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Comparison of ventral spotting in *Bombina variegata* between northern Slovakian localities

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Abstract. A comparison of ventral spotting of the yellow-bellied toad (*Bombina variegata*) was investigated in four localities (Kysucká vrchovina, Spišská Magura, Spišsko-šarišské medzihorie and Bukovské vrchy). The research complements previous results from 2014 and 2022 with the addition of new samples from 2022 and 2023. The resulting patterns were evaluated by principal component analysis (PCA) and differences between territories were examined. The most significant factors were related to spotting or morphology of individuals. The first component (PC1) is related to melanism, to which the variables characterising spotting contribute significantly. Individuals from the Spišsko-Sarišské medzihorie region had the darkest overall colour (most patches, high proportion of B/Y, etc.). The second significant component (PC2) is related to the size of individuals. Size differentiation of *B. variegata* (PC2) showed significant differences between individuals from different areas. Individuals from Spišská Magura were larger than individuals from other regions. PC3 defines the size of spots; our findings revealed significant regional differences. The largest spots were observed in the Spiš-Šariš population. We did not evaluate PC4 further due to image processing errors which were considered to be methodological error. PC5 represents the number of dark patches. Individuals from Spišská Magura showed a higher number of dark patches than other populations. PC6 is related to variation in hind leg length. This means that it is also related to the size of individuals. Similarly, individuals from Spišská Magura were larger. As abdominal spotting is an aposematic colouration, playing a role in deterring predators but also in camouflage and sexual selection, these variations may represent local adaptations of the species. Individual size may correspond to differences in habitat quality, resource availability or other ecological factors affecting growth.

Key words: Bombina variegata, ventral spot pattern, adaptation, environment, digital image analysis

Introduction

Amphibian species are particularly sensitive to changes in habitat, particularly chemical composition of the environment, such as the impact of spraying during agricultural activities, the impact of urban expansion, and road construction (Decena *et al.* 2020; Luedtke *et al.* 2023). These changes and resulting barriers affect their ability to reproduce, cause genetic differentiation, and contribute to the propagation of genetic lineages or, conversely, to the reduction of gene flow in populations (Coleman *et al.* 2018). Another type of environmental change is driven by climate change, including rising temperatures, fluctuations in the rainfall cycle, flooding, drought, and the development of new pathogenic sources. Amphibians are particularly sensitive to all these changes, reflected by declining populations or changing habitats. Adaptation to new conditions is thus, the primary basis for the survival of the species.

The yellow-bellied toad, *Bombina variegata* (Anura: Bombinatoridae), is a species of amphibian widely distributed throughout Europe (Hubl *et al.* 1977). *B. variegata* is widespread in central and southern Europe (Fijarczyk *et al.* 2011), apart from the southwest. It occurs east of France to the Carpathian Massif, and from central Germany and southern Poland south to southern Italy and the Balkan. This species has attracted the attention of researchers for its unique ecological importance and behavioral adaptations. Their upper side (dorsal) is grey-brown, often with washed-out bright spots. Their undersides (ventral), including the insides of the limbs, fingers and toes, are grey-blue to black-blue with conspicuous bright yellow to orange spots or patches, usually covering more than half of the underside.

The aposematic colouration and ventral patterning of *B. variegata* is a defensive strategy that acts as a deterrent to potential predators, but it is also unique to each individual and a manifestation of phenotypic and genetic diversity in the population (Vallan *et al.* 2004). Several studies have been conducted to decipher the differences in colouration and abdominal pattern between individual species of frogs in the family Bombinatoridae (Coppin *et al.* 2003; Dufresnes *et al.* 2021; Scheele *et al.* 2014). *B. variegata* is a species of frog found in aquatic environments such as rivers, streams, wetlands and lakes. Its unique mottled body colouration is thought to be related to the habitats in which it occurs (Cayuela *et al.* 2022). This frog is well camouflaged at the bottom of ponds, allowing it to avoid predators. Its distinctive ventral colouration serves not only as a warning and visual decoy for predators, but also as camouflage (Stuckert *et al.* 2019). Its appearance also influences neighbouring species living in the same habitat. Colouration and patch pattern are important characters traditionally used to identify animals within taxa, but also to distinguish between populations and individuals (Carafa and Biondi 2004).

