Environmental constraints affect underground reproduction of the common toad (*Bufo bufo*)

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**Abstract.** The common toad (*Bufo bufo*) is a widespread species in Europe, with accidental occurrence in caves. This study reports the first case of breeding of *B. bufo* in a natural cave in the Carpathian Mountains. The breeding activity was observed at the end of May and the beginning of June. We identified pairs of common toads in axillary amplexus, egg strings and tadpoles up to a distance of 97 m inside the cave. We hypothesized that there is an active selection of some cave sites as breeding habitat and that the egg deposition is not randomly distributed inside the cave. In 25 sample points, we recorded ten cave environmental features and we assessed the relationship between egg strings presence and environmental features by performing a bias reduction in binomial-response generalized linear model. The results revealed that the strongest ecological factor determining the selection of breeding areas inside the cave was water flow velocity. The presence of light, although an important environmental feature, was not the most determining factor in breeding site selection, as toads successfully entered in completely dark environments to lay eggs in hydrologically favourable places. This study provides new information about the cave breeding of *Bufo bufo* and highlights the environmental features determining the common toad’s breeding site selection.

**Keywords:** amphibians, breeding, environmental features, selection, subterranean habitat, tadpoles.

**Introduction**

A large variety of organisms can be found in subterranean environments. They can be temporary visitors or permanent residents and have a different distribution within the cave environmental zones (Culver and Pipan, 2019). These zones, delimited mainly by the amount of penetrating light, are the entrance, twilight and dark zones (the dark zone is subdivided into transition, deep and stagnant air zones). The entrance zone is exposed to sufficient light for vascular plant life. The twilight zone is characterized by reduced light and extends from the entrance zone to the dark zone, where light is completely absent (Howarth and Wynne, 2022). Subterranean organisms must cope with the particular conditions of the underground environment (Culver and Pipan, 2019). The most adapted species inhabit the deepest parts of the cave (Lunghi and Manenti, 2020), which can be considered the most extreme (Lee et al., 2012), and relatively few animals are capable to reside in this zone (Howarth and Wynne, 2022). A larger number of species but less adapted to these environments are found in the entrance zone, with more food resources and more tolerable conditions (Lee et al., 2012).

The most used to date classification of subterranean organisms is Schiner’s classification (1854), later revised by Racovitza (1907).
The Schiner-Racovitza classification, based on the organism’s degree of adaptation to the subterranean environment, classifies organisms that occur in caves in troglobionts, troglophiles, and trogloxenes and was the starting point for subsequent classifications and redefinitions (Howarth and Moldovan, 2018). Troglobionts live only in the subterranean environment and are often characterized by profound morphological changes; they usually inhabit the deepest areas of caves. Troglophiles are able to live and breed in the subterranean environment, generally preferring areas near the cave entrance, but they also can live outside. They were subdivided into eutroglophiles for basically epigean species capable to maintain permanent hypogean populations, and subtroglophiles for species tending to inhabit a subterranean habitat but connected to epigean habitats for different biological functions (Sket, 2008). The category of troglobionts (sometimes troglobites) (Culver and Pipan, 2019) remained mostly unaltered, whereas the category of trogloxenes was much disputed, the most significant advancement being the trogloxene concept with exclusion of accidentals, namely animals without ecological connection with caves (Trajano and de Carvalho, 2017). The traditional classification does not take into account the complexity of ecological processes which are taking place, and in this context, the notions of trogloxene and accidental may not exactly represent the ecological processes that would cause the appearance of these species in caves. An ecological analysis of the relationships between non-strict cave dwelling species and habitat variables can allow to go beyond the typological classification and may lead to a more ecological classification of these organisms (Lunghi et al., 2014).

Apart from the obligate cavernicole species belonging to the Proteidae and Plethodontidae families (Romero, 2009), the occurrence of amphibians in caves and other underground habitats was often documented, especially for salamanders (Sequeira et al., 2001; Manenti et al., 2009; Ianc et al., 2012) but also for some anurans (Rosa et Penado, 2013; Koller, 2017; Russo et al., 2018). Although their presence in the subterranean environment can be accidental (Niemiller and Miller, 2009), there are studies indicating the preferences of some amphibian species for specific environmental features and active selection of the cave (Lunghi et al., 2014; Lunghi et al., 2018). Several studies document reproductive activity of some amphibian species in caves, such as Salamandra salamandra (Manenti et al., 2011; Manenti and Ficetola, 2013), Eleutherodactylus cundalli (Diesel et al., 1995), Discoglossus pictus (Faraone and Io Valvo, 2018).

