Effects of Mixed Mineral Supplementation in On-Farm Feeds on the Metamorphosis Development and Growth Performances of Frog (*Hoplobatrachus rugulosus*) Tadpoles

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ABSTRACT

The frog (*Hoplobatrachus rugulosus*) is widely cultured in Asian countries. Tadpoles in these cultures are prone to diseases and sensitive to environmental factors, but their metamorphosis and growth may be enhanced by applying minerals. Therefore, this study was conducted to determine the influence of mixed mineral supplementation in on-farm feeds on the metamorphosis development, growth performance, and production cost of *H. rugulosus* tadpoles. Four levels of mixed mineral supplementation (0%, 0.2%, 0.4%, and 0.6%) were fed to groups of 500 tadpoles stocked in cement ponds. Tadpoles were fed experimental feeds at 5% of their body weight for 35 days. The 0.2% supplementation group showed significantly greater average weight gain (3.84±0.15 g·tadpole^{-1}) and survival rate (81.00±0.47%) compared to the 0.6% and 0% groups ($p<0.05$), but was comparable ($p>0.05$) to the 0.4% group. Notably, other growth parameters (average daily weight gain, specific growth rate, and feed conversion ratio) did not differ significantly ($p>0.05$) among treatments. The groups receiving mineral supplementation showed significantly ($p<0.05$) higher average forelimb length than the control from day 20 until day 28, but not from days 30 to 36. Conversely, mineral supplementation did not affect hindlimb length throughout the experiment. Tadpoles receiving 0.2% supplementation completed metamorphosis faster than other groups, starting on day 26 and reaching 100% completion by day 34. This study demonstrated that feeding 0.2% mixed mineral supplementation to frog tadpoles accelerated the metamorphosis and improved their growth, albeit with a slight increase in production costs.

Keywords: Amphibian, Essential minerals, Frog farm, Frog life cycle, Thyroid hormone

INTRODUCTION

Frog (*Hoplobatrachus rugulosus*) are widely cultured in Asian countries (Nghia *et al*., 2023) because of their high nutritional values and delicious taste. Currently, the demand for frog consumption is increasing both domestically and internationally, while the production from wild and farm is insufficient (Department of Fisheries, 2021). For this reason, the intensive commercial farming of frogs has been developed. Although frogs can be cultured year-round, tadpoles are unable to reach metamorphosis in winter when the temperature is 15 °C or below (Goldstein *et al*., 2017). Plastic-covered greenhouses were set up to control the temperature, but slow growth, low survival rates, and prone to pathogens remain major problems in tadpole rearing. As a result, farmers use antibiotics and chemicals to prevent and treat diseases, which may cause drug-resistant pathogens and undesirable residues in the environment and frog meat. Thus, it is necessary to find cost-effective alternatives for environmentally friendly frog farming.

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Thyroid hormone is the fundamental morphogen controlling metamorphosis in amphibian larvae (Shi, 2000). This hormone is a more important factor in regulating amphibian metamorphosis than temperature factor, water quality, and feed. The urodele *Amblistoma mexicanum* must have its metamorphosis induced by thyroid treatment (Oofusa and Yoshizato, 1991). Thyroxine induces contraction of tadpole tails and requires iodine as a precursor. Iodine and sodium chloride in appropriate amounts are required for normal thyroid hormone function. Krishnapriya *et al*. (2014) reported that 2.0 ppm iodine was needed for *Philautus* sp. metamorphosis. Amphibians are susceptible to salinity since their skin, gills, and eggs are permeable and can absorb water and solutes from surrounding aquatic environments (Haramura, 2007). Embryos and tadpoles are more severely affected by water salinity than adults because they live fully in an aquatic environment. Salinity causes adverse effects on the growth, development, metamorphosis, survival, and behavior of tadpoles (Squires *et al*., 2010). On the contrary, Nakkrasae *et al*. (2016), using saline water made from pure sodium chloride, reported that low salinity (e.g., less than 4 ppm) enhanced survival and body weight of *H. rugulosus* tadpoles, and reduced the metamorphosis period.

