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Research Article

Effects of Salinity on the Egg Fertilization, Hatching, and Tadpole Growth and Survival Rates of the Thailand Frog (*Rana tigerina* Dubois, 1981)

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We studied the effects of salinity on the egg fertilization, hatching, and tadpole growth and survival rates of the Thailand frog (*Rana tigerina* Dubois, 1981), an introduced species that is commonly cultured in the Mekong Delta, Vietnam. Salinity levels of 2, 6, and 4% significantly affected the fertilization and hatching rates of the eggs, as well as the tadpole survival rates from the newly hatched to yolk sac exhaustion stages, respectively ($p < 0.05$). Notably, during the stage from yolk sac exhaustion to juvenile tadpole, a salinity of 3% resulted in an increase in the final weight of the tadpoles throughout the stage and a significant increase in Phase 1 (the tadpole stage from yolk sac exhaustion to the onset of foreleg emergence) and Phase 2 (the tadpole stage from the onset of foreleg emergence to the onset of hind leg emergence); however, it did not show a significant effect on tadpole survival rates in Phases 1 and 2 of this stage ($p > 0.05$). A salinity level of up to 5% showed no significant effect on the growth performance but caused a significant reduction in the survival rates of the tadpoles during this stage. We recommend that the artificial reproduction process of Thai frogs should be performed using salinities of <2%, with salinities of ≤2 and 3% being suitable for rearing tadpoles from the newly hatched to yolk sac exhaustion stage and from the yolk sac exhaustion to juvenile stage, respectively.

1. Introduction

Frog meat has high protein content, a high proportion of unsaturated fatty acids, complete and balanced amino acids [1], low lipid content, and low cholesterol [2], while also being a good source of minerals and amino acids [3]. In many African countries (e.g., Burkina Faso, Cameroon, Ghana, Guinea, Nigeria, and Namibia), frogs are consumed as an essential animal protein source [4, 5]. Frog meat is also a popular delicacy in Europe and is even consumed in countries where it is legally prohibited to hunt frogs [6] and even in Asia (Indonesia) [7]. Beyond food values, frogs have also been used in the leather industry, as souvenirs, as pets, and in traditional medicine [8, 9]. Frog production has been widely reported since the 20th century, yet frog product consumption has been well-known since the 16th century [7, 10, 11]. In the 1980s, frog legs consumed were 6500 tons per year globally, with Asian nations having the highest exports [12]. Advances in cultural technology have promoted the expansion of high-productivity frog farming under controlled conditions, both in terms of distinctive facilities for different life stages and control of water quality, nutrition regimes, and sanitation [13]; Olvera-Novoa [14, 15]. Currently, many major frog producers are based in Asian (China and Taiwan) and Latin American (Brazil and Mexico) countries, while Europe and the United States of America are the largest frog importers worldwide [7, 10, 11, 16, 17]. Frog species are commonly used as meat sources, including European green frog (*Pelophylax ridibundus* Pallas, 1771), East Asian bullfrog (*Hoplobatrachus rugulosus* Wiegmann, 1834), and Rana species [18].

The intrusion of salt into inland areas is increasing daily due to human activities such as salt production and deforestation, as well as salinization due to climate change (linked to sea level rise) [19–21]. Over the past years, the effects of climate change have also substantially affected the Mekong Delta Region in Vietnam, especially sea level rises in low-lying areas at the mouth of the delta [22]. In 2012, around 620,000 ha in the Mekong Delta Region were affected by salinity intrusion—roughly 16% of the total agricultural production area; it is estimated that by 2030, up to 45% of the region's agricultural area could be impacted, with coastal provinces such as Tien Giang, Tra Vinh, Soc Trang, Bac Lieu, Ca Mau, and Ben Tre experiencing the highest levels of impact [23]. Eslami et al. [24] predicted that salinity would rise by 2–2.5% on average, adding roughly 6% of salinity-affected areas by 2040 in all coastal provinces and increasing the total affected area by 10–25% by 2050 of the Mekong Delta area. Since February 2020, the intrusion of saline water (at a concentration of 4 g/L) into the main rivers in the Mekong Delta has occurred at a distance of 50 to 130 km, which is a substantial increase compared to the same period in 2016 [25]. Salinization threatens the aquatic ecosystem, particularly the lives of freshwater aquatic species [26, 27]. Amphibians are particularly osmotically sensitive organisms because their skin, gills, and eggs are permeable and can absorb water and solutes from the surrounding aquatic environment [28, 29], but some species are innately or adaptively salinity tolerant [30]. Hopkins and Brodie [31] conducted a literature review and presented data on 144 species in 28 families of amphibians in saline waters on all continents except Antarctica and recommended that the majority of observed species require additional research.

