicted, the minimum required acclimation period for increasing salinity tolerance in *F. cancrivora* tadpoles is shorter than that in reported non-euryhaline tadpoles. The mechanisms are suggested to be mediated by expression of NKA proteins, as these transmembrane proteins are responsible for maintaining intracellular homeostasis and driving other transporter pathways. These physiological mechanisms are ecologically relevant for tadpoles adapted to living in brackish water, where water salinity of their habitats may vary frequently and widely because of evaporation and rainfall. Gordon et al. (1961) suggested that spawning and tadpole metamorphosis of *F. cancrivora* were synchronized by a period of heavy rain, because high salinity negatively impacts survival and metamorphosis of tadpoles and they require dilute media for breeding and tadpole growth. Nevertheless, acclimated progressively to increasing salinity enhances salt tolerance, hence tadpoles exhibit a flexible salinity threshold of metamorphosis and would not depend on the timing of raining to facilitate metamorphosis (Gordon and Tucker, 1965; Hsu et al., 2012; Wu et al., 2014). The current finding may add substantially to our understanding of the physiological mechanisms and adaptive processes of salt tolerance in amphibians, required to expect rates of physiological adaptation to the pace and intensity of environmental change and to explore the factors that influence amphibian distributions in brackish wetlands (Albecker and McCoy, 2017).

Declarations of interest

None

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