RESEARCH ARTICLE **(Open Access)**

# Effects of temperature on in-vitro produced embryos and larval development of Albanian water frog, *Pelophylax shqipericus*

BLERTATURANI*,VALBONA ALIKO

Department of Biology, Faculty of Natural Sciences, University of Tirana, Tirana, Albania

*Correspondence author: E-mail: blertaturani@yahoo.com

**Abstract**

The temperature is a determining factor for the successful development of the larvae in amphibians. The plasticity during metamorphosis is important especially in populations of amphibians living in Mediterranean climate zones with high temperatures and relatively low precipitations, were the risk of pond desiccation is high. To study the effect of water temperature on the embryonal and larval development of *in-vitro* reproduced Albanian water frog, *Pelophylax shqipericus*, two batches of approximately 100±20 eggs (stage 14-16, Gosner) were raised in the laboratory at four different temperature regimes ($3^0$C, $17^0$C, $20^0$C and $25^0$C), with 12:12 photoperiod. To follow the process of metamorphosis, three important key events were evaluated: budding of the hind limbs (stage 1), the formation of the anterior (stage 2) and posterior limbs (stage 3), and the reduction and disappearance of the tail (stage 4). Development time and stage were strongly affected by temperature variation. The results showed a lengthening of development time with decreasing temperatures and a better survival rate of *P. shqipericus* with high temperatures. Values of temperature of $19^0$C-$20^0$C resulted to be optimal for larval development, while very low temperatures (t=$3^0$C) and very high ones (t=$25^0$C) strongly impede larval development. These results demonstrate that temperature is one of the most important factors that strongly affects the success of embryonic and larval development of *P. shqipericus* reproduced *in-vitro*.Since the Albanian water frog, is a native and an endangered species whose known populations are currently in decline, its successful *in-vitro* reproduction will contribute to conservation of the specimen.

**Keywords**: *Pelophylax shqipericus*, *in vitro* reproduction, development, temperature.

## 1. Introduction

Populations of ectotherm animals have a strong dependence on ambient temperature because they do not have an efficient mechanism for physiological thermoregulation [5]. Climatic variation is an important selective factor for life history trait differentiation, so populations of ectotherms are expected to diverge in their thermal optima for development and growth if they are exposed to different temperature environments. Amphibians have been widely used as model systems for the study of physiological ecology and temperature adaptation due to the easy manipulation of tadpoles in control experiments [9]. In temporary ponds, time to metamorphosis is influenced by temperature and duration of the larval stage is highly plastic, especially in semi– arid environments where rainfall and pond duration are unpredictable [16; 22]. In these environments, larvae need to accelerate metamorphosis when ponds dry out and delay the process when ponds dry later in the season. When pond duration is extremely short larvae should be the most adaptive strategy. It would be to express not a plastic but a quick canalized development [22]. Predation and pond desiccation have been identified as major causes of larval mortality in permanent and transient aquatic environments, respectively [6; 7; 21; 15]. In anuran populations, larger metamorphosis may exhibit higher terrestrial survival since they can cope with different stressors such as predators and desiccation [17; 1]. Smith–Gill and Berven (1979) [20] considered that low temperatures retard differentiation more than growth and increase the stage– specific size. Therefore, larvae growing at cold temperatures have prolonged developmental periods but they may increase their size at metamorphosis. This phenomenon is considered a general rule for ectotherms [3; 4]. This trade–off in fitness, mediated by the influence of temperature, largely conditions the

expression of an optimal phenotype at metamorphosis in amphibians and other aquatic organisms with complex life cycles [8; 20; 19; 11].

The minimum temperature required to achieve this larval metamorphosis is 20°C, which is a temperature higher than the room temperature more tolerable in other types of anurian in Europe [14; 12; 13]. The increase is related to the length of larval development [23] as favorable conditions for differentiation are also favorable for growth [20]. The results suggest that temperatures below 20°C are too low to allow a normal larval development and normal growth, while higher temperatures can promote conditions of growth and development.

