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Thermal tolerance limits and effects of temperature on the growth and development of the green toad, *Bufotes viridis*

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Abstract. There is growing concern that climate change will have significant impacts on environmental quality and natural resources including amphibians. Among some data available on the effects of temperature changes on anurans, most studies have concentrated on adult individuals, and information is lacking on the effects of changed temperatures on the developmental patterns of anurans, including *Bufotes viridis*. To identify the range of temperature tolerance in *B. viridis*, we studied the effect of five temperatures (8, 15, 18, 25, 30°C) on the growth and development of the eggs of this species. Egg masses of *B. viridis* were collected from its natural habitat and placed in a designed aquarium with heating and cooling systems supplying a range of temperatures between 0 and 40°C. Sizes were measured and developmental stages identified relative to age to the early feeding tadpole stage (Gosner stage 25) at increments of temperature. The numbers of abnormal embryos and larvae were recorded at every temperature and thermal tolerance was recorded. A direct correlation was found to exist between temperature and growth and developmental rates. The results also confirmed that the developmental rate decreases with decreasing temperature. Larvae at stage 25 showed a significant difference in length only between eggs incubated at 8 and 30°C, respectively. It is concluded that developing eggs have a temperature tolerance from 12 through 25°C, and temperatures outside this range will reduce their survival rate.

Key words. Anura, Amphibia, Bufonidae, cooling and heating system, embryo, growth rate, tadpoles.

Introduction

Over recent decades, climate change has become a major concern because of its impact on the biology, distribution, and abundance of microorganisms, animals, and plants (Darrow et al. 2004, Blaustein et al. 2010). Ectothermic animals are very sensitive to ambient temperatures because they cannot regulate their body temperature physiologically (Jarosik et al. 2004). Changing environmental temperatures therefore affect rates of cellular, biochemical, and physiological processes as well as growth rate and development (Zuo et al. 2012).

Over recent years, data on declining biodiversity has been documented, and in most areas, amphibians declined more than other taxa (Beebee & Griffiths 2005, Blaustein et al. 2010). Amphibians are biological indicators, and their decline is a warning sign of environmental perturbation (Beebee & Griffiths 2005, Burggren & Warburton 2007). They have permeable skin, eggs without shells, and a complex life cycle, for which reason they are vulnerable to the change of many biotic and abiotic factors (Wells 2007, Lopez-Alcaide & Macip-Riosi 2011, Li et al. 2013). Although there are several reasons for amphibian decline, the recent warming of Earth plays a significant role in their physiological interaction with the environment and has affected their phenology, distribution and survival (Carey & Alexander 2003, Corn 2005, Blaustein et al. 2010, Li et al. 2013). In response to global warming, some amphibians may breed earlier in the season (Blaustein et al. 2010). Change in breeding periods may lead to species having overlapping breeding periods, resulting in increased competition for food and breeding sites (Blaustein et al. 2010). Also, temporal shifts in and shortened hibernation periods could result in amphibians experiencing problems with finding food (Blaustein et al. 2010) as they become active at a time when prey is not yet abundant, thus contributing to their global decline. Rising temperatures have an impact on those amphibians in particular that live in threatened habitats such as temporary ponds and those that live in arid and semi-arid zones characterized by a general lack of water bodies. Temporary ponds are also exposed to destruction and are unstable habitats (Blaustein et al. 2010). At these sites, larvae have a limited period available for completing their metamorphosis before the pond will have evaporated and this will lead to their having to

go onto land at a reduced body size (Sanuy et al. 2008, Blaustein et al. 2010).

