Effects of Antimicrobial Peptides on the Growth and Development of Rana zhenhaiensis Tadpoles: Postprint

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Date: 2018-12-24T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of dietary supplementation with different concentrations of antimicrobial peptides on the growth and development of Rana zhenhaiensis tadpoles. Five hundred Rana zhenhaiensis tadpoles (Gosner stages 26~27) were selected and randomly divided into 5 groups, with 5 replicates per group and 20 individuals per replicate. Each group was fed experimental diets supplemented with 0, 1, 2, 4, and 8 mg/g antimicrobial peptides, respectively. The experiment continued until all Rana zhenhaiensis tadpoles completed metamorphosis. The results showed: 1) Antimicrobial peptide concentration was significantly negatively correlated with the developmental duration of Rana zhenhaiensis tadpoles ($P<0.001$), but not significantly correlated with tadpole body length ($P=0.324$); 2) The average metamorphosis rate of Rana zhenhaiensis tadpoles was 94.6%, with no significant difference among groups ($P=0.549$) and no significant correlation with antimicrobial peptide concentration ($P=0.434$); 3) Antimicrobial peptide concentration was significantly positively correlated with metamorphosis time as well as body length and body weight of Rana zhenhaiensis tadpoles ($P<0.001$); 4) Antimicrobial peptide concentration was not significantly correlated with the jumping distance of metamorphosed Rana zhenhaiensis froglets ($P=0.578$); 5) Antimicrobial peptide concentration was significantly negatively correlated with superoxide dismutase activity in the thigh muscle of metamorphosed Rana zhenhaiensis froglets ($P<0.001$). These results indicate that antimicrobial peptide supplementation decreased the developmental rate of Rana zhenhaiensis, prolonged metamorphosis time, while simultaneously increasing the size of metamorphosed individuals, thereby enhancing the fitness of juvenile frogs.

Full Text

Abstract

This experiment was conducted to investigate the effects of dietary antimicrobial peptides at varying concentrations on the growth and development of *Rana zhenhaiensis* tadpoles. Five hundred *R. zhenhaiensis* tadpoles at Gosner stages 26–27 were randomly allocated into five groups, each consisting of five replicates of 20 individuals. The groups were fed experimental diets supplemented with antimicrobial peptides at concentrations of 0, 1, 2, 4, and 8 mg/g. The trial continued until all tadpoles had completed metamorphosis. The results showed: (1) antimicrobial peptide concentration was significantly negatively correlated with tadpole developmental stage ($P<0.001$), but not significantly correlated with body length ($P=0.324$); (2) the average metamorphosis rate was 94.6%, with no significant intergroup differences ($P=0.549$) and no significant correlation with antimicrobial peptide concentration ($P=0.434$); (3) antimicrobial peptide concentration was significantly positively correlated with metamorphosis time, body length, and body mass ($P<0.001$); (4) the correlation between antimicrobial peptide concentration and jumping distance of newly metamorphosed froglets was not significant ($P=0.578$); and (5) antimicrobial peptide concentration was significantly negatively correlated with thigh muscle superoxide dismutase (SOD) activity in newly metamorphosed froglets ($P<0.001$). These findings indicate that antimicrobial peptide supplementation reduced developmental rate and prolonged metamorphosis time in *R. zhenhaiensis* while simultaneously increasing body size at metamorphosis, thereby enhancing juvenile fitness.

Keywords: antimicrobial peptides; *Rana zhenhaiensis*; tadpoles; metamorphosis; superoxide dismutase

Introduction

With the rapid development of aquaculture, intensive and high-density farming has become the dominant production model. This approach has led to increasing disease problems caused by viruses and bacteria, severely affecting production efficiency and making disease prevention and treatment critical issues in frog farming [1]. For decades, antibiotics have been considered an unparalleled solution, contributing significantly to economic growth as performance enhancers [2]. However, excessive antibiotic use has raised serious concerns about drug resistance and residues in animal products, threatening human health. Consequently, the search for alternatives to conventional antibiotics—particularly antimicrobial peptides—has become a major focus in molecular biology and biochemistry research [1,3-4].