The term "spot pattern" or "colour pattern" refers to a mosaic of coloured spots of varied sizes and shapes arranged in a particular position in relation to each other (Zakharova *et al.* 2022). The principle of mapping is to examine the colouration of individuals and compare the spots, the distribution of patterns, their position and connections. The pattern of spots in *B. variegata* frogs is genetically determined and linked to specific genes that affect the production and distribution of pigments in the skin. Studies show that individual populations of *B. variegata* can have different genotypes within these loci (Souter *et al.* 2011), resulting in colour variability and characteristic spots. Overall, this represents a colour polymorphism determined by multiple genes that have a major influence on the spotted pattern of the frog (Stuckert *et al.* 2019; Hantzschmann *et al.* 2020).

Colour patterns and body characteristics are highly variable in amphibians (Hoffman and Blouin 2000). Based on previous research (Zakharova *et al.* 2022), we hypothesized that environmental conditions may be a crucial factor influencing colouration and patterns of ventral spotting in this species. Therefore, this research focused on changes in ventral spotting patterns in relation to location of occurrence. The main objective of this study is to determine whether the coloration and pattern of abdominal spotting in yellow-bellied toads differs among populations from different territories.

Research on the yellow-bellied toad could provide new insights into how differences in spotting in this species are influenced and conditioned by the environment (Clemente-Carvalho *et al.* 2017; Di Cerbo and Biancardi 2020). Understanding the importance of habitats *B. variegata* chooses to live in can support conservation efforts (Snow and Witmer 2010). The results of this work could contribute to the creation and improvement of strategies for monitoring amphibian species in a non-invasive or minimally invasive way, whereby it is possible to record the shape of the pattern on the body of the toads (Renet *et al.* 2019).

Material and Methods

Sampling

The results of this study include data from previous surveys undertaken between 2013 and 2021 (Karaščáková 2014; Zakharova 2022), as well as new samples collected in 2022 and 2023. The sampling sites were divided into four units (Fig. 1): Kysucká vrchovina (2018: n = 47; 2021: n = 20; 2021: n = 15), Spišská Magura (2020: n = 29; 2022: n = 5), Spišsko-šarišské medzihorie (2022: n = 29) and Bukovské vrchy (2013: n = 113; 2023: n = 115). In total, we included 113 complete samples from Karaščáková (2014), 100 samples from Zakharova (2022) and 164 samples from the current study.

Individuals were captured by hand or with a small net. Basic measurements (body length, head width, hind leg length, thigh length) and weight were measured for each individual. Sex was determined based on external sexual characteristics. Males were identified by thicker forelimbs with nuptial pads. Females were identified by the absence of nuptial pads and a larger body with a significantly larger abdomen and thinner forelimbs. Juveniles were distinguished from adults based on their smaller size and the absence of nuptial pads. Each individual was then released from the capture site to its original location.

Photographs of the abdominal region of each individual were taken to examine the ventral part, emphasising stable fixation of individual in order to obtain the most accurate measurement.

Photographs were taken in the field with a non-specific photographic camera.

Pattern analysis of yellow-bellied toad

Abdominal spot pattern analysis was performed using the analysis program Image-Pro Plus 6.0 (2D image analysis software from Media Cybernetics Inc.) (IPro). Photos went through several stages of editing to achieve proper results. In the first step, the image samples were edited using the Photos application from the 2023 Microsoft Windows suite. This phase involved aligning and cropping the image to the required format, including potential colour correction for easier manipulation in IPro, which was necessary in situations where the image contained unwanted reflections. Shading with filters was used to correct the image. The colour spectrum of the analysed section was modified in IPro, in addition to inversion of the image, and pigment marking (marking the specific colour of the pixels - in other words, black spots designated as the object of interest). Through this process, all pixels of the same colour were labelled and connected to investigated locations according to the manual spot plotting plan. After selecting parameters to be studied, the program