Among amphibians, most data about the impact of temperature on biological processes has been obtained from anurans. Their sizes render them easy to maintain in aquaria, they are easy to rear and well understood physiologically, and, most importantly, the ease of obtaining their temperature-sensitive developmental stages (eggs and embryos) make them suitable for laboratory studies (Flury 1972). The effect of temperature on the embryonic stages has been studied in different anuran species (e.g., *Rana pipiens* by Atlas 1935, *Rana aurora* by Licht 1974, wood frog by Darrow et al. 2004). However, there is less information available on Bufonidae (e.g., *Bufo valliceps* by Volpe 1957, *Bufo clamita* by Sanuy 2008) and a lack of information on *Bufotes viridis*. Bernal & Lynch (2013) also studied the impact of temperature on the embryos of six anurans species with different modes of reproduction, but none of them were Bufonidae.

The green toad, *Bufotes viridis* (formerly known as *Pseudepidalea* or *Bufo viridis*), is one of the most widespread Old World amphibian species. It belongs to the family Bufonidae, which is one of the most species-rich (with more than 350 species) and widely distributed amphibian families (Degani et al. 2013). Considering the importance of temperature on each stage of the amphibian life cycle, our main aim was to study the effects of different temperatures on the development and growth of *Bufotes viridis*, which lives in habitats sensitive to temperature changes (semi-arid zone and temporary ponds). Our secondary aim was to quantify its temperature tolerance limits for growth and survival and assess the effects of temperature on survival rates in this species. In Iran, this species is native to Fars Province, and we expected it to be exposed to highly variable temperatures in accordance with the nature of the environment described in materials and methods, which may affect the survival rate of developing eggs. Therefore, identifying the temperature thresholds for the survival of *B. viridis* eggs is important.

Material and methods

Study species and study site

Bufotes viridis is a terrestrial and nocturnal toad that depends on the availability of water during its reproductive season. This species spawns in ephemeral ponds and stagnant waters (Gurkan & Hayretdg 2012). *Bufotes viridis* is widely distributed from eastern France and Italy to Central Asia. It is also found in Northern Africa. In Iran, the green toad has been observed from below sea level to 4600 m above (Baloutch & Kami 2007).

The study area (Bajgah, 52°35' E, 29°43' N) is a semi-arid zone located 10 km from Shiraz city, Iran, within an agricultural field. The mean annual temperature is about 14°C (January = -11.5°C and July = 39°C), and the mean annual precipitation amounts to 420 mm (Bajgah meteorological station). The annual breeding season of *B. viridis* lies between late February and late April, i.e., from just before spring to early summer (Nokhbatolfoghahai 2009).

Egg collection, incubation at different temperatures, and fixation

In this study, the effects of temperature on size and developmental period were examined from the fertilized egg to Gosner (1960) stage 25 in *B. viridis*. Amplecting adult animals were collected at night, brought to the laboratory, and kept in an aquarium suitable for spawning. To establish a thermal gradient along which the spawn could be kept at various temperatures, a thermostat-controlled (with an error of 0.5°C) glass aquarium was designed, consisting of 18 individual chambers and two extra chambers that contained the heating and cooling elements (Fig. 1). The two extra chambers each had a large surface area of glass in contact with the first three individual chambers on both sides, with each containing 7.5 litres of water. These two sources of cooling and heating, respectively, had enough capacity to conduct heat and cooling through the glass across the rest of the compartments in which the eggs were left to develop. These sources created a linear gradient between them through thermal exchange. To create a stable temperature, both the heater and the chiller were fitted with a thermostat each, while the aquarium was covered with a lid to prevent the loss of heat. The system was pre-tested for 24 hours to ensure the temperature within each compartment remained stable. The total dimensions of the aquarium were 120 × 45 × 30 cm. Each individual chamber (15 × 15 × 20 cm) had its own air supply by means of an aeration stone that oxygenated and subtly mixed the water. At the beginning of the experiment, before heating and cooling, 150 fertilized eggs at stage 9 of development were transferred to each chamber (containing 1.5 l of dechlorinated water) and left to continue their growth. Then the chilling and heating systems were turned on. In order to prevent any temperature shock to the samples and adjust the samples to the new temperature, temperatures were adjusted gradually to the target values. Heating and chilling systems were set to create a range of temperatures between 0 and 40°C. Temperatures of the chambers were measured every 8 hours using a digital thermometer throughout the experiment. Tadpoles at feeding stage were fed with fresh lettuce (Amanat Behbahani et al. 2014). Depending on the developmental stage of the embryos, their developmental stages and the duration of development at each stage were observed and recorded every 10 to 15 hours,. These parameters were measured and analysed for five different temperatures (8, 15, 18, 25 and 30°C). In a separate experiment, eggs were also reared at 0 and 40°C in the chambers of the heating and chilling systems, respectively. At various stages of development for each specific temperature, 10 samples were taken for fixation to examine morphological and biometrical patterns. Fixation of samples was carried out for most Gosner (1960) stages, from approximately late cleavage (stage 10) to a larva at stage 25. At some specific stages,