However, due to high price of pure sodium chloride (50 g, price of 6.92 USD), commercially available iodized salt is an alternative to be used for enhancing growth and development of young tadpoles. The purpose of this study was to determine the effects of the mixed mineral feed supplementation (sodium chloride, iodine, calcium, magnesium, and iron) on metamorphosis development, growth performances, and production cost of frog culture.

MATERIALS AND METHODS

Animals and study area

Tadpoles of *Hoplobatrachus rugulosus* were obtained from a frog farm at Maejo University, Chiang Mai, Thailand. After the larvae developed to an average weight of 0.1 g·tadpole^{-1}, they were moved into twelve 1-m diameter cement ponds with 10 cm of water depth and 40 cm of pond height (Figure 1). Aeration was provided using 1 set of air-stone in each pond.

Formulated feed and feeding

The experimental diet was basically a commercial floating pellet for frog larvae with

Figure 1. Experimental cement ponds used for tadpole rearing during the experiment.

40% protein and 10% fat, mixed with mineral supplementation at 0, 0.2, 0.4, and 0.6% (Table 1). 10 g agar powder·L^{-1} of water was used as a binder, allowed to air dry, and stored at -20 ºC until use. The tadpoles received 10% feed of their body weight daily, divided into 3 feedings at 9:00 a.m., 12:00 p.m., and 3:00 p.m. The total amount of feed provided was recorded daily for each treatment and used for FCR calculation.

Proximate analysis of mixed mineral supplementation in the experimental diets

The four diets were dried at 80 °C before determining the proximate composition according to standard methods of analysis (AOAC, 1995). Crude protein content was analyzed using the Kjeldahl method with a conversion factor of 6.25 to convert total nitrogen into crude protein. Ash content was acquired by heating the sample overnight in a furnace at 525 °C, and determining the content gravimetrically. Crude fat was extracted using petroleum ether at 160 °C and measured gravimetrically after oven drying (80 °C) the extract overnight. Crude fiber was extracted using 1.25% of H_2SO_4. Samples for mineral analysis were subjected to acid digestion and analyzed trough inductively coupled plasma optical emission spectrometry (ICP-OES) (AOAC, 2000).

Experimental conditions and details

A Complete Randomized Design consisting of four experiments with three repetitions each was applied in this study. Tadpoles (an initial average weight was 0.11±0.01 g·$tadpole^{-1}$) were raised at a density of 500 individuals·m^{-2} for a 35-day feeding trial. Samples of 20% tadpoles were taken from each pond and pooled weighed weekly. Then measurement was made for forelimb, and hindlimb length (mm·$tadpole^{-1}$), and tail length (mm·$tadpole^{-1}$). The process of complete metamorphosis was assessed daily by observing front limb appearance until the entire absorption of tails and a juvenile frog development. Water exchange of 50–70% was done weekly. Water quality parameters were maintained in the following ranges: temperature of 27–28 °C, dissolved oxygen of 4–6 mg·L^{-1}, pH of 6.5–8, alkalinity of 100–150 mg·L^{-1} and total ammonia <0.04 mg·L^{-1}. The growth performance parameters were calculated using the following formulae:

Average weight gain (g·$tadpole^{-1}$)
= Final weight-initial weight

Specific growth rate (SGR) (%·day^{-1})
= (Ln Final weight-Ln initial weight) × 100 / Days

Table 1. The composition analysis of the experimental food (% dry weight).

	Mixed mineral supplementation			
	Control	**0.20%**	**0.40%**	**0.60%**
Mixed mineral supplementation	-	0.20	0.40	0.60
Sodium chloride (NaCl, %)	-	0.195	0.390	0.585
Iodide (I^-, mg·kg^{-1})	-	0.080	0.160	0.240
Calcium (Ca^{2+}, mg·kg^{-1})	-	0.030	0.060	0.090
Magnesium (Mg^{2+}, mg·kg^{-1})	-	0.002	0.004	0.006
Iron (Fe, mg·kg^{-1})	-	0.010	0.020	0.030
$[Fe(CN)_6]^{4-}$ (mg·kg^{-1})	-	0.020	0.040	0.060
The composition analysis of the experimental food				
Crude protein	39.49	39.52	39.80	39.75
Crude fat	10.55	10.39	10.18	10.27
Crude fiber	2.84	2.84	2.42	2.73
Ash	9.48	9.74	9.45	9.63