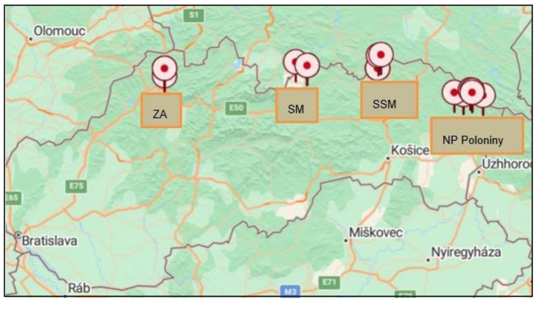

Fig. 1. Sampling sites 2014 - 2023. Map includes the locations divided into 4 larger areas: ZA - Kysucká vrchovina, SM - Spišská Magura, SSM - Spišsko-šarišské medzihorie, NP Poloniny – Bukovské vrchy (Developed with maps from: www.bing.com, 2024).

automatically extracts the necessary data to create a matrix for further evaluation and analysis. In IPro, basic data were selected, including object area (total area of black spots; excluding holes), spot perimeter (sum of perimeter - perimeter of all black spots; average perimeter - average perimeter of black spots), number of black spots, average sphericity (circularity). From these values, other variables characterising the abdominal pattern were calculated, including: per cent black (ratio of total black spot area to total calculated abdominal area), per cent yellow (expressed as the difference between total abdominal area and % black), B/Y ratio (ratio of black to yellow), RMPA (ratio of average black spot content to average black spot perimeter). A detailed description of the analysis procedure is given in Zakharova *et al.* (2022).

Statistical analysis

Statistical evaluation of ventral spotting was performed in Statistica Ver. 12 (TIBCO Software Inc, USA). Principal component analysis (PCA) was used to assess the main variables associated with abdominal spotting. The most significant components associated with abdominal spotting patterns and morphological parameters of frogs and their association with localities were evaluated by one-way ANOVA (at a 95% confidence level $p < 0.05$).

Results

Using principal component analysis (PCA), we identified 6 main principal components (PC) that collectively represent almost 90% of the variability in the data (Table 2). PC1 – Melanism. The variables contributing most to this component are the total area of black spots, average area of black spots, black color %, black to yellow ratio, RMPA, which have an increasing tendency, while the variable yellow color has a decreasing tendency. This most significant component is represented by individuals with a darker overall colour (melanic individuals). PC2 – Animal size; the main variables of this factor are head width, body length, and weight. This factor represents a group in which these variables increase or decrease, that is, the manifestation of the factor is in the size of the individuals. PC3 – Size of spots. The variables contributing to this component are perimeter mean and perimeter sum, (i.e., the size and number of spots). The factor also expresses patterns in the spots. PC4 – Image quality. There is only one significant variable in this component – total abdominal area, (i.e., the section of image characterised by number of pixels). This number varies according to the quality of the photograph, the focal length, and other factors. As this is a parameter related to image quality, it was not included in further analyses. PC5 – Number of dark patches. This component contains the most significant single variable, (i.e., it represents a group of individuals with an increasing number of dark patches). PC6 – Leg length. The most significant variable in this component is the length of the hind leg (femur). This factor is also related to morphology, (i.e., the size of individuals). Differing factors were compared based on where monitoring was conducted.

PC1 differed significantly between the compared sites. The population of frogs from the Spišsko-šarišské medzihorie (S-ŠM) is the most melanic compared to the other monitored populations (Fig. 2), from the Spišská Magura (SM), Kysucká vrchovina (ZA), Bukovské vrchy (NP Poloniny) ($F(3, 253) = 9.5661$, $p = 0.00001$). Melanism did not differ significantly between sexes ($F(1,256) = 1.1816$, $p = 0.27805$).

Variables	PC 1	PC2	PC3	PC 4	PC5	PC6
Body length	0.26558	**-0.79182**	0.10311	-0.36836	0.045097	-0.18156
Head width	0.34040	**-0.77484**	0.07777	-0.14245	0.110781	0.16418
Hind limb (femur)	-0.23447	**-0.52653**	-0.07819	0.18658	-0.30206	**0.70880**
Weight	0.32788	**-0.76077**	0.03225	-0.36483	0.01268	-0.20554
Total abdominal area	0.66664	-0.24344	0.22673	**0.59918**	0.17043	-0.10971
Total area of black spots	**0.86588**	-0.05896	0.15837	0.41489	0.14862	-0.01237
Average area of black spot	**0.85601**	-0.01870	0.00773	0.27738	-0.23281	0.01394
Black color %	**0.80620**	0.36043	-0.06183	-0.33794	0.15903	0.17702
Yellow color %	**-0.80620**	-0.36043	0.06183	0.33794	-0.15903	-0.17702
Ratio B/Y	**0.75640**	0.25253	-0.06855	-0.36329	0.08658	0.24460
Number of dark patches	-0.52953	-0.15402	0.13755	0.24857	**0.60492**	0.19759
Mean roundness (circularity)	0.66135	-0.02386	-0.21340	0.15448	-0.46568	-0.09721
Perimeter mean	0.20349	-0.13404	**-0.93495**	0.12893	0.15561	-0.04176
Perimeter sum	0.19615	-0.12780	-0.93544	0.14204	0.15936	-0.04225
RMPA	**0.82857**	0.05283	0.43641	0.13109	0.09144	0.01580
Variance %	37.55	16.88	14.11	9.55	6.07	5.28