The Thailand frog (*Rana tigerina* [32], also known as *Hoplobatrachus tigerinus*, is a commercially important freshwater frog species with many superior characteristics, such as comparatively large size (500–750 g), high fecundity (around 6000 eggs per clutch), a successful breeding ability in ephemeral pools in human-modified habitats, and adaptability to changing climate conditions [33–36]. *Rana tigerina* is known as a species introduced to Vietnam, and it showed high adaptability to cultivation conditions in the Mekong Delta [32]. In recent years, this species has been domesticated to be proactive in terms of breeding stock as well as their ability to use artificial feed to farm intensively in the Mekong Delta coastal area of Vietnam. They have shown a quick growth rate, few diseases, and a well-developed domestic market. This has resulted in increased income for farmers in provinces such as Long An, Dong Thap, Tien Giang, Hau Giang, Vinh Long, and Tra Vinh [37]. However, no information is available on the salinity tolerance of the eggs and larvae of Thailand frogs that inhabit brackish areas. Therefore, the present study aimed to investigate the effects of salinity on the egg fertilization, hatching, and tadpole growth and survival rates of the Thailand frog as a basis for the development of artificial reproduction and rearing techniques for this species in saline intrusion conditions.

2. Materials and Methods

2.1. Materials. The eggs used for the experiments were obtained from the artificial reproduction of 12 broodstock pairs of frogs at the experimental hatchery of Tra Vinh University, Vietnam. The broodstock frogs included females that were at least 1 year old, healthy, without deformities, and with a mean weight of 400 g. The frogs were reared to sexual maturity in a 2 m^3 tank at a ratio of one male to one female. The frogs were fed an artificial feed containing 26% protein twice per day (7:00 and 17:00). The artificial pellet feed used for the experiments was purchased from GREENFEED Vietnam Corporation and had a protein content of 25–35% depending on the stage of frog growth.

Two types of water were used for the experiments: freshwater and saltwater. The freshwater was river water, from which the sediment was removed. The water was then disinfected with potassium permanganate ($KMnO_4$) at a dose of 50 mg/L and continuously aerated for 3 to 5 days. The saltwater consisted of seawater with a salinity of 30% that was treated with 30 ppm chlorine and aerated for 3 to 4 days. The two types of water were mixed to achieve experimental salinities.

Composite tanks with a volume of 0.5 m^3, a bottom area of 1 m^2, and a water depth of 15 cm were used in this study. The guide for the Care and Use of Laboratory Animals by the US National Research Council was followed.

2.2. Experimental Design. This study was conducted from December 2018 to April 2019 at the Aquaculture Experimental Hatchery of Tra Vinh University in Tra Vinh Province, Vietnam. Three one-factor experiments, with completely randomized designs and three repetitions for each, aimed to determine the effect of salinity on the egg fertilization, hatching, and tadpole growth and survival rates of the Thailand frog (*Rana tigerina* [32], and they were conducted consecutively as follows:

The first two experiments were at four salinities (0, 2, 4, and 6%), based on the research results on the frog species *Incilius nebulifer* and *Hoplobatrachus rugulosus* by Alexander et al. [38] and Nakkrasae et al. [39], respectively.

Experiment 1 aimed to investigate the effects of salinity on the fertilization and hatching rates of the Thailand frog eggs. Sexually mature broodstock frogs were used for artificial reproduction. The males and females in the sexual maturity period are distinguished by their following morphological characteristics: the males are smaller, slimmer, and darker in color than the females. Males possess swollen copulatory pads on the first inner finger and have vocal sacs that help them croak loudly, while females lack these features [40]. We chose females that had large, slightly pink bellies, a rough belly surface, and slow movement, while males had dark vocal sacs and two front limbs with genital spines. A luteinizing hormone-releasing hormone (LHRH) stimulant, an exogenous hormone commonly used to induce spermiation in anuran amphibians [41], was injected at a dose of 50 μg/kg for females, while the dose for males was half that of the females. We used 5 mg of DOM dissolved in 1 ml of 0.9% NaCl physiological saline solution +50 μg of powdered LHRH for 1 kg of the frog. This mixed solution is injected into the posterior thigh muscle of the frogs. The aim was to cause the frogs to lay eggs in unison. After being injected with the LHRH stimulant, each of the three pairs of broodstock frogs was gently placed into a 1 m^3 composite-spawning tank with a water depth of 20 cm at the experimental salinities, and we recorded the times when the frog laid eggs and the eggs hatched. After each frog laid eggs, a total of 100 eggs were randomly collected from each tank to analyze the fertilization rate. The incubation period for the observed eggs was 24 hours. Fertilization rate and hatching rate were calculated using the following equations:

$$\text{Fertilization rate}\,(\%) = \frac{\text{number of fertilized eggs}}{\text{number of observed eggs}} \times 100,$$

$$\text{Hatching rate}\,(\%) = \frac{\text{number of hatched eggs}}{\text{number of fertilized eggs}} \times 100. \tag{1}$$