In this study, we examined the influence of temperature on the development and growth of the *Pelophylax shqipericus*. In this study we focus on the response of larvae to increasing temperatures and analyze their metamorphic response.

## 2. Material and Methods

We examined the effects of temperature on size and time to metamorphosis on the embryonal and larval development of *in-vitro* reproduced Albanian water frog, *Pelophylax shqipericus*. Two batches of approximately 100±20 eggs were raised in the laboratory at four different temperature under a constant 12 hours light / 12 hours dark photoperiod. The temperature treatments were: $3^0$C, $17^0$C, $20^0$C and $25^0$C, under laboratory conditions and in the external environment ($16^0$C-$17^0$C).

The first group consisting of 10 larvae (2.5cm) we put in external environmental conditions (average temperatures of $16^0$C-$17^0$ C).

The second group consisted of 82 larvae (1.8cm) we put a $30^0$C temperature of the refrigerator.

The third group consisted of 20 larvae (2.5cm) we put in average temperature ($19^0$C-$20^0$C).

The fourth group consists of: 20 larvae (2.5cm) we put at $17^0$C.

The fifth group consist of: 30 larvae (2.5cm) we put in thermostat at temperature $25^0$C and in terms of lack of oxygen.

At the start of the experiment eggs were near Gosner stage 16 of development [10]. To follow the process of metamorphosis, three important key events were evaluated: budding of the hind limbs (stage 1), the formation of the anterior (stage 2) and posterior limbs (stage 3), and the reduction and disappearance of the tail (stage 4). Development time and stage were strongly affected by temperature variation. The results showed a lengthening of development time with decreasing temperatures and a better survival rate of *P. shqipericus*.

The larvae were kept in plastic containers; the water was changed every two days and the food thrown by little and periodically. The larvae were fed with lettuce even after the release of the legs, when became carnivorous, were fed with liver-based foods. During the experiment the stage of development was checked and photographed every 12 hours. The total body length (BL) of each larva was estimated using the program corelDRAW 11. The stage of development and B BL of larvae in each sample were measured on days 1, 5 and 19 of the experiment. Stage–size was measured for each spawn at each temperature condition, at stages: 16, 25 and 46 [10].

Due to development time differences between individuals of the same experimental container, the developmental stage was considered to have changed when 70% of larvae in the same sample had reached a particular stage. After the experiment was completed (experimental time: 150 days), the surviving larvae were returned to the pond where they were collected.

The relationships between the development phase (variable variables) and the time to set up each experiment were analyzed using the t-test applied. The significance level was defined for $p < 0.05$. All data are expressed as mean value.

## 3. Results and Discussion

The influence of temperature on embryonic and larval development. Table 1.1 reflect the duration of the process of development (metamorphosis) of larvae placed in its various temperature conditions. The duration of the metamorphosis is estimated at

days. During the process of metamorphosis were three major events, which focus on: training of the gems of the hind limbs (stage 1); the formation of the posterior limb (phase 2); front limb formation (stage 3) and the reduction and disappearance of the tail (step 4).

**Table. 1** The time of larval development depending on the temperature.

| | *Control (T=20 °C)* | *Group 1 (T=3 °C)* | *Group 2 (T=17 °C)* | **Group 3 (T=25$^0$C)** |
|---|---|---|---|---|
| Stage 1 | 9 | 27 | 10 | 0 |
| Stage 2 | 17 | 0 | 18 | 0 |
| Stage 3 | 27 | 0 | 28 | 0 |
| **Stage 4** | 32 | 0 | 35* | 0 |

The duration of development increases with the decrease of the temperature (see table 1 in). As so the duration of development increases with the decrease of the temperature (see table 1 in). As shown in the table, in a control group of larvae placed in average temperature 19$^0$C-20$^0$C, the time of completion of metamorphosis takes less than other groups, 32 days.