Average daily weight gain (ADG) (g $\cdot$tadpole$^{-1}\cdot$day^{-1})
= (Final weight-initial weight) / Days

Feed conversion ratio
= Total feed applied (g) / weight gain (g)

Survival rate (%)
= No. of tadpoles survived × 100 / Initial no. of tadpoles

Statistical analysis

Results are expressed as the mean values± standard deviation (SD). Data were analyzed using a one-way analysis of variance (ANOVA). A Duncan test was applied to determine significant differences between treatments. Significant differences were accepted at $p<0.05$. All statistical analyses were performed using SPSS statistic Bass 17.0 for Window EDU S/N 5065845 (SPSS Inc, Chicago, USA).

RESULTS

Average weight gain

The significant differences in average weights of tadpoles were noticed after 14-day of feeding (Figure 2). Tadpoles that received 0.2% and 0.4% mixed mineral supplementation exhibited the highest average weight gain (1.57±0.32 and 1.58±0.14 g·tadpole^{-1}, respectively) which were significantly higher than the 0.6% and the control groups (1.22±0.04 and 1.03±0.01 g·tadpole^{-1}, respectively). However, as the rearing proceeded, no noticeable differences were detected, on days 21 and 28. Significant differences were detected again at the end of the experiment, whereby the tadpoles received 0.2% mixed minerals supplementation exhibited the highest weight (3.84±0.15 g·tadpole^{-1}) although it was non-significant different with the group received 0.4% supplement (3.82±0.13 g·tadpole^{-1}) (Table 2 and Figure 2).

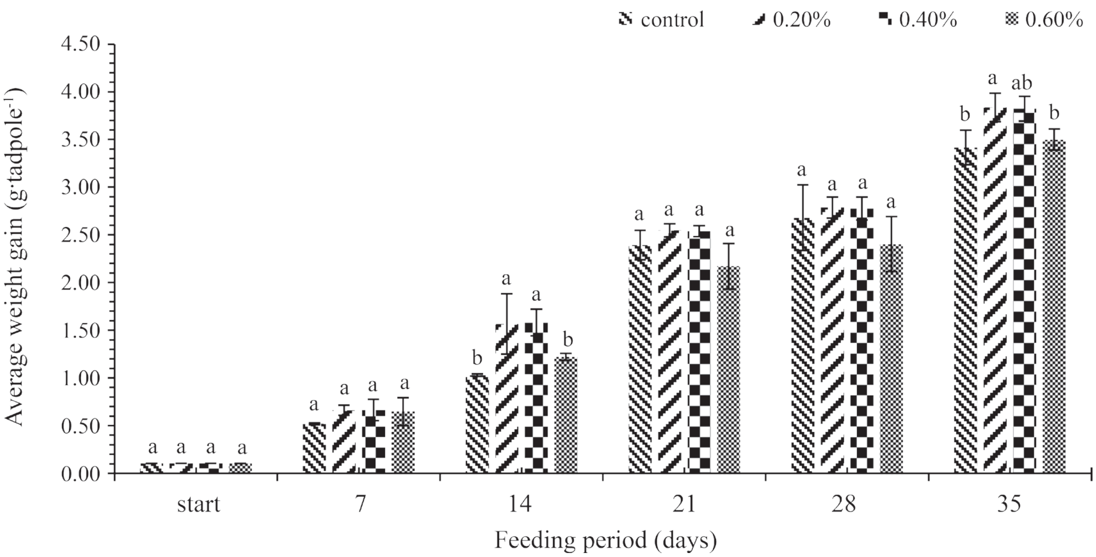

Figure 2. Histograms showing average weight gain (g·tadpole^{-1}) of the tadpoles fed diet supplemented with various levels of mixed minerals at various time points; Different lowercase letters above bars denote significant differences ($p<0.05$) among means within each time point; Error bars represent SD.