Antimicrobial peptides, also known as host defense peptides, are small molecules widely distributed in nature [5] with immunomodulatory properties. They constitute an important component of the innate immune system and can inhibit

various pathogens including bacteria, viruses, fungi, and parasites [6-7]. Novel functions of antimicrobial peptides have been reported, including antitumor activity [8], growth promotion [9], and immune enhancement [10-11]. Research on antimicrobial peptides as feed additives has primarily concentrated on livestock [12], poultry [1,13], fish [14-16], and shrimp aquaculture [17-18], while their effects on amphibian growth remain unreported.

Superoxide dismutase (SOD) is a key member of the enzymatic antioxidant system, functioning to scavenge superoxide radicals in vivo. It counteracts reactive oxygen species by directly eliminating superoxide anions, hydroxyl radicals, peroxyl radicals, and nitric oxide [19]. Oxidative stress and subsequent lipid peroxidation can exacerbate free radical chain reactions, disrupt cell membrane integrity, and activate inflammatory mediators [20-21]. Therefore, SOD activity reflects the capacity of the antioxidant enzyme system and serves as a crucial indicator of health status and immune competence, with potential applications in disease diagnosis and physiological monitoring.

Rana zhenhaiensis belongs to Amphibia, Anura. Its tadpoles primarily inhabit low-lying areas, temporary water bodies, and ditches [22]. Current research on this species has focused on life history characteristics [23-24] and extraction of skin antimicrobial peptides [25]. This study investigated the effects of different dietary antimicrobial peptide concentrations on tadpole growth and development to provide a foundation for the conservation, exploitation, and utilization of frog resources.

Materials and Methods

Experimental Materials

On December 28, 2016, five freshly laid egg masses of *R. zhenhaiensis* were collected from puddles on the Lishui University campus and transported to the laboratory. Eggs were incubated separately in opaque plastic tanks (700 mm × 500 mm × 400 mm, water depth 200 mm) maintained in a climate-controlled room at 20.00 ± 0.22 °C. Once tadpoles reached Gosner stages 26–27 [26], 500 individuals were randomly selected for the experiment. The antimicrobial peptide used was cecropin (National Medical Products Administration approval number H13021322) manufactured by North China Pharmaceutical Co., Ltd.

Experimental Design

Five hundred *R. zhenhaiensis* tadpoles at Gosner stages 26–27 were randomly divided into five groups, each with five replicates of 20 individuals. Antimicrobial peptide powder was added to a basal diet (bullfrog feed provided by Ningbo Tech-Bank Co., Ltd.; main ingredients: fish meal, soybean meal, cottonseed meal, rapeseed meal, wheat flour, fish oil, calcium dihydrogen phosphate, vitamin premix, mineral premix; guaranteed analysis: crude protein 40.0%, crude fat 3.0%, crude fiber 4.0%, crude ash 18.0%, moisture 12.0%, total phosphorus 1.0%, lysine 2.2%). The mixture was ground, passed through a 200-mesh

sieve approximately 20 times, and thoroughly mixed to produce powdered feed (required for tadpoles). The five groups received diets containing 0, 1, 2, 4, and 8 mg/g antimicrobial peptide, respectively.

Tadpoles were reared in plastic containers (18 cm × 13 cm × 6 cm, water depth 6 cm) placed in an artificial climate chamber at 20 ± 1 °C with a 12:12 light:dark photoperiod. Tadpoles were fed ad libitum every two days, with complete water replacement using aerated tap water before each feeding. Survival was monitored daily; any dead tadpoles were removed and replaced with size-matched individuals that had undergone tail clipping to maintain constant density. The experiment concluded when all tadpoles had completed metamorphosis.