The second group of larvae placed in average temperature 17$^0$C, the metamorfosis process was completed for approximately 35 days, and then for a short time longer. In the first group of larvae put in conditions of temperature 30°C, their average life expectancy was 30 days. During this time the larvae have not undergone any change, then the process of metamorphosis has not been completed within the duration of the experiment. The larvae are "falling" into a State of confusion. This group of larvae was placed in the thermostat, in terms of a high temperature 25$^0$C and in the absence of oxygen. Both of these factors have affected to have a short life, until they are dead. The third group of larvae had shorter life, 24 hours. This group of larvae was placed in the thermostat, in terms of a high temperature of 25$^0$C and lack of oxygen. Both these factors also influenced their short life . The relationship between the temperature and the time it takes for the metamorphoses is analyzed using t-test. Using this test proves that the value of p = 0.028, therefore the p value is p <0:05. Then, the result is significant, this would mean that the temperature is a determining factor in the length of the metamorphosis. The larvae placed in temperature 19$^0$C-20$^0$C were used as the control group because in this group the 70% of the larvae ended up the metamorphosis.

The Figure 1 shows the duration of larval development in different temperatures. As expected, in our study, development time increases with decreasing temperature.

The development time is very important to *P. shqipericus* as its reproduction is done usually in temporary ponds, with a higher risk for drying, due to the higher temperature variation and depth of water. The pond temperature plays an important role in determining the duration of the development of amphibians [14].

Our data also supported by data from other laboratories have shown that temperature 19$^0$C -20$^0$C is optimal for larval development and very low temperatures (t = 3$^0$C) and very high (t = 25$^0$C) are prohibitive for larval development. Several studies from other laboratories have shown that the development and growth of the larvae at low temperature is performed more slowly and the metamorphoses is more extended in time [8; 20; 11] etc.

This result is also supported by our data. Thus, the larvae kept at different temperatures are running the metamorphosis at different times.

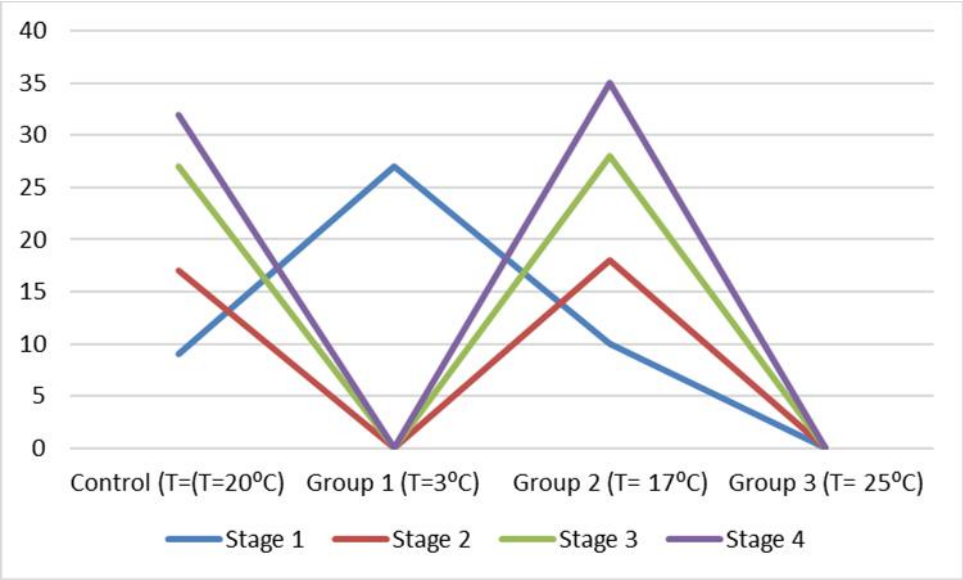


**Figure 1**. The duration of larval development (days).

During metamorphosis, we noticed that the larvae do not have altered their size, but have only highlighted the changes initially came out the hind limbs, then the front and finally the tail reduction. At the end of the larvae metamorphoses appears as a miniature version of the adult frog, which serves only to increase the size of the body. We note that the larvae at T = $19^0$C-$20^0$C are the first who have formed the hind limbs, more after the larvae in T = $17^0$C.