Table 2. Growth performances of the tadpoles fed diet supplemented with various levels of mixed minerals at various time points.

Growth performance	Mixed mineral supplementation			
	Control	0.20%	0.40%	0.60%
Average weight gain (g·tadpole^{-1})	3.42±0.18^{b}	3.84±0.15^{a}	3.82±0.13^{ab}	3.50±0.11^{b}
Specific growth rate (%·day^{-1})	0.09±0.01^{a}	0.10±0.01^{a}	0.10±0.01^{a}	0.10±0.03^{a}
Average daily weight gain (g·tadpole^{-1}·day^{-1})	0.09±0.01^{a}	0.11±0.01^{a}	0.11±0.01^{a}	0.10±0.01^{a}
Feed conversion ratio	1.99±0.25^{a}	1.90±0.17^{a}	1.93±0.09^{a}	1.93±0.10^{a}
Survival rate (%)	75.00±3.51^{c}	81.00±0.47^{a}	80.00±1.89^{ab}	78.00±2.16^{b}

Note: Mean±SD within each row, superscripted with different lowercase letters indicate significant difference ($p<0.05$).

Specific growth rate (SGR) and average daily weight gain (ADG)

The results indicated that both SGR and ADG of the tadpoles showed non-significant differences among treatments (Table 2). However, it is worth noting that mixed mineral supplementation tended to enhance growth of the tadpoles with SGR around 0.1 %·day^{-1} compared with 0.09±0.01 %·day^{-1} of the control, and ADG ranged between 0.10±0.01 and 0.11±0.01 g·tadpole^{-1}·day^{-1} compared with 0.09±0.01 g·tadpole^{-1}·day^{-1} in the control.

Feed conversion ratio

FCR of all treatments showed non-significant differences ($p>0.05$). Notably, the groups received mixed mineral supplements showed a tendency of lower FCR (1.90±0.17–1.93±0.10) as compare to the control (1.99±0.25) (Table 2).

Survival rate

In terms of survival rate, the tadpoles received mixed mineral supplementation had significantly ($p<0.05$) higher survival rates (78.00±2.16%–81.00±0.47%) than the control (75.00±3.51%), regardless of the concentrations. The survival rate was highest for the group with 0.2% supplement (81.00±0.47%) which was non-significant different ($p>0.05$) with the 0.4% group (Table 2).

Metamorphosis

In order to assess the developmental stages of the tadpoles, four indicators were evaluated: average hindlimb length (mm·tadpole^{-1}), average forelimb length (mm·tadpole^{-1}), average tail length (mm·tadpole^{-1}), and percentage of complete tadpole transformation (%). The results of the study are presented below.

Average hindlimb length

Hindlimbs were first observed at day 14 and their development proceeded, as seen from the increase of its length, as the rearing time progressed. However, significant differences among treatments were observed only at 14th day whereby, the tadpoles fed 0.6% mixed mineral supplementation exhibited the highest value of 13.94±2.09 mm·tadpole^{-1}, which differed significantly ($p<0.05$) compared to other groups. However, from day 16 and onward, there was no significant difference ($p>0.05$) among these groups, as depicted in Figure 3.

Average forelimb length

Forelimbs were first observed on day 20, which was later than the emerging of hindlimbs. In general, mixed mineral supplement did not show significant effects on forelimb length as compared to the control (Figure 4). Significant differences were observed only at days 20, 26, and 28 whereby the groups received 0.2% and 0.4% resulted in longer forelimb length. Notably, at the end of the experiment, forelimbs of the tadpole were around 11.29±0.70 mm·tadpole^{-1}.