2 or 3 samples were also fixed for scanning electron microscopy (SEM). Specimens for light microscopic examination were fixed in buffered neutral formalin or Bouin's fluid, and in 2.5% glutaraldehyde in phosphate buffer for SEM. The stage at which hatching occurred at each temperature treatment was also noted. Egg diameters and total body lengths (BL) of embryos and larvae at different stages were measured by an ocular graticule and correlations between size and temperature were studied.

Size and developmental stage relative to age

Body measurements (head–body length, total length and tail length) were taken of a series of embryos and larvae fixed in Bouin's solution and stored in 70% alcohol. Growth parameters including head–body length, total length, and developmental stage according to Gosner (1960) were measured at different times and stages. Sizes of embryos and larvae at a certain age were calculated from the average change in the length of larvae at different points of time. The developmental stages of embryos and larvae at certain ages were also calculated from the changes in developmental stages at different points of time.

Q_{10}-calculation for developmental rate

The Q_{10}-factor by which the growth and developmental rate increases when the temperature is raised by about ten degrees was measured at three temperature ranges (8–15, 15–25, and 18–30°C). Based on the equation below, R_1 is the measured developmental rate at temperature T_1, and R_2 is the measured developmental rate at temperature T_2.

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

Survival rate

After each part of the experiment, the percentage of larval abnormality was estimated, and the best range of temperature tolerance of larvae and embryos was determined from those that showed the highest survival rate (near 100%).

The survival percentage in each temperature chamber was estimated by the number of normal samples divided by the total number of samples multiplied by 100, using the following formula:

$$\text{Survival (\%)} = \frac{\text{Number of normal embryos and larvae} \times 100}{\text{Total number of embryos and larvae (normal + abnormal)}}$$

Scanning electron microscopy (SEM) preparation and examination

Glutaraldehyde-fixed specimens for scanning electron microscopy were post-fixed in 1% osmium tetroxide, stained in 0.5% aqueous uranyl acetate, dehydrated in an acetone series, then critical-point dried, and coated with gold with a Polaron SC 515. They were then examined with a JSEM 6400 scanning electron microscope. Morphological char-