The second experiment investigated the effects of salinity on the tadpole survival rate in the stage from newly hatched to yolk sac exhaustion. The 2400 newly hatched tadpoles obtained from Experiment 1 were randomly arranged into 12 Styrofoam boxes (0.5 m^3 each) at a density of 200 individuals per box. The number of dead tadpoles in each of the Styrofoam boxes was recorded daily. Observations were performed until the yolk sac was completely depleted. The survival rate of the tadpoles was calculated at the end of the experiment.

In the third experiment, the growth and survival rates of the tadpoles during the transition stage from yolk sac exhaustion to juveniles were investigated at three salinities (0, 3, and 5%) based on the research results on the frog species *R. sylvatica* and *Litoria ewingi*i by Langhans et al. [42] and Chinathamby et al. [43], respectively. The metamorphosis-based phases were observed according to the study by Gilbert [44]:

(i) Phase 1: the tadpole stage from yolk sac exhaustion to the onset of foreleg emergence
(ii) Phase 2: the tadpole stage from the onset of foreleg emergence to the onset of hind leg emergence
(iii) Phase 3: the stage from the onset of hind leg emergence to fingerling status

In Phase 1, 5400 tadpoles that were obtained from Experiment 2 were arranged at a density of 600 individuals per tank with a total observation time of 10 days. In Phase 2, the total of 3150 tadpoles that were obtained from Phase 1 was arranged at a density of 350 individuals per tank with a total observation time of 8 days. In Phase 3, the total of 1350 tadpoles that were obtained from Phase 2 was arranged at a density of 150 individuals per tank with a total observation time of 7 days.

At each phase, the tadpoles were randomly arranged into nine composite tanks (volume: 0.5 m^3; water depth: 15 cm) and fed snakehead-pellet feed that contained 42% protein, twice a day (7:00 and 16:00), with an amount equal to 5% of their body weight per day. Feeding was conducted until the tadpoles stopped consuming the food. Water spinach was attached to Styrofoam bars and placed on the surface of each tank to create the substrate and a more natural environment for the tadpoles. At the end of each phase (when approximately 70–80% of the tadpoles had metamorphosed), 20 individuals from each tank were weighed to determine their growth parameters. Also, the total number of tadpoles in each tank was obtained to calculate the survival rate. The daily weight gain (DWG), specific growth rate (SGR), and survival rate (SR) were calculated using the following equations:

$$\text{DWG}\left(\frac{\text{g}}{\text{day}}\right) = \frac{(\text{final body weight} - \text{initial body weight})}{\text{experiment duration}},$$

$$\text{SGR}\,(\%/\text{day}) = 100 \times \frac{(\ln(\text{final weight}) - \ln(\text{initial weight}))}{\text{experiment duration}},$$

$$\text{SR}\,(\%) = 100 \times \left(\frac{\text{final number of frogs}}{\text{initial number of frogs}}\right). \tag{2}$$

2.3. Statistical Analysis. All variables, such as fertilization rate, hatching rate, initial mean weight, final mean weight, DWG, SGR, and SR, were analyzed using a one-way analysis of variance (ANOVA) followed by the Duncan test to identify significant differences between the mean values (significance level: $p < 0.05$). The variance homogeneity was assessed by Levene's test, and the percentage data were transformed into arcsines prior to conducting statistics. The Statistical Package for the Social Sciences (SPSS) software for Windows version 20.0 was used to perform all statistical analyses in this study.

3. Results

3.1. Egg Fertilization and Hatching Rates of the Thailand Frog. The fertilization rates in the salinity range of 0 to 4% were relatively high (79.33–86.00%) and no significant differences were observed among them ($p > 0.05$), with values of 86.00, 79.33, and 82.67% at salinities of 0, 2, and 4%, respectively. However, the fertilization rate was markedly reduced (56.67%) at a salinity of 6% and was significantly different from the fertilization rate at other salinities ($p < 0.05$, $F = 27.85$, $\text{df} = 11$, Table 1). The hatching rates showed a tendency to decrease with an increase in salinity (Table 1). The highest hatching rate (87.52%) was observed at a salinity of 0%, while a significant decrease was recorded at 2% (71.60%). The hatching rate continued to decrease to extremely low values of 8.87 and 2.56% at salinities of 4 and 6%, respectively ($p < 0.05$, $F = 78.71$, $\text{df} = 11$ Table 1). This experiment showed that the salinities of 6 and 2%

Table 1: The egg fertilization and hatching rates of the Thailand frog (*Rana tigerina* [32]), at various salinities.