Meanwhile, the larvae at $3^0$C has not undergone any changes. Also we note that the larvae at T = $19^0$C-$20^0$C are the first who have formed the forelimbs, more after the larvae in T = $17^0$C. Note: larvae at T = $19^0$C-$20^0$C are the first that reduce the tail, and then the larvae at T = $17^0$C. All individuals have completed the metamorphosis with the same body size.

This result suggests that the population of *P. shqipericus* requires a minimum size to complete metamorphosis.

The Metabolic enzyme system has a sensitivity to high temperatures, particularly in their optimal range, then growth and development can take place only within the "window" heat [18]. The optimum temperature seems to be $19^0$C -$20^0$C, however *P. shqipericus* larvae have arrived more quickly to metamorphosis in this temperature. The different sizes of larvae at different temperatures for this phase can be explained, that the metabolism of growth factors is influenced by temperature [2].

To see the environmental impact on larval development period we put in the comparison process of metamorphosis for larvae amphibians groups kept in the same temperature, but in different environments (Table. 2). Completion time of metamorphosis is measured in days.

**Table 2.** Larval development time at the same temperature in different enviroments (external environment and internal environment).

| | Time of larval development |
|---|---|
| Group 1 (T = $17^0$C) (internalenvironment) | 35 days |
| Group 2 (T= $16^0$C – $17^0$C) (externalenvironment) | 39 days |

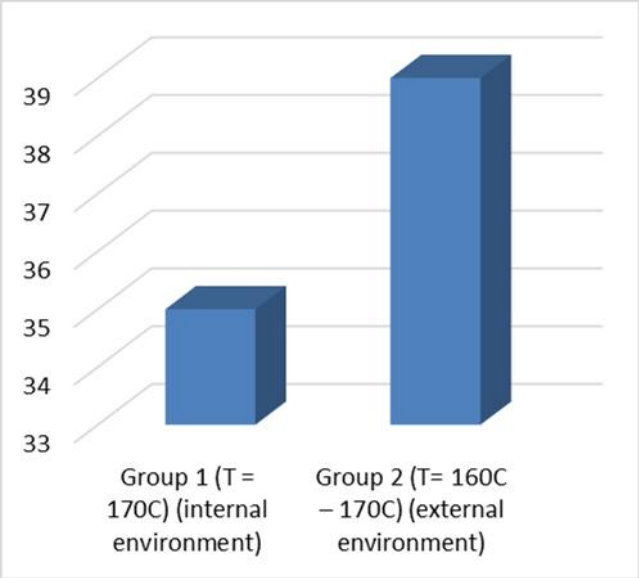


**Figure 2**. Larval development time at the same temperature in different enviroments (external environment and internal environment

From the graph (Figure 2) we can see that: the larvae puttedon the average temperature $17^0$C in the domestic environment, have concluded the proces of metamorphosis for about 35 days. The larvae putted on the temperature, of $16^0$C-$17^0$C in external environment, have concluded the proces of metamorphosis for about 39 days. So, time to complet the metamorphosis in this group last edseveral days more because wehave to consider that this group was put in terms of the external environment and during the day-night there was a severe temperature fluctuations and in this way the temperature played the role of a stressor to slow down the proces of metamorphosis.

The difference at the time of completion of metamorphosis between these two groups of study reveals also the birth of the hind limbs; front limb formation, as well as the reduction of tail.

The hind limbs that are formed more rapidly into the interior enviroment and the forelimbs were formed more quickly indoors.

## 4. Conclusions

The results showed a lengthening of development time with decreasing temperatures and a better survival rate of *P. shqipericus* with high temperatures. Values of temperature of $19^0$C-$20^0$C resulted to be optimal for larval development, while very low temperatures (t=$30^0$C) and very high ones (t=$25^0$C) strongly impede larval development. These results demonstrate that temperature is one of the most important factors that strongly affects the success of embryonic and larval development of *P. shqipericus* reproduced in-vitro.