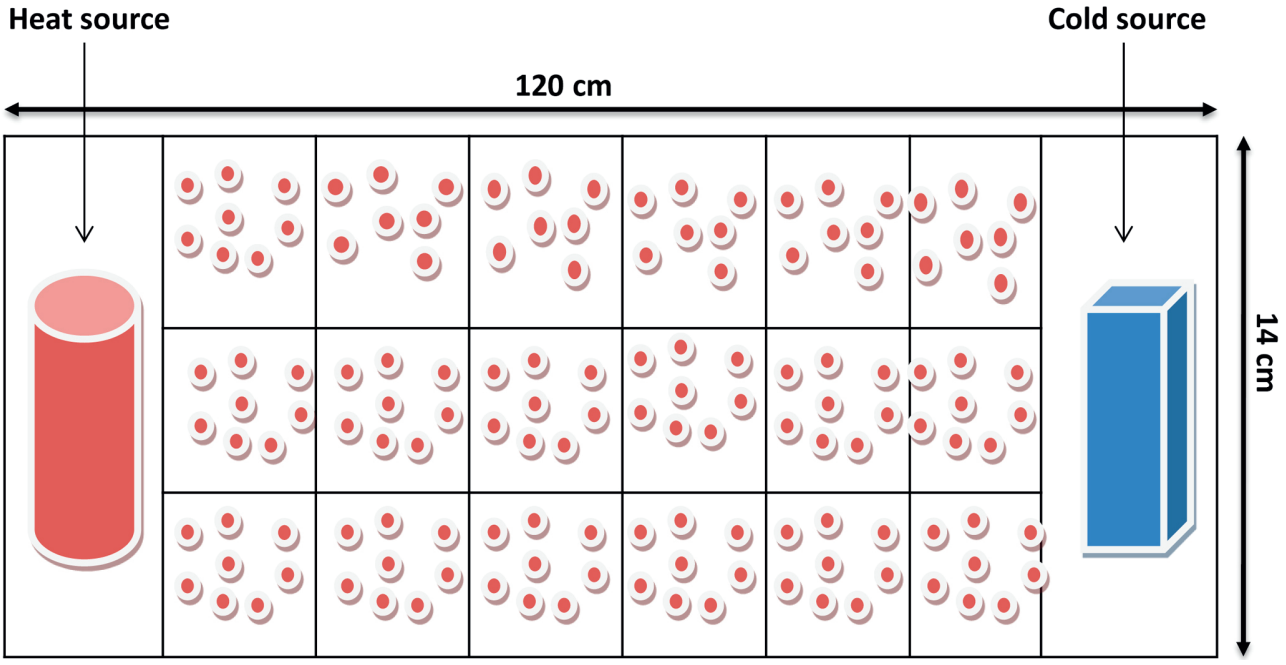

Figure 1. Design of a thermostat-controlled aquarium providing a heat gradient. Circles with red dots mark eggs.

acters of embryos and larvae including tail, cement gland and external gills were examined from different perspectives over a magnification range of 24–400× and recorded with Image-slave for Windows (Meeco Holdings, Australia).

Data analysis

The data obtained from the numbers and percentages of eggs, embryos and larvae were analysed with a One-Way ANOVA test, using SPSS version 11. Growth parameters such as embryonic and larval length, as well as developmental stage at a given point of time at a certain temperature were analysed through multiple comparisons and applying Tukey's procedure. All tests were conducted at a 5% level significance, and all data expressed as mean ± SD.

Results

Temperature, developmental stage and growth

Embryos developing at different temperatures (8, 15, 18, 25, and 30°C) showed a significant difference ($p < 0.001$) in the time required to reach stage 25. Developmental time decreased with increasing temperature (Fig. 2). At 8°C, eggs had developed to stage 25 after 280 hours, but after only 52 hours at 30°C. Table 1 shows the details of the Q_{10}-calculation for the rate of development between temperature ranges of 8–15, 15–25, and 18–30°C. The Q_{10} for these ranges are 0.29, 0.31, and 0.24, respectively. The size of the samples increased with increasing time in colder water and at each developmental stage. Based on Gosner's (1960) staging table, maximum development of the external gills was used as a landmark for stage 22, and the ratio of tail length to total length was used as a landmark for staging up to stage 22. We also used operculum appearance and external gill disappearance as landmarks for staging from stage 23 through 25, at which point of development the external gill will be completely covered by the operculum. We found significant differences in the total size at stage 22 between larvae that were reared at 8, 15, 18, and 25°C and those that were reared at 30°C (Tab. 2). Larvae kept at 30°C had the smallest length compared to the other experimental groups. Furthermore, at stage 25, significant differences in total size were only found between larvae reared at 8 and those raised at 30°C (Tab. 2).

