

## The Characterization and Activity of Protease Enzyme on Different Days of Tadpoles [*Pelophylax ridibundus* (Pallas, 1771)]

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Cite this article as: Sereflisan, H., Alkaya, A., Gokcek, K. (2019). The characterization and activity of protease enzyme on different days of tadpoles [*Pelophylax ridibundus* (Pallas, 1771)]. *Aquatic Sciences and Engineering*, 34(4), 112-115.

### ABSTRACT

In this study, protease enzyme activity of *Pelophylax ridibundus* (Pallas, 1771) tadpoles was determined during the larval period starting from 25 Gosner stage (1960). When the *P. ridibundus* tadpoles reached the 25 Gosner stage (1960) on the 22<sup>nd</sup> day, the sampling was continued until the metamorphosis was completed by sampling at 5-day intervals. Initial average weight and length of the tadpoles were 0.076 g and 6.42 mm, respectively. The tadpoles were fed with pellet feed the size of 2 mm and 39% crude protein content twice a day in the morning and afternoon. The differences in the protease activity between the 22<sup>nd</sup> day and the 62<sup>nd</sup> day of the metamorphosis were found statistically significant ( $P < 0.05$ ). It has been observed that the protease enzyme reached maximum level at the 32<sup>nd</sup> day. In the next stages, protease activity showed a tendency to increase and decrease up to metamorphosis. These results can be used as the basis for the most appropriate nutritional formulation for *P. ridibundus* in the larval stage.

**Keywords:** *Pelophylax ridibundus*, frog, acid protease, larval period, metamorphosis

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### Submitted:

18.05.2019

### Accepted:

10.09.2019

### Online published:

27.09.2019

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Available online at  
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### INTRODUCTION

Most adult amphibians are fed on insects, while a small part are carnivores because they eat live invertebrates. The anuran tadpoles are herbivorous and their diets may also include plant material as well as aquatic invertebrates. The functional development of digestive organs is very important in the digestibility of the tadpole. In particular, in the frog culture where artificial feeds with high protein values are preferred in their diet, the digestive enzyme system mainly contains protease, amylase and lipase (Klahan and Yuangsoi, 2012). In the larval period, which is the most important step in the feeding of frogs, there is little information about how these animals are fed. According to the needs of the larvae, the deficiency in feeding resulted in death with the emergence of larval diseases (Veiga et al., 1998; Seixas Filho et al., 1998). In other zoological groups, the rela-

tionship between the nutrition regime and the digestive system has been studied extensively and satisfactory results have been obtained by formulating the feed used to be manipulated (Seixas Filho et al., 2000). However, tadpole nutrition is far from establishing requirement standards that can be used by nutritionists. It is necessary to know the digestive physiology of these animals in order to identify the feed formulation in accordance with the nutritional requirements of the larvae at different stages of development. Furthermore, the effective use of the feed is directly related to the digestion process which is the basis of enzymes. In particular, the diets currently used in frog breeding have 40% crude protein content, but they contain the basic ingredients necessary for fish, but the needs for the tadpole frog is insufficient. In addition, nutritional habits and nutritional requirements of frogs vary at each stage of their development. Due to the structural changes that oc-

cur during the development of larvae, nutrients need to be higher and nutritional deficiencies should be avoided (Barbosa et al., 2005; Oliveira-Bahia, 2007).

Generally, the use of feeds with high crude protein values in frog cultivation has led to good performance both in the larval period (Carmona-Osalde et al., 1996; Albinati et al., 2000; Albinati et al., 2001; Hayashi et al., 2004) and in other developmental periods (Barbalho, 1991; Braga and Lima, 2001). In addition, 26.6% to 33.6% of crude protein levels were reported to be suitable for larval development (Barbosa et al., 2005), but the use of feeds with protein values of 22.5% may cause high mortality. For this reason, it is important to understand the relationship between the enzymes involved in digestion and the percentages of carbohydrates, proteins, and fats in the diet to increase meat production in frogs.

Studies report that adult amphibians fed with different diets do not exhibit any changes in the activity of intestinal enzymes, but alter the digestive performance of different diets used in feeding the tadpole (Toloza and Diamond, 1990; Sabat and Bozinovic, 1996). In addition, studies on the enzyme activity profiles of animals eating controlled diets are needed.