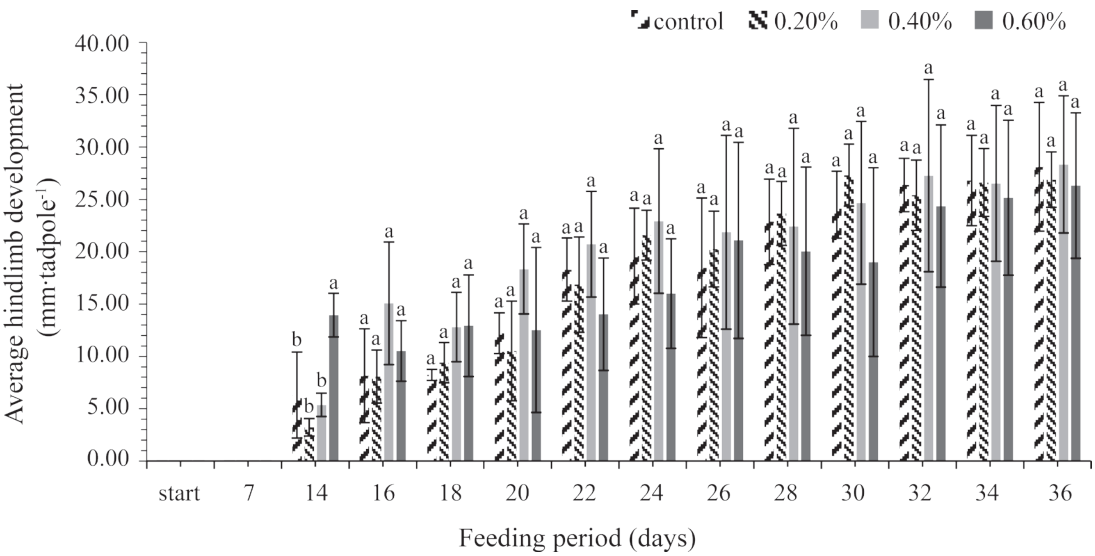

Figure 3. Histograms showing average length of hindlimbs (mm·tadpole^{-1}) of the tadpoles fed diet supplemented with various levels of mixed minerals at various time points; Different lowercase letters above bars denote significant differences ($p<0.05$) among means at each time point; Error bars represent SD.

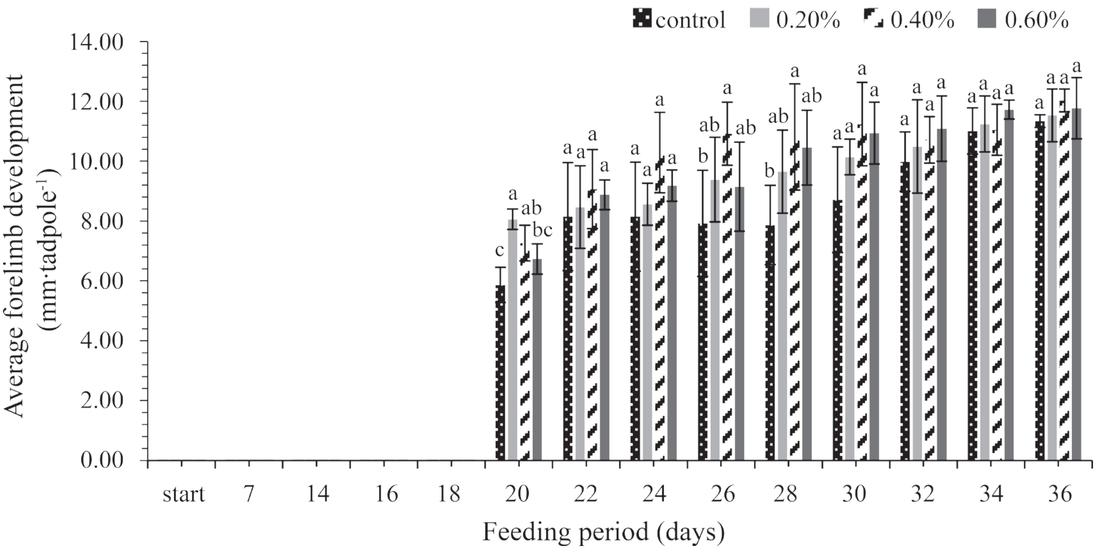

Figure 4. Histograms showing average forelimb length (mm·tadpole^{-1}) of the tadpoles fed diet supplemented with various levels of mixed minerals at various time points; Different lowercase letters above bars denote significant differences ($p<0.05$) within each time point; Error bars represent SD.