Temperature tolerance limits and survival rate

The majority of abnormalities (including a distorted tail, lack of cement gland development, abnormal mouth formation, and abdominal swelling) were distinguished at each larval stage. Developmental arrest was also observed during the early stages of development (Fig. 3). According to the percentages of normal larvae per temperature chamber at the end of the experiment, the optimal temperature range facilitating proper development of this species' eggs and larvae was found to lie between 12 and 25°C (Fig. 4). At optimum temperatures, no abnormalities were observed, and the survival rate was approximately 100%, whereas optimum range survival rates decreased outside of this range. Eggs reared at about 0 and 40°C exhibited no development and no embryos survived.

Discussion

Amphibians are amongst the most vulnerable animals as has recently been confirmed by the observation that their population decline is proportionally greater than in all other vertebrates (Beebee & Griffiths 2005, Blaustein et al. 2010). Although many factors such as chemical pollution, pathogens, and habitat destruction impact on the diversity of amphibians (Wells 2007, Lopez-Alcaide & Macip-Rios 2011, Li et al. 2013), temperature could have substantial direct and indirect effects on these organism

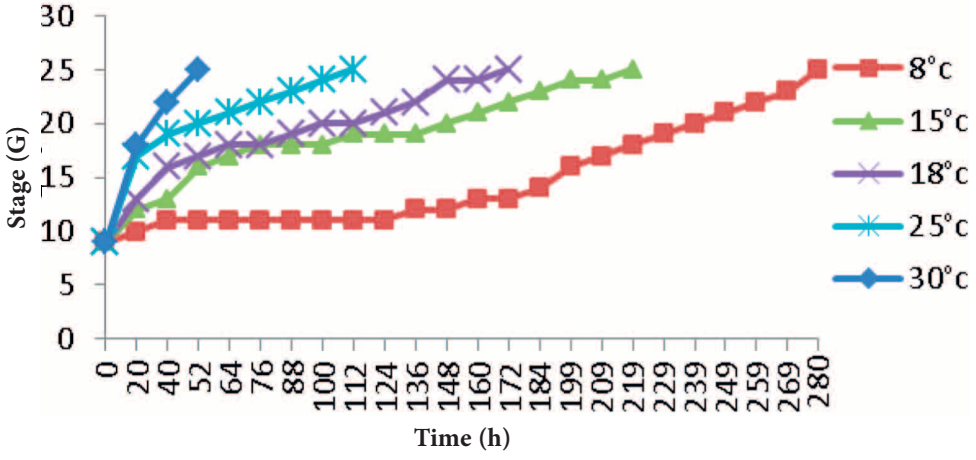

Figure 2. Graph comparing developmental stages relative to the age of eggs incubating at five different temperatures.

Table 1. Calculated Q_{10}-results for developmental rates of *B. viridis* eggs (R_1–R_2 at T_1–T_2 in hours/stage) in different ranges of temperature (T_1–T_2) from stage 10 through 25. R_1 is the measured developmental rate/growth rate at temperature T_1; R_2 is the measured developmental rate/growth rate at temperature T_2. The duration of development is given from fertilized egg to stage 25 (h), the rate of development in hours/stage.

Temperature	Duration	Rate of development	T_1–T_2 (°C)	R_1–R_2	Q_{10}
8	280	35.0	8–15	35–14.6	0.29
15	219	14.6	15–25	14.6–4.5	0.31
18	172	9.6	18–30	9.6–1.7	0.24
25	112	4.5			
30	52	1.7			

Table 2. Temperature effect on total size of *B. viridis* at stages 22 and 25. * – significant differences between larval size at T_1 and T_2.