The common toad (Bufo bufo) is considered to be trogloxene, with accidental occurrence in caves (Lunghi et al., 2014). It is a widespread species in the western Palearctic region (Cossu et al., 2017), inhabiting forests, shrubs, meadows, gardens, or arid areas. Most frequently it is found in forested habitats and it uses as breeding sites stagnant or slowly-flowing waters (Lanza et al., 2007). It lives a solitary life for most of the year, but it converges on its native pond during the breeding season (Sinsch, 1988). Bufo bufo is an explosive breeder with a short breeding period in early spring (Kyek et al., 2017); most individuals mate within a 1-2 week period (Hettyey et al., 2009). The choice of a breeding habitat can be influenced by environmental conditions as well as by the existence of alternative breeding habitats (Laurila, 1998).

Although its presence in caves has been reported as more or less occasional in several countries (Bologna, 1982; Uhrin and Lesinsky, 1997; Koller, 2017; Lunghi et al., 2017) the only records of common toad breeding in an underground habitat, to our knowledge, were reported in Italy, from artificial caves (Bonini et al., 1998; Angelini and Cari, 2004), but little more than the observation of reproductive activity is reported. Bonini et al. (1998) however reported that toads spawned in dark sections of caves.
the artificial tunnel and that the tadpoles metamorphosed 2 months later than those of surrounding populations in open habitats.

In this paper, we document the first report of the reproduction of the common toad in a natural cave for both the Continental Biogeographical Region of Europe and the Carpathian Mountains. There are scarce investigations of the factors promoting amphibian reproduction in caves (Rogowitz et al., 2001; Manenti et al., 2011) and, as far as we know, none is focused on common toad. Apart from reporting the first case of common toad breeding in a natural cave and providing new information about it, in this study, we test two hypotheses: (1) The common toad selection of the cave as breeding habitat is not random but it is related to inappropriate epigean aquatic habitats. (2) The egg deposition is not randomly distributed along the cave stream but it is related to environmental characteristics of the underground habitat. Furthermore, we made observations on tadpole behavior in the underground environment.

Materials and methods

Study area

The underground breeding of the common toad was observed in Peștera cu Apă Cave from Gârliștei Gorges (45°9.89′N and 21°51.21′E), in the southwest of the Romanian Carpathians. The cave is located in the Anina Mountains (fig. 1A), which are low mountains formed almost entirely of limestones. The main landforms are the elevated karst plateaus, devoid of surface water and dissected by deep gorges (Onac and Goran, 2019). The climate is characteristic of low mountains in the temperate zone. The slopes are covered with dense forest consisting predominantly of Fagus sylvatica with a rich shrub layer. Peștera cu Apă Cave is located on the right slope of the Gârliștei Gorges, in the steepest and narrowest sector (supplementary fig. S1A), at 320 m a.s.l. and 22 m relative altitude to the river. The cave has a single entrance of large dimensions: 8.2 m height and 5.6 m maximum width (supplementary fig. S1B). It is a linear, joint-controlled cave, formed by the water that infiltrates the limestones, with a length of 910 m. The cave is slightly ascending and is crossed by an outflow stream that can dry out during the dry season (Orghidan et al., 1984).

Survey methods

Five field surveys (FS) were performed from May 2021 to September 2021 which will be numbered chronologically. On May 29 (FS1), we noticed common toad breeding activity within the Peștera cu Apă Cave. Other field surveys were made on June 4 (FS2), June 12 (FS3), July 22 (FS4), and September 1 (FS5). The breeding activity was observed in distinct areas inside the cave. These areas, with different lengths, will be called breeding areas (BA) and will be numbered in ascending order from the entrance to the inside of the cave (fig. 1B). The cave was explored over a distance of 200 m, the deepest explorable part without speleological equipment. At 110 m from the cave entrance, a steep slope would have made the further advance of toads impossible, so the study was conducted within the first 110 m of the cave.

Within the selected area, we recorded the following environmental features: distance from the cave entrance,
illuminance, maximum height and width of the cave gallery, declivity of the cave floor, air temperature, air humidity, water temperature, water surface flow velocity, and water depth.