Average tail length

The study revealed that the average tail length of the tadpoles increased as the rearing time progressed, and significant differences were noticed since the 16th day of feeding. The longest tail length of 38.22±1.64 mm·tadpole^{-1} was recorded by tadpoles treated with mixed minerals supplementation at 0.4%, which was significant different ($p<0.05$) compared to tadpoles treated with mixed minerals supplementation at 0.2% and the control group, which recorded tail lengths of 33.68±0.33 and 32.19±2.65 mm·tadpole^{-1}, respectively. When compared to tadpoles treated with 0.6% mixed minerals supplementation which produced a tail length of 36.09±1.52 mm·tadpole^{-1}, there was no significant difference ($p>0.05$). The maximum tail length was noted on the 24th day of rearing at 0.2% mixed minerals supplementation of 45.76±0.99 mm·tadpole^{-1}, followed by tadpoles fed by the control group, mixed minerals supplementation 0.6% and 0.4% with values of 39.92±3.01, 36.49± 1.28 and 35.77±1.98 mm·tadpole^{-1}, respectively.

As the tadpoles progressed in the metamorphosis stage, the average length of the tadpoles' tails started to decrease until complete tadpole transformation. Tadpoles treated with mixed mineral supplementation at 0.2% had a mean tail length of 0 mm on day 28, following by the group received 0.4% mixed mineral supplementation (day 32), the control group (day 34), and 0.6% mixed mineral supplementation (day 36) (Figure 5).

The percentage of tadpoles completed metamorphosis.

Completed metamorphosis of the tadpoles was first observed on the 26th day of feeding. Tadpoles received 0.2% mixed mineral supplementation had the highest metamorphosis completion (40%), followed by tadpoles received 0.4% and 0.6% mixed mineral supplementation, both achieving a 20% metamorphosis completion. In the control group, tadpoles began completing metamorphosis on day 30 of rearing.

Furthermore, tadpoles received 0.2% mixed mineral supplementation was the first group that achieved 100% completion of metamorphosis (at day 34). Tadpoles received 0.4%, 0.6%, and the control group reached 100% conversion into juvenile frogs on the 38th, 40th, and 40th of rearing, respectively as shown in Figure 6.

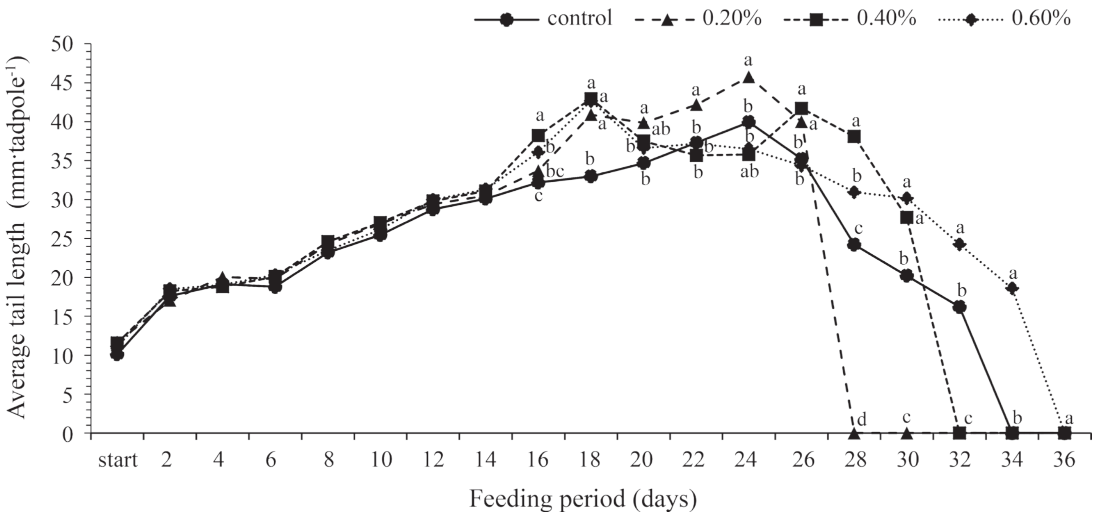

Figure 5. Average tail length (mm·tadpole^{-1}) of the tadpoles fed diet supplemented with various levels of mixed minerals at various time points; Different lowercase letters at each time point denote significant differences ($p<0.05$).