p	Mean size at T_2 (mm±SD)	Mean size at T_1 (mm±SD)	Temperature T_2	Temperature T_1	Stage
0.999	5.46±0.65	5.47±0.73	15	8	22
0.250	5.39±0.47		18		
0.742	5.42±0.75		25		
0.001*	5.13±0.42		30		
0.366	5.39±0.47	5.46±0.65	18	15	22
0.860	5.42±0.75		25		
0.001*	5.13±0.42		30		
0.919	5.42±0.75	5.39±0.47	25	18	22
0.001*	5.13±0.42		30		
0.001*	5.13±0.42	5.42±0.75	30	25	22
1.000	7.42±0.89	7.42±0.92	15	8	25
0.357	7.35±0.81		18		
0.999	7.38±0.93		25		
0.006*	7.23±0.86		30		
0.913	7.35±0.81	7.42	18	15	25
1.000	7.38±0.93		25		
0.720	7.23±0.86		30		
0.984	7.38±0.93	7.35±0.81	25	18	25
0.150	7.23±0.86		30		
0.068	7.23±0.86	7.38±0.93	30	25	25

as well. Even more at risk may be anuran species that live in arid and semi-arid regions and depend on temporary ponds for spawning (Sanuy et al. 2008, Blaustein et al. 2010). A problem is that research interests in climate change, and particularly the effects of temperature, have focused more on certain groups of marine organisms. Even where studies of high temperature have concentrated on freshwater organisms such as amphibians, the focus has usually been on adults or late-stage amphibian larvae, and information on the effects of temperature on the egg, embryo, and early larva is limited. Evaluations of the influence of temperature on animals should take into account all life stages, however. This is important because aquatic animals in general and water-dependent anurans at their developmental stages in particular are generally more susceptible to environmental changes than adults (Zweifel 1968, Licht 1974). It is obvious that survival during the early developmental stages contributes greatly to the population size of a species, as well as to community structure and biodiversity.

In our experiment, *B. viridis* eggs from early cleavage stage to stage 25 were exposed to a range of temperature levels (0–40°C). We chose these temperature levels because this species lives in ephemeral ponds and is exposed to highly variable temperatures in this specific environment (semi-arid regions with temperatures between 5 and 31°C during their active phase). Our results show that the optimum temperature range in which the eggs grew and de-