For measuring the distances of BAs, width and height of the gallery, we used a tape meter and a SNDWAY T40 laser meter (accuracy of ±2 nm). A TROTEC T210 thermometer (accuracy of 0.1°C and 0.1%) was used to measure air temperature and humidity at ground level. Care was taken to ensure that the human presence did not affect the temperature and air humidity measurements (Ferreira et al., 2015; Lunghi et al., 2020). The water temperature was recorded using a Hama T-350 digital thermometer with an external sensor (accuracy of 0.1°C), in the place where the water was deepest, at the bottom of the stream. Since the cave stream was shallow (mean water depth 5.3 cm), we considered that the measured temperature is characteristic of the entire water volume in the measurement points.

For each recording location, the water depth was measured every 10 cm along a transect across the stream. Further, the mean, minimum and maximum water depths were determined. The water surface flow velocity was determined by the float method (Dobriyal et al., 2017) because the water depth was too shallow to allow the use of the flowmeter. For each point, the velocity was calculated by measuring the time required for a float to traverse a 2 or 3 m long upstream sector, a length that represents 4 times the width of the stream in that area (Herschy, 2019). The procedure was repeated three times at each measuring point and the average water surface velocity was calculated (Herschy, 2019).

Illuminance measurements were made by using a portable lux meter Hanna HI97500. The minimum recordable light by the instrument was 1 lux, so it was not possible to measure the very low illuminance which was detected by the human eye between 40 and 50 m distance from the cave entrance. Thus, in the statistical analysis, light was expressed as a yes/no variable according to the presence/absence of light detected by the human eye, even if in this way we may lose part of the information. The slope of the cave floor was measured for 1 m long segments using the Clinometer application for Android (0.1° accuracy). The digital map of the cave was made, by scanning, georeferencing, and digitizing the analog cave map (Orghidan et al., 1984) and updated based on data collected by us on site.

The environmental features were recorded every 5 m, starting from the cave entrance. We selected a 5 m distance between recording points because it is long enough to apprehend the variation of the microclimatic variables (Ferreira et al., 2015) and short enough to capture relevant modifications in the morphology of the cave and hydrological parameters of the cave stream. Within the 5 m sector, we collected additional data if a breeding site fell into it. It was the case of BA2, at 56 m distance from the cave entrance, and BA3, at 97 m. Finally, environmental features were recorded in 25 points. The microclimatic parameters were recorded in every FS. The hydrological parameters were measured until the stream was dried out. The morphological features of the cave gallery were recorded once, on June 4 (FS2), when egg strings were observed in all breeding areas. The presence and abundance of adults and the presence of egg strings and larvae were recorded for each measurement point.

Outside the cave, we recorded the air temperature and the terrain slope. The air temperature was measured in the shadow, at 5 m distance from the cave entrance, on each field survey. The terrain declivity outside the cave was measured along the 30 m long access slope to the cave following the same procedure as inside the cave. Because the common toad can travel in mountainous regions up to 1 km during the breeding migration (Sztatecsny and Schabetsberger, 2005), we considered that an area with a radius of 1 km around the cave entrance would encompass the potential migration distance of the toads. So, we calculated the declivity for this area in ArcGIS Pro 2.3.0 software using a Digital Elevation Model (DEM) with 10 m spatial resolution. For the Gârlîşte River, the water temperature was recorded and the water-flow velocity was determined using a Geopacks Flowmeter and the 0.6 depth method (Herschy, 2019) in the sector in front of the cave.

Statistical analyses

Descriptive statistics of the measured environmental data were calculated and the variables were checked for normal distribution using Shapiro-Wilk test (Ghasemi and Zahediasl, 2012). The variable slope was transformed before analyses using logarithms to better fit the normal distribution. Although some outliers exist in slope values and water-flow velocity values, we didn’t remove them because our data set is small and because they represent a genuine variation of the measured variable (Zuur et al., 2010).

Due to our small sample size, we performed the Bias Reduction in binomial-response Generalized Linear Models (brglmFit) using the ‘brglm2’ R-package which reduces the bias resulting in cases of complete separation in the data or unbalanced data (Kosmidis et al., 2020). The presence of egg strings was considered the dependent variable while the other environmental features (distance from the cave entrance, presence of light, maximum height and width of the cave gallery, declivity of the cave floor, air temperature, air humidity, water temperature, water surface flow velocity, and water depth) were considered independent variables. A limitation of this model is that it doesn’t accommodate random effects (Bittner et al., 2022; Boyd et al., 2022).