Table 2. Principal components (PCs) of the variables, based on correlations. In bold are highlights significant variables, which contribute to the strength of the factor.

Animal size (PC2) characterizes morphometric differences between individuals in populations. There were significant differences in the size of individuals between sites (F (3, 254) = 4.2480, p = 0.00597), with the smallest individuals in the Bukovské vrchy and the largest in Spišská Magura (Fig. 3).

Another component that characterizes spotting is PC3 (size of spots). In this component, populations from different areas differed significantly (F (3, 254) = 6.8271, p = 0.00019). Larger patches were typical for the population from the Spišsko-šarišské mezdihorie region (Fig. 4). Populations from other territories tended to have smaller spots.

The total number of dark patches (PC5) also characterizes differences in spotting. Again, differences between areas were significant (F (3, 254) = 11.024, p = 0.00000). The least number of dark patches in the ventral spotting was recorded in the frog population from the Spišsko-šarišské medzihorie region and the highest number of patches in the Spišská Magura (Fig. 5).

Component (PC6) related to body size, in this case characterised by the length of the hind leg (femur). The size of the hind leg differed significantly between populations (F (3, 254) = 12.410, p = 0.00000). The population in the Bukovské vrchy had a leg length smaller than the other populations (Fig. 6). This confirms the dominant size parameters in PC2, where individuals from this population are smaller compared to the others.

Discussion

Individual variables related to frog morphology and spotting were evaluated by principal component analysis. These components are exclusively related to the pattern of spotting (PC1, PC3, PC5) or the size of individuals (PC2, PC6). The individual populations differed significantly in all components, (i.e., the compared populations differed both in the pattern of spotting and in size).

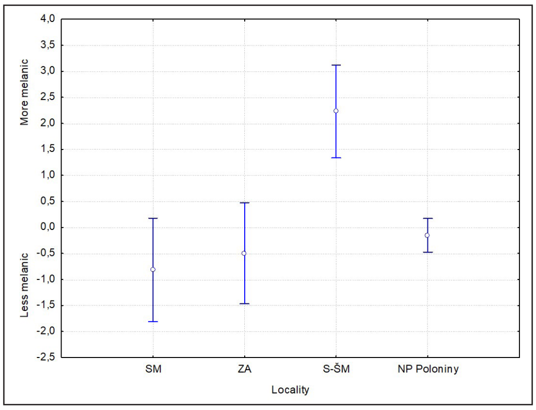

Fig. 2. Melanism (PC1) in relation to different sampling sites. The degree of melanism across four main environments indicated considerable dispersion, with groups SM (Spišská Magura), ZA (Kysucká vrchovina) and NP Poloniny (Bukovské vrchy) showing significantly lower levels of melanism compared to groups from S-ŠM (Spišsko-šarišské medzihorie). Data in factor coordinates are given in mean and SD - standard deviation (+/-).

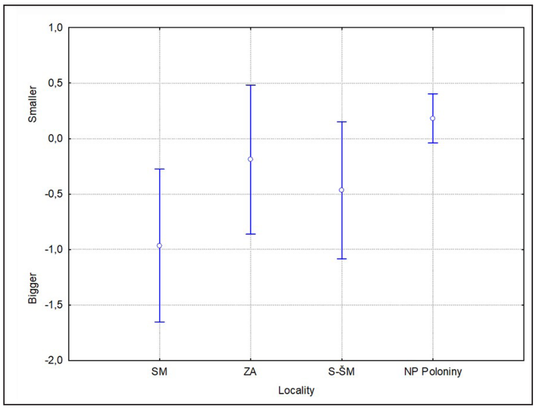

Fig. 3. Body size of frogs characterized by PC2. The largest individuals (negative direction of the y-axis) are from the territory of Spišská Magura and the smallest from the territory of Bukovské vrchy. SM - Spišská Magura, ZA - Kysucká vrchovina, S-ŠM - Spišsko-šarišské medzihorie, NP Poloniny - Bukovské vrchy. Data in factor coordinates are given in mean and SD - standard deviation (+/-).