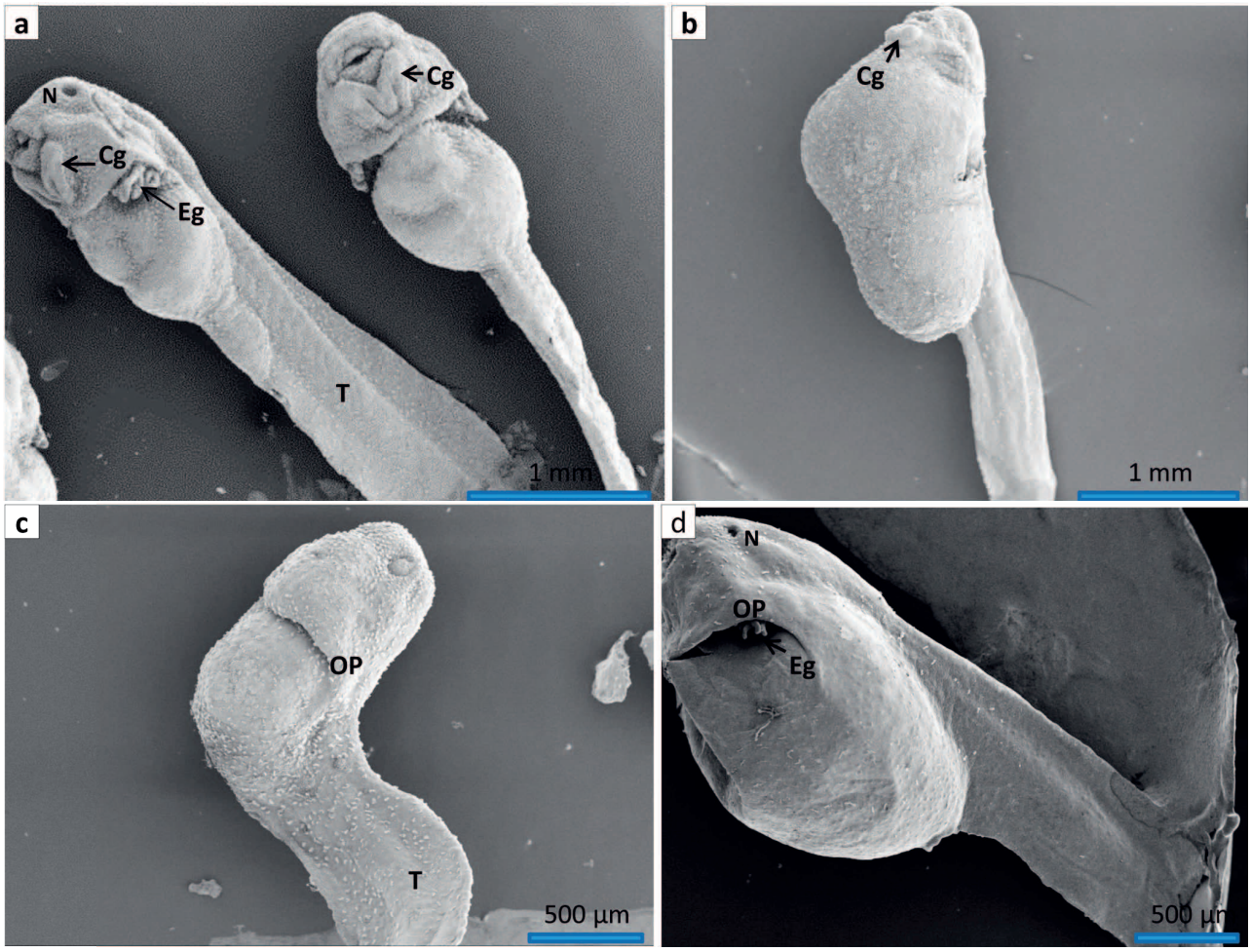

Figure 3. SEM photograph of *B. viridis* larvae at stage 22. A) Lateral and ventral view of normal larvae; B, C, and D) Larvae with abnormalities afflicting tail, cement gland, external gills, and abdomen. Cg – Cement gland; Eg – external gill; N – nostril; Op – operculum; T – tail.

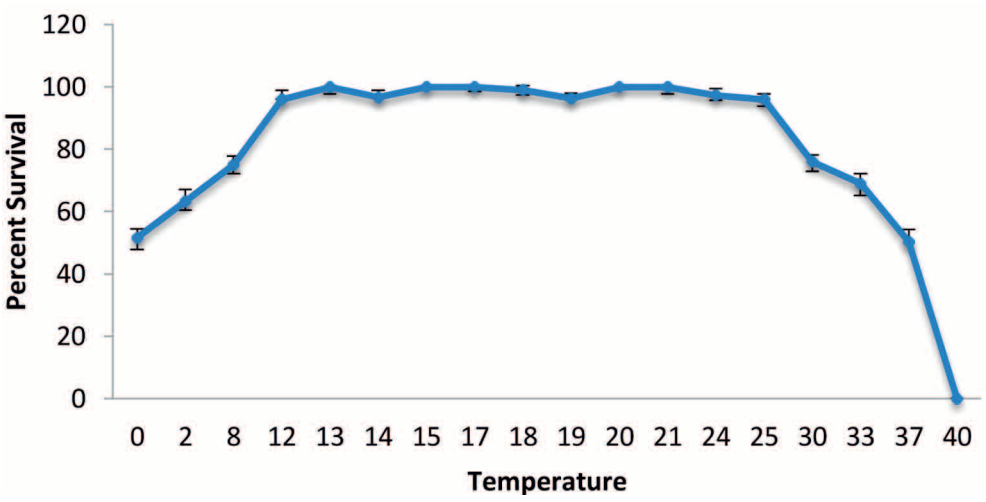

Figure 4. Survival in percent at different temperatures (range: 0–40°C) showing that the optimal temperature limits for development of *B. viridis* eggs and larvae lie between 12 and 25°C, within which the survival rate is nearly 100% and outside of which survival rate decreases.