Tadpole Morphological Measurements

Wet mass (±0.0001 g) was measured using an electronic analytical balance on days 1 and 18. Developmental stages were identified using a Nikon XTS30 stereomicroscope, and body length (snout-to-cloaca distance) was determined from photographs. Metamorphosis was defined as the emergence of forelimbs (Gosner stage 42), and metamorphosis time was recorded as the duration from experiment initiation to forelimb emergence. Metamorphosing individuals were transferred to plastic cups containing 5 mm of water, covered with 1-mm mesh gauze, and maintained in the climate chamber until tail resorption was complete (Gosner stage 46).

Morphological and Locomotor Measurements of Froglets

Upon completion of metamorphosis, all froglets were removed, surface moisture was absorbed with filter paper, and wet mass (±0.0001 g) and body length (snout-to-urostyle distance, ±0.01 mm) were measured using digital calipers. Locomotor performance was assessed two hours later. Froglets were acclimated at 20 ± 1 °C for 10–15 minutes, then immersed in 1% red food dye solution to stain their limbs. They were placed on white gauze-covered ground and stimulated to jump by gently touching the urostyle with a willow twig. Jumping distance was measured as the distance between successive hindlimb imprints. Each froglet was tested five times; the three longest jumps were measured (±0.01 cm) and averaged.

SOD Activity Assay

Five hours after locomotor testing, seven froglets from each group were selected for SOD activity analysis. Froglets were euthanized, and thigh muscle tissue was collected and stored at -80 °C. For analysis, samples were thawed, rinsed three times with pre-cooled Ringer' s solution, surface moisture was absorbed with filter paper, and 0.04 g (wet mass) was weighed. Tissue was homogenized in pre-cooled Ringer' s solution at a 1:9 mass-to-volume ratio on ice, then centrifuged at 3,000 r/min for 10 minutes. The supernatant was collected for analysis. Protein content was determined using the Coomassie brilliant blue

method. Thigh muscle SOD activity was measured using assay kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer' s instructions.

Statistical Analysis

All statistical analyses were performed using Statistica 10 software. Data normality (Kolmogorov-Smirnov test) and homogeneity of variance (F-max test) were verified before analysis. All data met parametric assumptions without transformation. Data were analyzed using ANOVA with multiple comparisons, regression, and correlation analysis. Descriptive statistics are presented as means ± standard error. Antimicrobial peptide concentration groups were coded as 0, 1, 2, 4, and 8. Different lowercase superscripts indicate significant differences ($P<0.05$), while identical superscripts indicate no significant difference ($P>0.05$).

Results

Initial tadpole wet mass was 20.93 ± 0.89 mg, body length was 8.45 ± 0.08 mm, and developmental stage was Gosner 26-27.

Effects of Antimicrobial Peptides on Tadpole Growth and Development

As shown in [Figure 1: see original paper]-A, antimicrobial peptides significantly affected developmental stage ($F_{4,244}=14.99$, $P<0.001$; 0ab, 1a, 2bc, 4cd, 8d) and body length ($F_{4,244}=14.99$, $P<0.002$; 0a, 1ab, 2b, 4b, 8ab). Antimicrobial peptide concentration was significantly negatively correlated with developmental stage ($r=-0.40$, $F_{1,247}=46.20$, $P<0.001$), indicating that higher concentrations resulted in lower developmental stages. As shown in [Figure 1: see original paper]-B, the correlation between antimicrobial peptide concentration and body length was not significant ($r=0.06$, $F_{1,247}=0.98$, $P=0.324$).

Effects of Antimicrobial Peptides on Metamorphosis Rate, Time, Body Length, Body Mass, and Jumping Distance

The average metamorphosis rate across all groups was 94.6%, with no significant intergroup differences ($F_{4,20}=0.78$, $P=0.549$) and no significant correlation with antimicrobial peptide concentration ($r=0.16$, $F_{1,23}=0.63$, $P=0.434$). As shown in [Figure 2: see original paper]-B, antimicrobial peptides significantly affected metamorphosis time ($F_{4,485}=66.24$, $P<0.001$; 0d, 1b, 2c, 4a, 8a), with a significant positive correlation between concentration and metamorphosis time ($r=0.49$, $F_{1,488}=152.41$, $P<0.001$), indicating that higher concentrations prolonged metamorphosis.