Salinities (%)	Fertilization rate (%)	Hatching rate (%)
0	86.00 ± 2.00^{a}	87.52 ± 5.69^{a}
2	79.33 ± 3.06^{a}	71.60 ± 15.01^{b}
4	82.67 ± 5.03^{a}	8.87 ± 2.68^{c}
6	56.67 ± 5.03^{b}	2.56 ± 4.44^{c}

Values are presented as mean ± SD. Values with different superscript letters (a, b, c) in the same column show a significant difference ($p < 0.05$).

significantly affected the fertilization and hatching rates of Thailand frog eggs, respectively.

3.2. Survival Rate of Thailand Frog Tadpoles during the Stage from Newly Hatched to Yolk Sac Exhaustion. The tadpole stage from newly hatched to yolk sac exhaustion had a duration of 4 days. On the first day, the tadpole survival rates ranged from 79.33 to 85.67%, with no significant differences observed among the various salinity levels ($p > 0.05$) (Table 2).

The survival rate on the second and third days ranged from 57.67 to 72.67% and 57.00 to 72.33%, respectively, and the variations did not show a trend for the investigated salinities (Table 2). However, at the end of the stage (on the fourth day), an effect of salinity on tadpole survival was observed since the survival rate tended to decrease as the salinity increased. The highest survival rate (67.33%) was observed at a salinity of 0%, followed by a survival rate of 55.67% at 2%, and no significant difference was observed between them. However, the survival rate markedly decreased to 50 and 20% at salinities of 4 and 6%, respectively, with both of these groups being significantly different from the control salinity (0%) ($p < 0.05$, $F = 24.09$, df = 11, Table 2). This indicates that the survival rate of the yolk sac-exhausted tadpoles was significantly reduced at salinities of 4% or greater.

3.3. Growth and Survival Rates of Thailand Frog Tadpoles during Their Transition from Yolk Sac Exhaustion to Juveniles

3.3.1. Growth Performance. Initial mean tadpole weight was 0.34 g/individual at all salinity levels (Table 3). At the end of Phase 1, the final mean weights ranged from 1.47 to 1.57 g/individual. Furthermore, the final mean weights of 1.57 and 1.56 g/individual at salinities of 3 and 5%, respectively, were equivalent, and both were significantly higher than that observed at a salinity of 0% (1.47 g/individual) ($p < 0.05$, $F = 18.56$, df = 8, Table 3). In Phases 2 and 3, the greatest final mean weight was observed at a salinity of 3% (3.98 and 5.61 g/individual, respectively). The final mean weights at salinities of 0 and 5% were comparable at 3.17 and 2.99 g/individual, respectively, in Phase 2 and weights of 4.98 and 4.77 g/individual, respectively, in Phase 3 ($p > 0.05$). These results indicate that a salinity of 5% did not significantly affect tadpole growth during Phase 1 but significantly affected tadpole growth during Phase 2 ($F = 14.46$, df = 8, Table 3) and Phase 3 ($F = 34.48$, df = 8, Table 3).

The representative growth parameters (DWG and SGR) varied only slightly, and no significant differences were observed for any of the phases when the salinity was increased from 0 to 5% ($p > 0.05$), except in Phase 2, where DWG ($p < 0.05$, $F = 8.24$, df = 8, Table 3) and SGR ($p < 0.05$, $F = 46.40$, df = 8, Table 3) were significantly higher at a salinity of 3% than at salinities of 0 and 5%. DWG ranged from 0.083 to 0.087 g/day in Phase 1, 0.186 to 0.226 g/day in Phase 2, and 0.127 to 0.134 g/day in Phase 3, while SGR varied from 0.110 to 0.113%/day in Phase 1, 0.112 to 0.223%/day in Phase 2, and 0.030 to 0.036%/day in Phase 3 (Table 3).

3.3.2. Survival Rate. The survival rates of Thailand frog tadpoles during the transition stage from yolk sac exhaustion to juvenile were continuously the highest at 0% salinity (80.83% (Phase 1), 56.38% (Phase 2), and 58.89% (Phase 3)), followed by 3% salinity (75.00% (Phase 1), 49.71% (Phase 2), and 44.89% (Phase 3)). However, in Phases 1 and 2, no significant differences in tadpole survival rate were observed between the salinities of 0 and 3% ($p > 0.05$). Furthermore, in all of the phases, the tadpole survival rate was markedly decreased at a salinity of 5% and continuously reached significantly lower values when compared to those observed at lower salinities (61.11% (Phase 1) ($p < 0.05$, $F = 14.73$, df = 8, Figure 1), 32.48% (Phase 2) ($p < 0.05$, $F = 16.40$, df = 8, Figure 1), and 20.00% (Phase 3) ($p < 0.05$, $F = 106.68$, df = 8, Figure 1)). These results demonstrate that the tadpole survival rate remained stable at a salinity of 3% during Phases 1 and 2; however, the tadpole survival rate during Phase 3 was significantly reduced at salinities of 3% and higher.