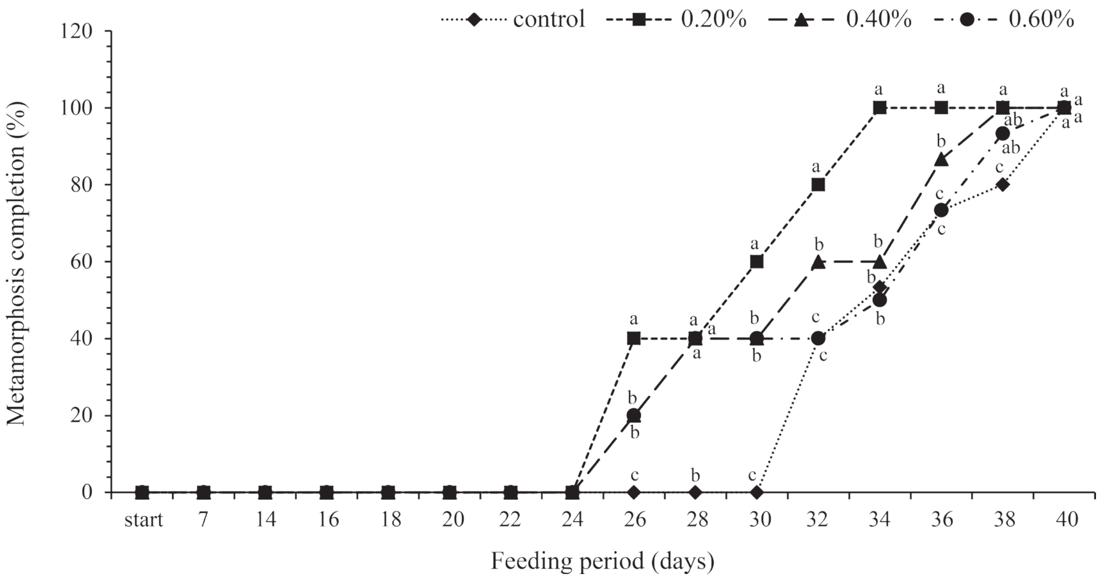

Figure 6. Average tail length (mm·tadpole^{-1}) of the tadpoles fed diet supplemented with various levels of mixed minerals at various time points; Different lowercase letters at each time point denote significant differences ($p<0.05$).