Before performing the multivariate analysis, we tested the multicollinearity of the independent variables using correlation analysis to identify the least-correlated sets of variables, setting the threshold for collinearity at $r > 0.7$ (Ficetola and de Bernardi, 2004). First, we built an initial model with all the selected variables after the data exploration. Next, the best reliable model was selected by applying an automated model selection procedure using the ‘MuMin’ R-package (Barton, 2017). Thus, the initial model was simplified automatically by removing non-significant variables, based on Akaike information criterion corrected for small samples (AICc). For each model, the difference between AICc of a candidate model and the AICc of the best model ($\Delta$-AICc), and the probability for a model to be the best one among candidates ($AIC \text{ weight} = w_j$) were calculated. Models were ranked using AICc and the model with the
Environmental constraints and cave reproduction of *Bufo bufo*

Table 1. Adult individuals, egg strings and larvae observed during the field surveys (FS1 – May 29, FS2 – June 4, FS3 – July 22, FS4 – September 1).

<table>
<thead>
<tr>
<th>Breeding area (BA)</th>
<th>Field survey (FS)</th>
<th>Pairs in amplexus</th>
<th>Isolated adult individuals</th>
<th>Egg strings</th>
<th>Larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA 1</td>
<td>FS1</td>
<td>1</td>
<td>2</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>FS2</td>
<td>1</td>
<td>1</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS3</td>
<td>A</td>
<td>1</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS4</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
</tr>
<tr>
<td></td>
<td>FS5</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
</tr>
<tr>
<td>BA 2</td>
<td>FS1</td>
<td>2</td>
<td>3</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS2</td>
<td>A</td>
<td>2</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS3</td>
<td>A</td>
<td>1</td>
<td>A</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS4</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS5</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>BA 3</td>
<td>FS1</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>FS2</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>FS3</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>FS4</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
</tr>
<tr>
<td></td>
<td>FS5</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
<td>Ad</td>
</tr>
</tbody>
</table>

P = present; A = absent; Ad = absent, but the cave stream was dried.

lowest AICc and highest weight was considered the best model supported by the given data set (Hurvich and Tsai, 1989; Johnson and Omland, 2004). To assess the amount of variation explained by each model we calculated Tjur’s $R^2$ coefficient of determination for binary outcomes (Tjur, 2009). In the results are presented only the models with Δ-AICc smaller than 4 (Burnham and Anderson, 2001).

Although the GLM is mainly used as a predictive modelling tool, we used it for explanatory modelling (Shmueli, 2010), namely to test our hypotheses regarding the association between the common toad breeding site selection inside the cave and the environmental features. All statistical analyses were conducted in R, through R studio (R Version 4.2.0; RStudio Team Version 2022.2.2.485).

Results

The breeding activity of toads was observed inside the cave in three breeding areas. The first breeding area (BA1) was located between 15 and 26 m from the cave entrance, at the beginning of the twilight zone. The second breeding area (BA2) stretched between 45 and 57 m from the entrance, in the transition zone. The third breeding area (BA3) was located deeper, between 96 and 97 m from the entrance, at the beginning of the deep zone.

In FS1 the breeding activity was observed only in BA1 and BA2. In FS2, a new breeding area (BA3) was identified much deeper inside the cave (fig. 1B). We noticed breeding pairs only during the first two FS. On FS1 the pairs were present both in BA1 and in BA2, whereas on FS2 they were present only in BA1. During FS3 no pairs were present anymore, only isolated adults, and on FS4 and FS5 no adults were present in the cave. The overall total number of individuals observed in the cave was 18: eleven adults were observed on FS1, with a male biased sex ratio of 2.66; five adults on FS2, and two adults on FS3 (table 1).