3.4. Water Quality Parameters. The recorded water quality parameters included a temperature range of 24.63 to 27.32°C, a pH range of 7.17 to 7.41, an NH_4 range of 4.11 to 6.67 mg/L, and an NH_3 range of 0.05 to 0.08 mg/L (Table 4).

4. Discussion

4.1. Water Quality Parameters. The monitored water quality parameters included in this study were temperature (24.63–27.32°C), pH (7.17–7.41), NH_4 (4.11–6.67 mg/L), and NH_3 (0.05–0.08 mg/L). Since all of these parameters were within the appropriate ranges for the survival of frog eggs and tadpoles [45], they had little impact on the experimental frogs.

4.2. Fertilization and Hatching Rates of Thailand Frog Eggs. Variations in aquatic habitat salinity levels affect eggs, tadpoles, and adult frogs differently. Eggs and tadpoles are extremely sensitive because they are limited to the ephemeral aquatic environment where they develop. However, adult frogs may only be slightly affected by variations in salinity levels because they can escape to alternative, favorable locations [46, 47]. The results of the present study show that a salinity range of 0 to 4% did not significantly affect the egg fertilization rate. Nevertheless, when the salinity was increased to 6%, the fertilization rate significantly decreased

Table 2: The tadpole survival rate (%) of the Thailand frog during the stage from newly hatched to yolk sac exhaustion, at various salinities.

Salinities (%)	Day 1	Day 2	Day 3	Day 4
0	82.00 ± 1.00^{a}	72.67 ± 2.51^{a}	72.33 ± 2.52^{a}	67.33 ± 1.15^{a}
2	79.33 ± 7.76^{a}	57.67 ± 10.01^{b}	56.33 ± 10.97^{b}	55.67 ± 11.55^{ab}
4	85.67 ± 8.73^{a}	61.33 ± 5.50^{ab}	58.33 ± 8.72^{b}	50.00 ± 7.00^{b}
6	80.17 ± 7.12^{a}	71.67 ± 7.57^{a}	57.00 ± 9.26^{b}	20.00 ± 4.36^{c}

Values are presented as mean ± SD. Values with different superscript letters (a, b, c) in the same column show a significant difference ($p < 0.05$).

Table 3: The growth performance of the Thailand frog during the tadpole transition stage from yolk sac exhaustion to juvenile, at various salinities.

Salinities (%)	Initial mean weight (g/individual)	Final mean weight (g/individual)	Daily weight gain (g/day)	Specific growth rate (%/day)
The tadpole stage from yolk sac exhaustion to the onset of the emergence of the hind legs				
0	0.34 ± 0.13^{a}	1.47 ± 0.03^{b}	0.083 ± 0.005^{a}	0.110 ± 0.002^{a}
3	0.34 ± 0.13^{a}	1.57 ± 0.01^{a}	0.085 ± 0.003^{a}	0.111 ± 0.002^{a}
5	0.34 ± 0.13^{a}	1.56 ± 0.03^{a}	0.087 ± 0.002^{a}	0.113 ± 0.001^{a}
The tadpole stage from the onset of the emergence of the hind legs to the onset of the emergence of the forelegs				
0		3.17 ± 0.04^{b}	0.195 ± 0.003^{b}	0.112 ± 0.006^{b}
3		3.98 ± 0.41^{a}	0.226 ± 0.021^{a}	0.223 ± 0.019^{a}
5		2.99 ± 0.02^{b}	0.186 ± 0.002^{b}	0.113 ± 0.001^{b}
The stage from the onset of the emergence of the forelegs to fingerlings				
0		4.98 ± 0.15^{b}	0.129 ± 0.014^{a}	0.030 ± 0.003^{ab}
3		5.61 ± 0.07^{a}	0.134 ± 0.018^{a}	$0.036 \pm 0.004a^{a}$
5		4.77 ± 0.14^{b}	0.127 ± 0.009^{a}	0.033 ± 0.001^{ab}

Values are presented as mean ± SD. Values with different superscript letters (a, b) in the same column show a significant difference ($p < 0.05$).