Although there are studies on the study of the enzymatic activity in amphibians (Etkin, 1968; Leone et al., 1976; Braga et al., 2004; Braga et al., 2006; Oliveira-Bahia, 2007), studies on the relationship between nutrition levels and growth in the literature have been reported to be inadequate (Barbosa et al., 2005). In some studies, the effects of some variables on larval growth, such as the period of light (Bambozzi et al., 2004), stock density (Hayashi et al., 2004) and protein levels (Carmona-Osalde et al., 1996) were investigated.

Metamorphosis, period of major post-embryonic transformations that involving morphological, physiological, biochemical, and behavioral changes. Also, changes the lifestyle of frog larvae. Metamorphosis in amphibians is the development of structures and functions that are important in the transformation of larval structures into beneficial structures in adults (Duellman and Trueb, 1986).

Morphological changes are observable changes, such as the formation of the lung, the development of the dermal glands of the eyelids, the formation of the mouth and tongue, and the differentiation of the digestive system (Bonneville, 1963; Bonneville and Weinstock 1970; Thibaudeau and Altig, 1988; Paulson and Robinson 1995; Rovira et al., 1993). Proteolytic enzymes play an important role in performing these morphological and physiological changes before and during metamorphosis. Proteolytic enzymes are used as energy reserves in metamorphosis as a result of feeding of living organisms. In addition, the feeding strategies formed by knowing the physiological events occurring in the larval period and the changes due to the days in the activities of these enzymes will accelerate the larval development and increase the survival rate.

The aim of this study was to determine the protease enzyme activity of *Pelophylax ridibundus* (Pallas, 1771) tadpoles fed with a protein ratio of 39% starting from 25 Gosner stage (1960) during

the larval period. The results of the study will light on obtaining the most suitable feed ration for *P. ridibundus*, which is an economically important species.

## MATERIAL AND METHODS

This study was carried out to determine protease enzyme activity on *P. ridibundus* tadpoles grown in the frog production facility of Aydıncık in Mersin, Turkey. Larval tadpole sampling was performed on the 22<sup>nd</sup> day with the Gosner 25 (1960) level at 5-day intervals and this process was continued until the metamorphosis was completed. In this study, 6 m<sup>2</sup> ponds were used in the cultivation of tadpole larvae. The water of the ponds was changed 50% once a day and the temperature of the ponds was measured as 23.0±1.0°C during the study period. Initial average weight and length of the tadpoles were 0.076 g and 6.42 mm, respectively. The tadpoles were fed with pellet feed the size of 2 mm and 39% crude protein content twice a day in the morning and afternoon (Table 1).

**Table 1.** Chemical analysis of formulated pellet food for *P. ridibundus* tadpoles

Content	Percent (%)
Moisture	4.54
Crude Protein	39
Fat	22.88
Ash	14.74

**Basic composition:** fish meal, chicken meal and fish oil.

Pellet food content analyzes used in the feeding study were conducted in Iskenderun Technical University Marine Sciences and Technology Faculty Aquaculture Laboratory. The tadpole samples for analysis were stored in the Hettich freezer at -80°C.

### Determination of protease activities

Total protease activities of *P. ridibundus* tadpoles was measured as described by Walter (1984), using casein (10 mg ml<sup>-1</sup>) in 50 mM Tris-HCl buffer at pH 9 as the substrate. The mixtures including extracts of tadpoles were incubated with the substrate and then the reaction was stopped by addition of 500 µl trichloroacetic acid (TCA) (120 g L<sup>-1</sup>). The absorbance was recorded at 280 nm. All measurements were carried out in triplicate. The soluble protein concentrations of *P. ridibundus* tadpole were determined according to Brasford (1976).

### Statistical methods

Data was analyzed by using SPSS 17.0 statistics software. Comparisons were made using a one-way ANOVA test. Then, a Post-Hoc Tukey multiple-comparison test was used for significant differences at the P<0.05 level.

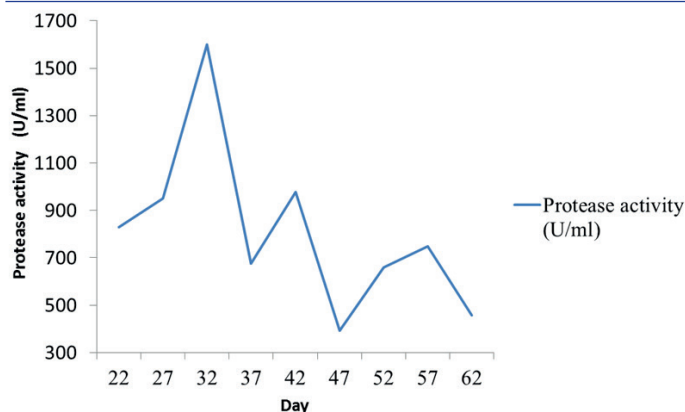
## RESULTS AND DISCUSSION

The results of the analysis of protease enzyme activities with *P. ridibundus* tadpoles are given in Table 2. The differences between the 22<sup>nd</sup> day of protease activity larvae and the 62<sup>nd</sup> day of the metamorphosis were found statistically significant (P<0.05). It has