## 6. References

1. Altwegg R and Reyer H U: **Patterns of natural selection on size at metamorphosis in water frogs**. Evolution 2003, 57: 872–882
2. Álvarez D and Nicieza G: **Effects of temperature and food quality on anuran larval growth and metamorphosis.** Functional Ecology 2002, 16: 640–648.
3. Atkinson D: **Temperature and organism size – a biological law for ectotherms?** Advances in Ecological Research 1994, 25: 1–58.
4. Atkinson D: **Ectotherm life–history responses to developmental temperature. Animals and Temperature.** Phenotypic and Evolutionary Adaptation 1996: 183– 204 (I. A. Johnston and A. F. Benett, Eds.). Cambridge Univ. Press
5. Brattstrom B H: **A preliminary review of thermal requirements of amphibians.** Ecology 1963, 44: 238–255.
6. Brockelman W Y: **An analysis of density effects and predation in Bufo americanus tadpoles.** Ecology 1969, 50: 632–644.
7. Calef G W: **Natural mortality of tadpoles in a population of Rana aurora.** Ecology 1973, 54: 741–758.
8. Etkin W: **Metamorphosis.** In: Physiology of the Amphibia: 427–468 (J. A. Moore, Ed.). New York Academic Press, New York, 1964.
9. Feder M E and Burggren W W: **Environmental physiology of the amphibians.** Chicago, London, The

Univ. of Chicago Press., Brockelman, 1992.

10. Gosner K L: **A simplified table for staging anuran embryos and larvae with notes on identification.** Herpetologica 1960, 16: 183–190.

11. Hayes T Chan and Licht P: **Interactions of temperature and steroid on larval growth, development, and metamorphosis in a toad (Bufo boreas).** Journal of Experimental Zoology 1993, 299: 206–215

12. Laugen A T, Laurila A and Merilä J: **Latitudinal and temperature dependent variation in embryonic development and growth in Rana temporaria.** Oecologia 2003a, 135: 548–554.

13. Laugen A T, Laurila A, Räsänen K and Merilä J: **Latitudinal countergradient variation in the common frog (Rana temporaria) development rates–evidence for local adaptation.** Journal of Evolutionary Biology 2003b, 16: 996–1005.

14. Loman J: **Temperature, genetic and hydroperiod effects on metamorphosis of brown frogs Rana arvalis and R. temporaria in the field.** Journal of Zoology 2002, 258: 115–129.

15. Newman R A: **Effects of density and predation on Scaphiopus couchii tadpoles in desert ponds.** Oecologia 1987, 71: 301–307.

16. Newman R A: **Developmental plasticity of Scaphiopus couchii tadpoles in an unpredictable environment.** Ecology 1989, 70: 1775–1787.

17. Reques R and Tejedo M: **Reaction norms for metamorphic traits in natterjack toads to larval density and pond duration.** Journal of Evolutionary Biology 1997, 10: 829–851.

18. Randall D, Burggren W and French K: **Eckert Animal Physiology: Mechanisms and Adaptations.** WH Freeman and Go, New York 1997.

19. Semlitsch R D, Scott D E and Pechmann J H K: **Time and size at metamorphosis related to adult fitness in Ambystoma talpoideum.** Ecology 1988, 69: 184–192.

20. Smith–Gill S J and Berven K A: **Predicting amphibian metamorphosis**. American Naturalist 1979, 113: 563–585.

21. Smith D C: **Factors controlling tadpole populations of the chorus frog (Pseudacris triseriata) on Isle Royale, Michigan.** Ecology 1983, 64: 501–510

22. Tejedo M and Reques R:. **Plasticity in metamorphic traits of natterjack tadpoles: the iteractive effects of density and pond duration**. Oikos 1994, 71: 295–304.

23. Wilbur H M and Collins J P: **Ecological aspects of amphibian metamorphosis.** Science 1973, 182: 1305–1314.