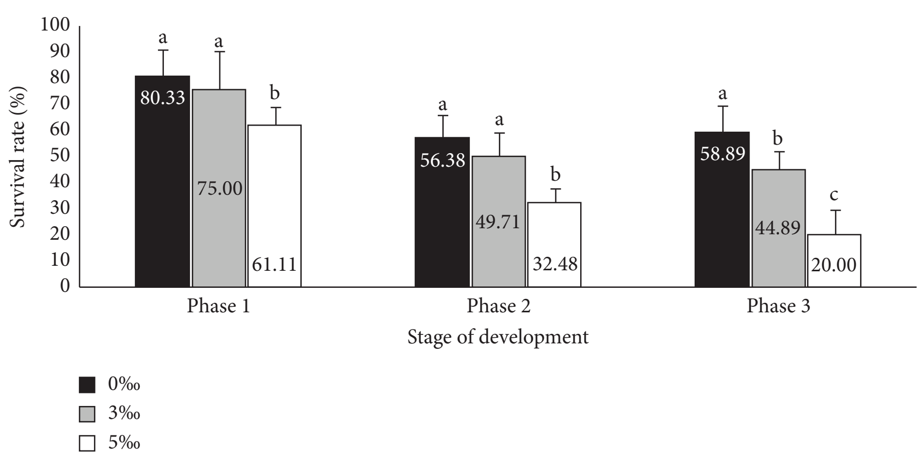

Figure 1: The survival rate of the Thailand frog (*Rana tigerina* [32]) during the tadpole transition stage from yolk sac exhaustion to juvenile, at various salinities. (The bars with the different letters (a, b, and c) show a significant difference ($p < 0.05$)).

Table 4: The water quality parameters (mean ± SD).

Salinities (%)	Temperature (°C) 7:00	Temperature (°C) 14:00	pH	NH_4 (mg/L)	NH_3 (mg/L)
0	24.63 ± 0.55	27.32 ± 1.062	7.41 ± 0.20	4.11 ± 1.69	0.66 ± 0.03
3	24.67 ± 0.62	27.24 ± 1.131	7.17 ± 0.28	4.44 ± 1.93	0.05 ± 0.10
5	24.77 ± 0.33	27.26 ± 1.125	7.33 ± 0.24	6.67 ± 3.69	0.08 ± 0.07

(to 65.9%) when compared to that at the control salinity of 0%. Moreover, the egg hatching rate significantly decreased with increases in salinity to 2, 4, and 6%. At salinities of 4 and 6%, the hatching rate was extremely low (8.87 and 2.56%, respectively). Haramura [29] observed that salinity levels higher than 5% were fatal for all studied *Buergeria japonica* eggs; however, the mortality risk of the embryos was lower when they were exposed to salinity levels of 1 or 2%. Moreover, Gosner and Black [48] demonstrated that high salinities dehydrate eggs, cause deformities in embryos, and may affect egg-hatching capacity. These results agree with those of other studies, which showed that salinity was an important factor in the mortality of amphibian eggs. Other studies demonstrated that even a slight increase in salinity caused a significant decrease in the egg survival rate [29, 49–51]. Although a salinity range of 0 to 4% did not affect egg fertilization rate in the present study, the results indicate that to achieve production efficiency, a salinity of less than 2% is suitable for the entire artificial reproduction process for the Thailand frog.

4.3. Survival Rate of the Tadpole Stage from Newly Hatched to Yolk Sac Exhaustion. The variation in larval survival rates of the Thailand frog did not reveal a trend for the investigated salinities during the first 3 days. However, by the fourth day (the end of the stage), the larval survival rates tended to decrease with increasing salinity and were significantly reduced at salinities greater than or equal to 4%. It is worth noting that the larval survival rate at a salinity of 6% significantly decreased (29.7%) when compared to the survival rate at a salinity of 0% (control). These findings could be explained by the fact that Thailand frog larvae require a period of adaptation to the external environment after hatching, with stability being demonstrated on the fourth day [52–56] (Albecker and McCoy, 2017).