During FS1 egg strings were present in BA1 and in BA2 (table 1) but in BA3 egg strings were first observed during FS2. So the last place where egg deposition took place was BA3, which is located much deeper inside the cave. Laying eggs at a distance of 97 m inside the cave represents a breeding record for the common toad in the underground environment. The egg strings were noticed in nine measurement points (36% of all 25 sample points). The water depth in the sites where eggs were present ranged between 5 and 17.35 cm and the water flow velocity was very low, with a maximum value of only 0.15 m/s. The descriptive statistics of the environmental features on the sites where eggs were present and on the sites without eggs are presented in table 2. The light was detected in 77.77% of the points where egg strings were...
Table 2. Comparison of the environmental features between the sites where eggs were present and sites without eggs. For each variable are reported the mean value, the standard deviation (SD) and the range.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Eggs present</th>
<th>Eggs absent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(range)</td>
</tr>
<tr>
<td>Water depth (cm)</td>
<td>8.22 ± 3.76</td>
<td>4.32 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>(17.35-5.00)</td>
<td>(7.50-2.92)</td>
</tr>
<tr>
<td>Water velocity (m/s)</td>
<td>0.06 ± 0.04</td>
<td>0.30 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>(0.15-0.01)</td>
<td>(0.72-0.07)</td>
</tr>
<tr>
<td>Water temperature (°C)</td>
<td>11.49 ± 0.21</td>
<td>11.24 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>(11.8-11.10)</td>
<td>(11.90-10.80)</td>
</tr>
<tr>
<td>Air temperature (°C)</td>
<td>13.60 ± 0.75</td>
<td>13.20 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>(14.60-12.40)</td>
<td>(14.80-12.30)</td>
</tr>
<tr>
<td>Air humidity (%)</td>
<td>65.40 ± 4.20</td>
<td>68.70 ± 5.10</td>
</tr>
<tr>
<td></td>
<td>(72.6-60.2)</td>
<td>(75.20-58.70)</td>
</tr>
<tr>
<td>Floor declivity (°)</td>
<td>1.17 ± 0.43</td>
<td>6.40 ± 7.16</td>
</tr>
<tr>
<td></td>
<td>(2.00-0.50)</td>
<td>(22.00-1.00)</td>
</tr>
<tr>
<td>Gallery max. height (m)</td>
<td>7.21 ± 1.79</td>
<td>5.91 ± 2.53</td>
</tr>
<tr>
<td></td>
<td>(9.59-3.57)</td>
<td>(9.59-2.60)</td>
</tr>
<tr>
<td>Gallery max. width (m)</td>
<td>4.43 ± 1.52</td>
<td>3.44 ± 1.56</td>
</tr>
<tr>
<td></td>
<td>(6.70-1.75)</td>
<td>(6.70-1.70)</td>
</tr>
<tr>
<td>Distance from the entrance (m)</td>
<td>38.40 ± 26.70</td>
<td>63.20 ± 34.30</td>
</tr>
<tr>
<td></td>
<td>(97.00-10.00)</td>
<td>(110-0)</td>
</tr>
</tbody>
</table>

present. Along BA1, the illuminance decreased from 30 to 9 lux. In BA2, the light was detected only in the first part, between 45 and 50 m from the cave entrance, where the illuminance was below 1 lux. Beyond 50 m from the cave entrance, the light could not be detected anymore, so in BA3 it was dark. In the measurement points where no eggs were present, the light was detected in 38.46% of cases.

Eggs were predominantly arranged on one row per string, but also on two rows per string. In the sectors with shallower water and higher flow velocities, the egg strings were more expanded in the direction of the flow, extending over distances of more than 4 m, while in BA3, characterized by deeper water (mean depth 17.3 cm) and very low flowing speed (0.01 m/s), the egg strings were disposed rather in large loops, over a distance of only 1 m.

During FS1 there were no tadpoles yet in BA1, only in BA2, so the breeding activity started in BA2. In BA1 tadpoles were first observed during FS2. In BA1 and BA2, apart from the larvae of *B. bufo*, larvae of fire salamander (*Salamandra salamandra*) were found. On FS4, the water in BA1 and BA3 was dried out and in BA2 were present only tadpoles. On FS5, the cave stream was completely dried, which indicates that the tadpoles died.

As we expected, most of the environmental features recorded inside the cave were correlated. There was a strong negative correlation between the distance from the cave entrance and air temperature ($r = -0.9681$), water temperature ($r = -0.9692$), maximum gallery height ($r = -0.8924$), and a strong positive correlation with the air humidity ($r = 0.9865$). The gallery width was moderately correlated with the distance from the cave entrance ($r = 0.5201$). Water flow velocity showed a strong positive correlation with the declivity of the cave floor ($r = 0.8717$).

The initial model included the variables water flow velocity, water depth, and light presence. The best AICc model resulting after performing the model selection process included the water flow velocity and the water depth, but only the
Table 3. Best AICc models relating the presence of egg strings (dependent variable) to environmental features. For each model, the degree of freedom (Df), AICc, Δ-AICc, \( w_i \) and Tjur’s R² are reported. For each variable, the estimate, standard error (SE) and significance (\( P \)) are reported. Significant \( P \)-values are marked in bold.