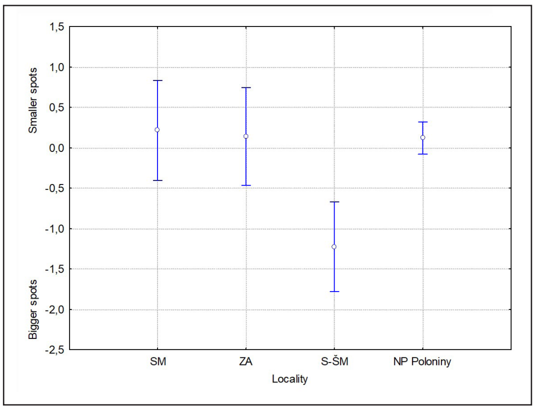

Fig. 4. The size of spots (PC3) varies between populations, the largest spots (negative direction of the y-axis) are in the population in the Spišsko-šarišské medzihorie. SM - Spišská Magura, ZA - Kysucká vrchovina, S-ŠM - Spišsko-šarišské medzihorie, NP Poloniny - Bukovské vrchy. Data in factor coordinates are given in mean and SD – standard deviation (+/-).

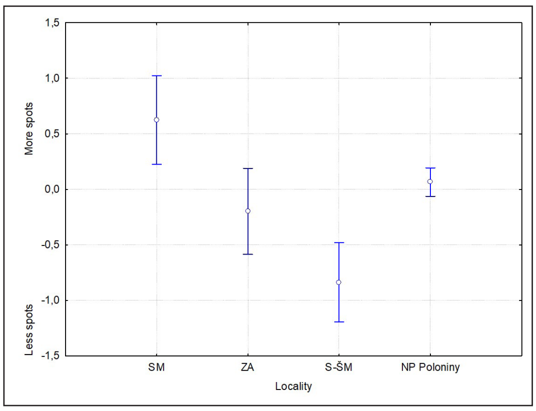

Fig. 5. Number of dark patches (PC5) shows highly significant variation between sites. The population from Spišská Magura had the highest number of spots. SM - Spišská Magura, ZA - Kysucká vrchovina, S-ŠM - Spišsko-šarišské medzihorie, NP Poloniny - Bukovské vrchy. Data in factor coordinates are given in mean and SD - standard deviation (+/-).

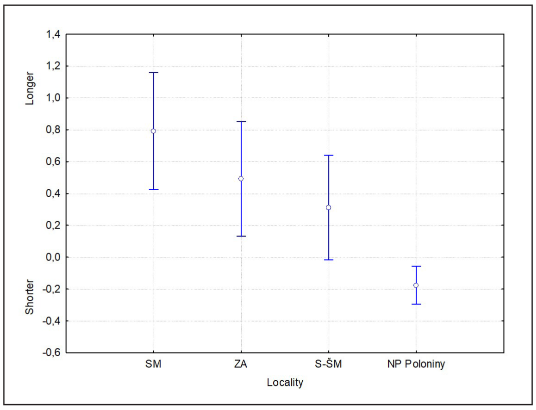

Fig. 6. The length of the hind leg (PC6) varies between sites. Individuals in the Bukovské vrchy population had significantly shorter legs than individuals from other populations. SM - Spišská Magura, ZA - Kysucká vrchovina, S-ŠM - Spišsko-šarišské medzihorie, NP Poloniny - Bukovské vrchy. Data in factor coordinates are given as mean and SD - standard deviation (+/-).