**Table 2.** The changes observed in protease activities (mean±standard error (SE))

Day	Protease Activities (U/ml)
22	830.50±64.19 <sup>bc</sup>
27	950.31±62.31 <sup>c</sup>
32	1600.22±12.61 <sup>d</sup>
37	675.76±13.94 <sup>b</sup>
42	977.17±14.79 <sup>c</sup>
47	392.08±25.08 <sup>a</sup>
52	659.93±40.72 <sup>b</sup>
57	739.36±32.86 <sup>b</sup>
62	451.24±17.38 <sup>a</sup>

In all lines, means with different superscripts are significantly different from each other (P<0.05)



**Figure 1.** Protease activity on different days.

been observed that the protease enzyme reached maximum level at the 32<sup>nd</sup> day, increasing from the 25 Gosner stage (1960) (day 22). After that, the increases and decreases in protease enzyme activity were detected until metamorphosis (Figure 1).

Santos et al. (2016) with study *Lithobates catesbeianus* showed that the increase in the amount of digestive enzymes before metamorphosis is important to increase the amount of metamorphosis thanks to the increase in the energy needed for the growth of the living organism detected. In our study with *P. ridibundus* tadpoles, the highest protease activity was found on day 32 (1600.22±12.61 U/ml) since the beginning of nutrition that this increase continued until the metamorphosis and the digestive system were completed. With the growth slowed after 30 days, the amount of energy required for development will decrease and the enzyme activity will be physiologically limited by the tadpoles (Seixas Filho et al., 2010). During the study period 32-37. (675.76±13.94 U/ml), 42-47. (392.08±25.08 U/ml) and 57-62. (451.24±17.38 U/ml) days, the amount of protease was decreased.

Seixas Filho et al. (2010) reported that the tadpoles of *Rana catesbeiana* were fed with 28% crude protein, the amylase enzyme was more active between 30 and 45 days and the tadpoles were fed

with mostly content of carbohydrate diets. In our study, a decrease in protease activity was observed in 32<sup>nd</sup>-37<sup>th</sup> days, although there was an increase again in protease activity from the 37<sup>th</sup> day (Figure 1). *P. ridibundus* tadpoles reached the metamorphosis stage on the 42<sup>nd</sup> day and the process ended on the 62<sup>nd</sup> day. As a result of our study, the amount of protease obtained from Oliveira-Bahia (2007) was similar to the results of the study.

The use of feeds with high crude protein values in frog culture has led to good performance in both larval stages (Carmo-Osalde et al., 1996; Albinati et al., 2000, 2001; Hayashi et al., 2004) and other developmental periods (Barbalho, 1991; Braga & Lima, 2001). In our study, tadpoles of *P. ridibundus* were fed with a pellet food containing 39% crude protein. Seixas Filho et al. (2010) reported that bullfrog tadpoles fed commercial frog feed with 28% crude protein content obtained good results in terms of enzyme activity.

Klahan and Yuangsoi (2012) reported that *Rana rugulosa* in 45-day tadpoles were more prone to protease activity than those with 60-day tadpoles. In our study, the 42-day *P. ridibundus* tadpoles (977.17±14.79 U/ml) were found to have more protease than the 62-day (451.24±17.38 U/ml) individuals. In this study, it was determined that the protease amount of *P. ridibundus* tadpoles (451.24±17.38 U/ml) decreased in the last period of the metamorphosis phase (62<sup>nd</sup> day). The reason for this decrease in the amount of protease is due to the maintenance of a number of morphological changes such as the consumption of metabolic energy in the loss of the tail and the tadpoles consuming a smaller amount of food in the last stages of metamorphosis than in previous periods (Gonçalves et al., 2015).

## CONCLUSION

As a result, *P. ridibundus* tadpoles fed with 39% crude protein were found to have more protease activity at the beginning of the larval development period than in other periods. In our study, it was emphasized that appropriate dietary formulations should be formed so that it can be consumed effectively by frogs on different days of the larval period.

**Ethics Committee Approval:** This study was carried out accordance with animal welfare and the ethics of trial.

**Conflict of Interest:** Author has no conflict of interest to report.

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