As shown in [Figure 3: see original paper]-A and [Figure 3: see original paper]-B, antimicrobial peptides significantly affected body length ($F_{4,446}=14.78$, $P<0.001$; 0c, 1bc, 2bc, 4b, 8a) and body mass ($F_{1,445}=11.20$, $P<0.001$; 0b,

1a, 2a, 4a, 8a) of newly metamorphosed froglets. Antimicrobial peptide concentration was significantly positively correlated with both body length (r=0.33, F1,489=55.72, P<0.001) and body mass (r=0.24, F1,488=28.04, P<0.001), indicating that higher concentrations produced larger froglets.

As shown in [Figure 4: see original paper], antimicrobial peptides did not significantly affect jumping distance (F4,453=0.91, P=0.458), and the correlation between concentration and jumping distance was not significant (r=0.03, F1,456=0.31, P=0.578), indicating no detectable effect on locomotor performance.

Effects of Antimicrobial Peptides on Thigh Muscle SOD Activity

As shown in [Figure 5: see original paper], antimicrobial peptides significantly affected thigh muscle SOD activity (F4,155=15.39, P<0.001; 0bc, 1ab, 2cd, 4a, 8d), with a significant negative correlation between concentration and SOD activity (r=-0.30, F1,138=13.71, P<0.001), indicating that higher concentrations reduced SOD activity.

Discussion

For anuran amphibians with complex life histories, growth rate and body size at metamorphosis are critical determinants of fitness in the terrestrial phase [23]. When tadpoles encounter favorable environmental conditions, they may delay metamorphosis to maximize growth, whereas exposure to harsh conditions can accelerate development to exit the aquatic environment sooner [27]. Our results demonstrate that under experimental conditions, antimicrobial peptide concentration was significantly negatively correlated with developmental stage and positively correlated with metamorphosis time, indicating that higher concentrations slowed tadpole development and reduced growth rate. Furthermore, antimicrobial peptide concentration was significantly positively correlated with body length and mass at metamorphosis, suggesting that higher concentrations produced larger juveniles with greater environmental fitness. Larger body size in anurans confers performance advantages in metabolic rate and stress tolerance, jumping ability (greater distance, endurance, and power), foraging (higher attack frequency and capture success, broader prey size acceptance), and predator avoidance [28-29].

These beneficial effects for aquaculture differ from findings in other vertebrates. Dietary supplementation with 3.4–3.7 mL/kg composite antimicrobial peptides promoted broiler growth [13], low concentrations improved growth performance in koi carp [14], 100 mg/kg enhanced growth in grouper [16], and 100 mg/kg NT-6 antimicrobial lipopeptide significantly improved growth in *Litopenaeus vannamei* [30]. These discrepancies suggest that both peptide type and species identity influence optimal dosage, and that for *R. zhenhaiensis*, high concentrations reduced developmental rate but increased metamorphic size and individual fitness.

The significant negative correlation between thigh muscle SOD activity and antimicrobial peptide concentration indicates that low concentrations enhanced SOD activity while high concentrations were inhibitory [14], contrasting with some previous studies. For example, dietary composite antimicrobial peptides increased piglet SOD activity in a concentration-dependent manner [31-32], and 200–250 mg/kg supplementation increased serum SOD activity in *Carassius auratus* var. Xiangyun [33]. The SOD activity patterns observed here suggest that within the tested concentration range, dietary antimicrobial peptides provided more favorable conditions for tadpole growth and development.

Conclusion

Antimicrobial peptide supplementation reduced developmental rate and increased metamorphosis time in *Rana zhenhaiensis* while simultaneously increasing body size at metamorphosis, thereby enhancing juvenile fitness.

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Note: Figure translations are in progress. See original paper for figures.