Dickman and Christy [47] performed a study on the green and golden bell frog (*L. aurea*) and observed a similar pattern. The tadpoles of *L. aurea* developed in salinities of up to 4% seawater (SW) without apparent adverse effects but indicated a salinity threshold (above which significant mortality occurred) within a range of 5.5–10% SW. Chinathamby et al. [43] also noted that a salinity of 16% SW significantly decreased the survival of *L. ewingii* tadpoles such that 39% of the tadpoles at a salinity of 16% SW survived metamorphosis—compared to 92% survival in freshwater. According to the study by Edwards [57], under salinity changes in habitats, many aquatic animal species have the regulative ability to alter the ion concentration and osmotic pressure of their bodily fluids to suit those in the external environment. Nevertheless, these regulatory mechanisms involve energy expenditure. When salinity changes are beyond the regulative ability of an animal, it will ultimately lead to its death. Amphibians are sensitive to habitat variations due to their skin permeability and their poor osmoregulatory capacity during the aquatic larval stage when they are highly dependent on the resources available in the aquatic environment [46, 58, 59]. The osmoregulatory process in amphibians is mediated by endocrine hormones from their adrenal glands, and corticosterone (CORT) and aldosterone (ALDO) are prime candidates [60, 61]. CORT in aquatic vertebrates increases with salinity exposure [62–66] and is linked to ionization and osmotic regulation [67–69]. Similar CORT changes in amphibian larvae are known to result in a reduction in the resources available for their growth, development, and water retention, which are thought to support survival [30, 60, 65, 70, 71]. Therefore, CORT may be used as a biomarker of physiological stress and an increase in the risk of negative outcomes in amphibian health as these animals are exposed to salinity [71]. Meanwhile, ALDO is known to stimulate sodium uptake in the kidneys and skin [72]. ALDO in amphibians often decreases when exposed to saline conditions [60, 73], presumably to reduce sodium uptake [30]. In addition, it is recorded that the ALDO of Northern leopard (*Rana pipiens*) frog larvae marginally decreased with increasing salinity and was positively correlated with stress-induced waterborne CORT, and CORT may be a substrate for ALDO synthesis [61]; so it also seemed difficult to decouple the roles of CORT and ALDO in the osmoregulatory process [61]. The Cuban treefrogs (*Osteopilus septentrionalis*) in the Gosner stages could respond to high salt levels by decreasing aldosterone secretion when they were exposed to salinities of 1.0, 2.5, and 3.5%, and the regulation process of osmotic functions in high salt concentrations must involve tradeoffs in their growth and development [60, 74]. There were no strong differences in survival among salinity treatments of salamanders (*Ambystoma mavortium*) in a NaCl range of 32–4000 mg/L for 24 days, demonstrating that salamanders were physiologically challenged but could tolerate the experimental concentrations. However, salinity reduced dry mass, snout-vent length, and body condition while increasing the water content of larvae [30]. Our study has not investigated the role of hormones in the osmotic regulation of *R tigerina*. Yet, salinities greater than 4% may have had a strong effect on the ion regulation and osmoregulation of the tadpoles, thereby resulting in significantly higher mortality when compared to lower salinities (tadpole survival rate significantly decreased to 20% at 6% salinity). Furthermore, Chinathamby et al. [43] also doubted that yolk sac-exhausted tadpoles would be hungry and eat not-yet-exhausted or weaker tadpoles. This would also decrease the survival of tadpoles. The trend of decreased survival under increased salinity has also been observed in several other anurans, such as *Rana cancrivora* [43] and *Bufo calamita* [75].

4.4. Growth and Survival Rates during the Tadpole Transition from Yolk Sac Exhaustion to Juvenile. No significant differences were observed among the final weights of the tadpoles in Phase 1 between salinities of 3 and 5%, with both being significantly higher than those at a salinity of 0%. However, in Phases 2 and 3, the final mean weight of the tadpoles at a salinity of 3% was significantly higher than those at salinities of 0 and 5%. Furthermore, the final mean weights of the tadpoles at a salinity of 0% were consistently higher than those at a salinity of 5%, with no significant differences observed.

Richter-Boix et al. [76] showed that among animals with complex life cycles, the most common adaptations to changes in environmental quality are to self-modify their growth and developmental rates. Amphibian larvae that inhabit high-risk environments with predators or rapid desiccation often have increased growth rates to promptly reach metamorphosis and thereby avoid mortality [77, 78]. For example, the spadefoot toad has an extremely short larval stage to manage the rapid drying of ponds in desert environments [79–82], while the American green tree frog (*Hyla cinerea)* has been shown to survive in brackish marshes with salinities higher than normal amphibian tolerance levels and has exhibited adaptive strategies in response to salinity, such as accelerated larval growth rates and shorter larval periods than freshwater populations [83]. This developmental pattern is often moderated according to perceived risk [76, 84–86]. The results of the present study suggest that a more rapid life cycle is likely an adaptation to minimize tadpole mortality in environments with highly variable salinities. The tadpoles in Phase 1 appeared to have increased growth to tolerate salinities of 3 and 5%; thus, their final weights at these salinities were significantly higher than those observed at a salinity of 0%.