<table>
<thead>
<tr>
<th>Model structure</th>
<th>Estimate</th>
<th>SE</th>
<th>( P )</th>
<th>Df</th>
<th>AICc</th>
<th>Δ-AICc</th>
<th>( w_i )</th>
<th>Tjur’s R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water velocity</td>
<td>-19.51</td>
<td>9.41</td>
<td>&lt;0.05</td>
<td>3</td>
<td>13.5</td>
<td>0.00</td>
<td>0.42</td>
<td>0.787</td>
</tr>
<tr>
<td>Water depth</td>
<td>0.82</td>
<td>0.56</td>
<td>&lt;0.142</td>
<td>2</td>
<td>14.2</td>
<td>0.73</td>
<td>0.29</td>
<td>0.709</td>
</tr>
<tr>
<td>Water velocity</td>
<td>-26.69</td>
<td>10.20</td>
<td>&lt;0.01</td>
<td>2</td>
<td>14.2</td>
<td>0.73</td>
<td>0.29</td>
<td>0.709</td>
</tr>
<tr>
<td>Water depth</td>
<td>1.61</td>
<td>0.79</td>
<td>&lt;0.05</td>
<td>3</td>
<td>16.1</td>
<td>2.57</td>
<td>0.12</td>
<td>0.716</td>
</tr>
<tr>
<td>Light presence</td>
<td>4.00</td>
<td>2.14</td>
<td>0.062</td>
<td>3</td>
<td>16.1</td>
<td>2.57</td>
<td>0.12</td>
<td>0.716</td>
</tr>
<tr>
<td>Water velocity</td>
<td>-12.05</td>
<td>9.20</td>
<td>0.191</td>
<td>4</td>
<td>16.4</td>
<td>2.95</td>
<td>0.10</td>
<td>0.774</td>
</tr>
<tr>
<td>Water depth</td>
<td>0.85</td>
<td>0.61</td>
<td>0.158</td>
<td>4</td>
<td>16.4</td>
<td>2.95</td>
<td>0.10</td>
<td>0.774</td>
</tr>
<tr>
<td>Light presence</td>
<td>1.53</td>
<td>1.90</td>
<td>0.422</td>
<td>4</td>
<td>16.4</td>
<td>2.95</td>
<td>0.10</td>
<td>0.774</td>
</tr>
<tr>
<td>Water velocity</td>
<td>-22.70</td>
<td>9.51</td>
<td>&lt;0.05</td>
<td>3</td>
<td>17.0</td>
<td>3.46</td>
<td>0.08</td>
<td>0.699</td>
</tr>
<tr>
<td>Light presence</td>
<td>0.81</td>
<td>1.45</td>
<td>0.580</td>
<td>3</td>
<td>17.0</td>
<td>3.46</td>
<td>0.08</td>
<td>0.699</td>
</tr>
</tbody>
</table>

Water flow velocity showed a significant interaction in the model. The probability of egg presence decreased with the increase of water flow velocity (table 3). The variance explained by this model reported as Tjur’s R² was 0.787. The egg presence was also associated with water depth, but the model containing this variable has much lower support, given the data (\( w_i = 0.12 \)), than the best AICc model (table 3).

Discussions

The breeding activity of common toad was observed in Peștera cu Apă Cave at the end of May and the beginning of June. The common toad breeding season in Romania occurs generally in late March and early April (Botnariuc, 1960) but there is a variation in the egg laying period in geographical regions at different latitudes and altitudes (Hemelaar, 1988). Populations appear to be adapted to their climates and the reproduction shifts according to the local climatic conditions (Sparks et al., 2007).

In the study area, the only surface waters are Gârliște River and a few small karst springs. This is characteristic in karstic areas, where the surface waters are scarce and the water from precipitation or melting snow easily infiltrates underground. The Gârliște River has a high value of the riverbed slope (28.9 m/km for the entire gorge sector), so the water flow velocity is high. Moreover, the hydric regime of the river is characterized by early high water flows in March and April, followed by floods during May and June (Badea et al., 1983). Thus, the egg laying period of common toad coincides with the period with the highest water-flow velocity and the highest flow of the Gârliște River. In the sector corresponding to the cave entrance, water-flow velocity ranges between 1.85 m/s and 0.24 m/s, with a mean value of 1.03 m/s. So, the Gârliște River is a high-velocity running water, which is not appropriate for common toad breeding. It is known that the common toad usually uses stagnant or low flow velocity waters (Buckley, 2001; Lanza et al., 2007; Baker et al., 2011).