DISCUSSION

The present study showed that mixed mineral supplementation at 0.2% in feed enhanced the growth, metamorphosis rate, and survival rates of *Hoplobatrachus rugulosus* tadpoles, but these enhancement effects were compromised at higher concentration of the supplements. A hormone released by the thyroid gland is a signal for frog metamorphosis. A reduction in the developmental rate and termination of metamorphosis may be attributed to lower levels of thyroid hormone in tadpoles (Gomez-Mestre *et al*., 2004). This hormone requires the presence of iodine in water for its production. Iodine is essential for thyroid hormone synthesis, which plays a crucial role in amphibian metamorphosis. If the water in which tadpoles are growing is deficient in iodine, the tadpoles will fail to grow and cannot become adult frogs. Inorganic iodine and its compounds, iodoform and potassium iodide, greatly accelerate metamorphosis of tadpoles (Swingle, 1919), leading to the development of adult frog characteristics. This study showed that tadpoles fed with 0.2% mixed mineral supplementation had significantly higher average weight gain and survival rate. This agrees with the results of Cabanilla-Legasp *et al*. (2021) where rabbitfish (*Siganus guttatus*) larvae fed iodine-supplemented *Artemia* increased the endogenous thyroid hormone levels which in turn stimulated the metamorphosis and their growth. However, excessive iodine levels can be toxic, leading to thyroid gland dysfunction and developmental issues in frogs. These mixed minerals applied in this study contain sodium chloride, iodine, calcium, magnesium, and iron. It is necessary for the larvae of all terrestrial, avian, amphibian, and aquatic animals to receive adequate levels of minerals, particularly iodine and sodium chloride, to facilitate thyroid hormone production. Thyroid hormones play a vital role in stimulating the development of various organs, promoting growth, and enabling normal tissue functions. Additionally, thyroxine, a hormone induced by thyroid hormone, contributes to the shrinking of tadpoles' tails, a process that relies on the presence of iodine. Apart from iodine, high salinity levels can disrupt osmoregulation in amphibians, affecting their ability to maintain proper internal balance of salts and water. This can lead to stunted growth, developmental abnormalities, and even death in frog larvae during metamorphosis. Nakkrasae *et al*. (2016) reported that low salinity (<4‰) improved the body weight, survival rate, and shortened the time taken to reach metamorphosis of the Chinese edible frog, *H. rugulosus*. However, in this study, tadpoles received 0.6% (0.06 ‰) mixed mineral supplementation showed lower growth efficiency compared to the others, possibly due to excessive concentrations of iodine and sodium chloride. The effects seem to depend on the dose, developmental stage, and possibly species specific factors. High doses of exogenous treatments could have negative effects on growth, survival, and development. In addition, calcium is important not only for bone structure but also for nerve and muscle function. Wilbur and Collins (1973) reported that the large variation in length of larval period and body size at metamorphosis typical of a particular species of amphibian cannot be directly explained by differences in dates of hatching or egg sizes. Many environmental factors including cold, salt, or food availability possibly influence the size at metamorphosis as well. Tadpoles living in either hypoosmotic, or hyperosmotic environments struggled to maintain homoeostasis of body fluids by switching more energy to osmoregulation rather than growth (Fielder *et al*., 2005).

A 100% transformation rate was observed on the 28th day of raising tadpoles fed with 0.2% mixed mineral supplementation. However, an excessive concentration of iodine and sodium chloride possibly have a synergistic effect leading to incomplete metamorphosis, as observed in a study by Nakkrasae *et al*. (2016), showing that water salinities above 5 ppm were toxic to both eggs and hatchling larvae of the Chinese edible frog, *H. rugulosus*. At 6 ppm salinity, tadpoles exhibited a cessation of metamorphosis, a reduction in growth, and failure to transform into juvenile frogs. The elevation of plasma salt levels has been reported to inhibit the metamorphosis in other anuran species (Uchiyama and Yoshizawa, 1992; Christy and Dickman, 2002). *Epidalea calamita* tadpoles' exposure to high salinity displayed a triiodothyronine (T_3) reduction, leading to a metamorphosis retardation (Gomez-Mestre *et al*. 2004). The decrease in metamorphic hormones, such as T_3, might have been possibly caused by the increased secretion of prolactin, a vital hormone that aided to maintain water and salt balance in amphibian (Brown and Brown, 1973). While salinity and iodine can have significant effects on overall frog metamorphosis and growth, they may not necessarily have a direct impact on the specific lengths of forelimbs and hindlimbs during frog development. Forelimb and hindlimb development in frogs are controlled by intricate genetic and hormonal processes that can be influenced by various factors, but the effects of salinity and iodine on these specific limb lengths may be more indirect or subtle. Factors such as nutrition, environmental temperature, and genetic factors play a more direct role in determining limb lengths in frogs. Salinity and iodine are more likely to affect overall growth, development, and metamorphosis in frogs rather than specifically targeting forelimb and hindlimb lengths.

CONCLUSIONS

Various minerals are required for the metamorphosis of frog tadpoles. Tadpoles fed with 0.2% mixed mineral supplementation exhibited the best metamorphosis development, average weight gain, and survival rate. This feed additive finding will be used to improve a tadpole development and frog production for frog farmers.

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