Rose [87] emphasized that ecologists have viewed the relationship between tadpole growth and development as an adaptive strategy for coping with restricted resources and other selection pressures in habitats. Although tadpoles could speed up development to possibly adapt to environmental change [77, 78], individual growth rates are also affected by many biotic and abiotic factors, such as density, saltwater, and feed [88–90]. Several studies showed that the growth rate of tadpoles was reduced via negative density dependence, even under other environmental pressures that were supported to promote rapid metamorphosis [88, 89]. High concentrations of salt water are lethal to amphibians [31]; however, at sublethal levels, salt water has been shown to reduce the growth rate and size of tadpoles at metamorphosis [47, 83, 91, 92]. Although certain species may compensate for decreased growth if they are free from salt stress [93], others cannot [94]. The effects of salinity vary depending on the species and life stage [95, 96]. Gomez-Mestre et al. [97] reported that an observed sublethal lower body mass for tadpoles in high-salinity conditions (16% SW) was most likely due to the reallocation of energy from somatic growth to increased osmoregulation. The tadpoles of *B. calamita* showed similar patterns of reduced body mass when subjected to increasing salt concentrations [97]. In addition, Albecker et al. [98] demonstrated that ion pumps, glycerol osmolytes, cytoskeletal components, and a suite of other genes are upregulated in response to the salt of tadpoles. Szeligowski et al. [99] also demonstrated that salinity induces morphological changes in tadpole gills that reduce the efficiency of respiration, osmoregulation, and feeding. The energetic cost of expressing and maintaining molecular machinery in response to salt is exacerbated by less efficient gill morphology. In the present study, a salinity of 5% slowed the growth of Thailand frog tadpoles in Phases 2 and 3, resulting in their final mean weights in both of these phases at a salinity of 5% being significantly lower than those at a salinity of 3% and also lower than those at a salinity of 0% (nonsignificant difference). These results may be due to the fact that the osmotic regulation of the tadpoles at a salinity of 5% during Phase 1 had a negative impact on their survivorship, which reduced their growth in Phases 2 and 3. This theory is supported by the tadpole survival rate results. The tadpole survival rate was constantly highest at a salinity of 0% in all the phases and that increasing the salinity up to 3% did not reduce the final mean weights of tadpoles in all phases or the survival rates of tadpoles in Phases 1 and 2. Nevertheless, tadpole survival rates were significantly reduced at salinities of 3 and 5% in Phase 3, which indicated that tadpole survivorship deteriorated at salinities of ≥3% in this phase, resulting in increased mortality. In previous studies, several other frog species exhibited a similar trend; for example, the lower survival and delayed development of tadpoles of *Fejervarya limnocharis* [92], *Litoria ewingii* [43], and *L. aurea* [47] under high salinity conditions. Researchers have revealed that responses that enable an organism to grow and reproduce under variable habitats will be favored by natural selection, provided that the responses are heritable and that the costs do not outweigh the benefits of the reactions [100]. However, when habitats exceed the tolerance of the species, no adaptations can occur, which result in the death of the organism [101]. Moreover, tadpoles of *R. tigerinus* are known to be carnivorous, preying upon other anuran larvae and zooplankton [33, 102–104], so living in high salinity habitats not only leads to low survival and the delayed development of tadpoles but may also increase the risk of cannibalism, larger tadpoles consuming smaller tadpoles, which contributes to a decreased tadpole survival rate. This suggests that it is necessary to train the yolk sac-exhausted tadpoles to consume industrial feed to avoid cannibalism [53].

As a conclusive remark, salinity levels of 2 or 6% significantly affected the fertilization and hatching rates of the eggs, respectively, while a salinity of 4% significantly affected tadpole survival rates in the stage from newly hatched to yolk sac exhaustion. In the tadpole stage from yolk sac exhaustion to juvenile, a salinity of 3% resulted in an increase in the final mean weight of tadpoles throughout this stage and a significant increase in Phases 1 and 2 of the stage. Moreover, no significant effects on the tadpole survival rates were observed at a salinity of 3% in Phases 1 and 2. An increase in salinity to 5% did not affect growth performance but caused a significant reduction in tadpole survival. It is recommended that the process of artificial reproduction for the Thailand frog be performed at salinities of <2%, with salinities of ≤2 and 3% being suitable for rearing tadpoles in the stages from newly hatched to yolk sac exhaustion and from yolk sac exhaustion to juvenile, respectively. Notably, it is necessary to provide sufficient external feed for tadpoles throughout the rearing stages to avoid cannibalism and improve the survival rate. [105].

Data Availability

The data supporting the findings of this study are available within the article.

Disclosure

Huynh Kim Huong and Cheng-Ting Huang are considered joint first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Huynh Kim Huong and Cheng-Ting Huang contributed equally.

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