Although there are more than 50 caves in Gârliștei Gorges, Peștera cu Apă Cave is the only one crossed by an emerging stream with sectors of low running water, almost stagnant, in the first 100 m, suitable for common toad breeding. The best model showed that the strongest ecological factor determining the selection of the subterranean breeding areas was the water flow velocity (table 3). The toads preferred to lay eggs in places where the water flow velocity was low and where the water depth was high (table 2). This fact supports also our assertion that Gârliște River has too high water flow velocity to serve as breeding habitat for the common toad.

Our result suggests that light, although an important environmental feature for surface...
organisms was not the most determining factor in the breeding site selection. As we expected, in most cases, the eggs were laid in places where the presence of light was recorded, but there are still 22.23% of cases where eggs were laid in dark locations. In 38.46% of illuminated sample points, there were no egg strings. Usually, in the illuminated places where no eggs were laid, either the water speed was very high, or the water depth was very low (or both simultaneously). Moreover, the toads managed to move in the dark to lay eggs in hydrologically favourable places. This is the case of the deepest part of BA2, but especially the case of BA3, which was reached after a 46 m journey through total darkness, in rough terrain. Although trogloxene species that use caves prefer more illuminated places located near their entrance (Howarth and Moldovan, 2018), or at least where the light is present, in this case, the common toad went deeper inside the cave and the breeding activity continued at the beginning of the deep zone, which is in accordance with the observations of Bonini et al. (1998). So, the obtained models are consistent with the phenomena observed in the field and show that the presence of favourable hydrologically factors prevailed over the presence of light. This fact underlines the importance of water flow velocity and water depth in breeding site selection and explains the presence of breeding activity in all the environmental zones of the cave.

Both air and water temperatures are important for breeding activity and tadpole development (Reading, 2010). The recorded air temperature was over 12°C (table 2), much higher than the activity threshold temperature for the common toad, which has been described as about 6°C in southern England (Reading, 1998). However, toads appear to be more sensitive to water temperature than to air temperature (Gittins et al., 1980). The water temperature recorded in the breeding areas in Peștera cu Apă Cave was above 11.1°C, which is higher than the water temperatures recorded in Belarus where breeding occurred at water temperatures above 6°C (Drobenkov et al., 2006) or in the Czech Republic where the first eggs were laid at water temperatures exceeding 5-8°C (Kovar and Brabec, 2007). The air humidity was higher than outside, being an advantage of this habitat because the water loss through the skin of amphibians is reduced (Wells, 2007). All the climatic parameters in the cave were favourable for the breeding activity. In addition, their variation is much smaller inside the cave than outside, the underground habitat having greater climatic stability.

During the field surveys, we noticed that the more recently hatched tadpoles showed, in general, a more inactive behaviour, some of them being almost immobile. Studies revealed that the daily cycles of illumination influence the activity of tadpoles (Beiswenger, 1977; Griffiths et al., 1988). Thus, *B. bufo* tadpoles are predominantly active during the day, while at night they are less active, remaining still or swimming only for short distances (Griffiths et al., 1988). Considering that in the cave the illumination decreased gradually reaching complete darkness in the final sector of BA2 and BA3, it is probable that the activity level of the larvae was also influenced by the lack of light.

Larval survival depends on several factors such as food, competition and predatory behaviour, temperature, and also the risk of pond drying out (Griffiths, 1997).

In BA1 and BA2, fire salamander larvae were also present. This is the first evidence of the cohabitation of *B. bufo* and *S. salamandra* larvae in subterranean waters. Unlike *B. Bufo*, cave breeding of *S. salamandra* is a more common phenomenon that is reported in many karst areas (Manenti and Ficetola, 2013; Manenti et al., 2017). Although the *S. salamandra* larvae populated the same pools and they were much larger, we did not notice them acting as predators on the common toad larvae, possibly because the common toad larvae have chemical toxins that make them unpalatable (Alvarez and Nicieza, 2009), but if the predator shows a high level of hunger, even an unpalatable prey may be consumed (Gunzburger and Travis, 2005).
We have observed that in some places tadpoles were grouped in aggregates. The aggregation behaviour of the tadpoles has been interpreted in terms of defence against predators or cooperative social foraging (Alvarez and Nicieza, 2009). Although algae are generally the major source of the tadpoles’ diet, they also feed on bacteria (Bardsley and Beebee, 1998), detritus (Diaz-Paniagua, 1989), possibly arthropods that fall into the water, or dead conspecifics (Bonini et al., 1998). In caves, however, the trophic resource is generally limited but, in regularly flooded caves, there is a periodic food supply (Hüppop, 2012). During rainy periods, the food supply can be abundant, and after the depletion of reserves, food scarcity occurs (Moldovan et al., 2018). During the laying of eggs, the water level was higher in Peștera cu Apă Cave. When the water level dropped and the pools began to dry out, the pressure on food resources increased. The increase in larval density is followed by increased intraspecific competition for food and cannibalism. In this situation, intraspecific competition can have benefits at the population level, allowing certain larvae to develop at the expense of others, to metamorphose faster and leave the pond before it dries out (Griffiths, 1997).