veloped normally was between 12 and 25°C. Outside this range, survival was reduced and the frequency of abnormalities increased, and no eggs developed at 40°C. The laboratory conditions did not allow us to test for the effects of temperatures lower than 0°C. High temperatures reduce the ability of water to hold dissolved oxygen, therefore, if the absolute oxygen content is lower than the requirement of the animal living in it, then it will have a negative effect on the survival of eggs and larvae (Darrow et al. 2004, Blaustein et al. 2010). On the other hand, enzymatic processes are temperature-sensitive and have an optimum temperature range for functioning normally (Sanuy et al. 2008). Some abnormalities are related to an abnormal genotype of the egg and defects in fertilization (Johnson 1964) and are not related to temperature, however. Laboratory studies have shown that species have distinct temperature tolerances that are correlated with habitat conditions and environmental temperature (Bachmann 1969, Bernal & Lynch 2013) and are species-specific (Bachmann 1969). For example, Volpe (1957) demonstrated that the optimum temperature range for development in *Bufo valliceps* is 20–33°C, which differs from our results for *Bufotes viridis*.

Temperature affects all physiological processes in amphibians, for which reason growth and developmental rates are also depend on temperature (Alvarez & Nicieza 2002, Zuo et al. 2012). In our study and within the range of temperatures we tested, developmental periods were inversely correlated to temperature, with increased temperatures reducing developmental periods. This is important for a species that reproduces in ephemeral ponds and is exposed to highly variable temperatures, because eggs and larvae that are to develop late in the breeding season when temperatures are high in this manner decrease their developmental period and so increase their chances of survival. Lillie & Knowlton (1897, as cited in Bachmann 1969) showed a negative correlation between temperature and developmental period in *Ambystoma tigrinum*. Duellman & Trueb (1994) pointed out that the developmental rate was dependent on temperature. Sanuy et al. (2008) studied effects of temperature on embryonic and larval forms of *Rana clamitans* and found that a decrease in temperature caused an increase in developmental period.

Our study showed a significant reduction in total size of larvae at stage 22 in water of 30°C compared to other temperatures, however, the decrease in size between other temperature groups was not significant. At stage 25, larvae kept at 30°C were significantly smaller than larvae kept in water of 8°C. Alvarez & Nicieza (2002) and Wells (2007) indicated that growth and developmental period are temperature-dependent, but development is more sensitive to temperature change than growth. Therefore, larvae that were raised at low temperatures stopped developing although they continued to grow. The authors thus concluded that larvae developing at low temperatures are larger than those that were reared at high temperatures (Alvarez & Nicieza 2002, Wells 2007). This rule is important for the larvae of ectothermic animals, as developing in warm temperatures will lead to smaller metamorphs (Jarosik et al. 2004, Cvetkovis et al. 2008, Kingsolver & Huey 2008, Zuo et al. 2012). The next study should continue our experiments to adult stage for testing this rule for *B. viridis*. Different sizes at 8C and 30°C were related to thermal tolerance limits. Another suggestion could be that at 30°C, larvae might have a higher metabolic rate, leaving less energy available for growth (especially since they all had about the same yolk size). Since the population of a species has a distinct optimum thermal tolerance and larvae in this range will have similar morphological traits (Bachmann 1969), *B. viridis* larvae living within their optimum thermal tolerance limits (between 12 and 25°C) did not exhibit any significant differences in total size. Normal development up to stage 25 in this range of temperatures does not guarantee tadpoles to reach metamorphosis. In addition, air temperatures of about 40°C in summer (the time when tadpoles approach metamorphosis) are considerably higher than the maximum temperature that facilitated normal development in our experiment. Therefore, further study is needed to identify the minimum and maximum temperatures that tadpoles need to successfully metamorphose. It would also be interesting to study this species' adaptive plasticity regarding the drying up of their spawning ponds.

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