The water level decreased gradually so that on FS4, the water was present only in BA2. Dead larvae appeared on the edges of the pool as well as on the boulders emerging from the water as a result of its partial drying. The more active larvae were found predominantly in areas with greater depth, less exposed to an immediate risk of drying, while the larvae from the periphery showed less movement, many being almost immobile. We noticed interlarvar cannibalism, especially in the central areas of the pool. Several larvae with more dynamic behaviour clustered next to a smaller and more inert larva, possibly dead, feeding on it. Cannibalism has been found in many amphibian species, including *Bufo bufo*, in various combinations of developmental stages during their ontogeny such as adult-juvenile, tadpole-tadpole, tadpole-egg (Vlcek et al., 2013). One of the main causes of larval cannibalism is the drying of water in which a large number of individuals are living (Vlcek et al., 2013) as well as the limited food resources (Kovacs and Sas, 2009). These conditions of environmental stress occurred in Peștera cu Apă Cave and they contributed to the observed interlarvar cannibalistic interaction.

The development of tadpoles usually takes 2-3 months in external environment (Botnariuc, 1960) but in the subterranean environment tadpoles metamorphosed later (Bonini et al., 1998), hence these tadpoles in Peștera cu Apă Cave did not succeed to accomplish their metamorphosis before drying out of the water (supplementary fig. S2).

The considerable importance of this subterranean aquatic habitat for common toads breeding is also suggested by the fact that they are crossing a rough terrain to reach the cave. The slope values calculated for the area that encompasses the approximate breeding migration distance of the common toads range between 1° and 56.38°, with an average declivity of 32.5°. The terrain declivity of the slope just below the cave entrance ranges between 18.9° and 35.4° and has a mean value of 27.31°. Despite the steep slopes, the toads managed to reach the cave to breed in the subterranean pools. This indicates that the common toad actively chose this environment for its reproduction. This is in accordance with Sztatecsny and Schabetsberger (2005) who reported that in Alps, the common toads had overcome even 45° slopes to reach their preferred habitats. Although there is a steep slope to be climbed, we need to consider that migrating toads are highly motivated to reach the breeding place (Pail et al., 2020) and endogenous factors, such as motivation (Sinsch, 1987), can overlap exogenous factors, like environmental features, overcoming their combined effect (Sinsch, 1988).
Final considerations

The sporadic presence of accidental species in underground habitats has most often been correlated with the existence of passive mechanisms, such as accidental falls or drifts. In the case of species commonly considered trogloxenes that occur in caves, an analysis of the relationship between the species and the habitat is important to assess whether their presence in the cave is accidental or is the result of habitat selection (Lunghi, 2020). Identifying such a selection would suggest a more active importance of the cave environment for these species as well (Lunghi et al., 2014). As the epigean water bodies in the study area are not favourable for common toad breeding, we believe that there is a selection of this subterranean aquatic habitat, based on more suitable conditions for its breeding. This is also supported by the fact that toads make a considerable effort to reach the cave, climbing a high declivity slope.

Considering this environmental context, but also the fact that the common toad has a high pond fidelity (Reading et al., 1991; Daversa et al., 2012), its return to this subterranean habitat for breeding is very probable. The limitation of our study is that breeding activity of the common toad in the cave was recorded just for a year and we need to conduct further investigations to assert if this is a common behaviour in this site or a sporadic or isolated event.

This study increases the understanding of the breeding habitat selection by the common toad and shows the importance of cave water as breeding habitat for the common toad in the study area. It suggests the potential importance of underground karstic waters for the reproduction of the common toad in areas with surface water scarcity or with fast-running waters. In the context of climate change, karst waters inside caves could represent possible breeding habitats for the common toad but also for some other amphibians in regions threatened by aridization and acidification, an important ecological challenge for amphibians.

Supplementary material. Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.2354455

References


Environmental constraints and cave reproduction of *Bufo bufo*