The spottnig patterns

Spotting patterns are intrinsically linked to an animal's habitat and play a crucial role in survival, species interactions, and adaptation to environmental change. The phenotypic expression of individuals in a population is generated by spatial heterogeneity in the environment, which selects for gene flow and genetic drift (Le Sage *et al.* 2021). As spotting varied between the populations studied in our research, we suspect that the dominant genes in each population are regulated by environmental conditions. Research confirms that environmentally sensitive genotypes generate alternative phenotypes, which is one manifestation of phenotypic plasticity (DeWitt and Scheiner 2004; Van Buskirk 2017). Phenotypic plasticity represents morphological and/or physiological adaptations or responses in an individual's behaviour in response to environmental stimuli and changes (Price *et al.* 2003). Ecological environmental conditions are fundamental to the response of species to the establishment of adaptive conservation strategies (Rissler 2016).

Our results confirmed that the overall pattern of spots and colouration has common features, (i.e., the size (area) of spots determines their number and overall colouration). The population of frogs from the Spišsko-šarišské medzihorie region had the darkest overall colour pattern (PC1), with the lowest number of spots (PC5) and the largest area of spots (PC3). The population of frogs from Spišská Magura was dominated by yellow colouration (PC1), the number of spots was high (PC5), but their size was small (PC3).

The breeding ponds where individuals from Spišská Magura were captured were located on forest roads that were not shaded by vegetation, or the nearby tall vegetation was located on the northern side of the pond. As a result, these localities were lightly shaded or completely unshaded. On the contrary, the capture sites in the Spišsko-šarišské medzihorie were in forested area, where they were surrounded by tall vegetation. Sunlight was diffuse and the ponds were heavily shaded. Although we did not correlate environmental conditions with colouration and spotting, we hypothesize that habitat shading (or lack of it) is the main selective factor shaping patterns of spotting and colouration in *B. variegata*. Dark colouration is important for camouflage in dark habitats. This form of colouration (crypsis) helps animals to blend into their environment, making it difficult for predators to detect them (Bauer 2023).

Melanism is a phenomenon in which organisms show increased production and accumulation of melanin, the pigment responsible for dark colouration. The production of melanin, which is responsible for the dark colouration of amphibian skin, varies with developmental stage, temperature, and light (Fukuzawa and Bagnara 1989). Pigmentation in amphibians is a complex process influenced by many factors, including genetics, environment, and physiology (Fernandez and Bagnara 1993). Melanism is linked to temperature of the environment. Skin melanisation in *Rana chiricahuensis* increased at low temperatures (4° C) and decreased at elevated temperatures (25° C) (Fernandez and Bagnara 1993).

The dark colouration of frogs is a key factor in thermoregulation, the process by which an organism maintains its body temperature within certain limits, even when the ambient temperature is vastly different. This adaptation is particularly important for ectotherms such as frogs, which rely on external heat sources to regulate their body temperature. Darker individuals can absorb heat from their environment more efficiently, which is beneficial in colder climates or during colder parts of the day (Laumeier *et al.* 2023). Because dark frogs absorb heat more efficiently, they can warm up faster than lighter frogs. This can be crucial for activities such as foraging and escaping predators. Differences in colour brightness between frog populations suggest local adaptation. Darker species are more common in cooler areas (northern), supporting the idea that dark colouration is advantageous for thermoregulation in these environments (Alho *et al.* 2010; Laumeier *et al.* 2023).

In addition to overall darker or lighter colouration, patterns of spots can indicate local adaptation of each population. Variation in specific patterns between populations from different regions has also been confirmed for *Bombina bombina* in Lithuania (Pupins and Pupina 2009). Variability in spot patterns and colouration can play a key role in camouflage, communication, thermoregulation, and even mate selection in specific habitats, or as a warning signal to predators (Rafińska 1991; Dorigo *et al.* 2012).

Bright colouration and contrasting ventral colours (aposematic) and patterns can be used as warning signals to potential predators, indicating that the frog may be toxic (Barnett *et al.* 2018). Research has shown that the size of dark spots on a bright background can influence predator behavior. Larger spots tend to be more effective in deterring predators, suggesting that spot size is a key factor in the evolution of aposematic signals (Preißler and Pröhl 2017).

Size of body

The fact that body size was expressed on independent components (PC2, PC6) suggests that factors other than colour and mottling influence it. The availability of food resources is essential for the growth of amphibians; body size of frogs increases with abundance of resources. When food resources are scarce, individuals grow to smaller sizes, directly influenced by population density, increasing competition for food (Crnobrnja-Isailović *et al.* 2012). Body size can vary within a population from year to year as environmental climatic conditions and food resources change. There is a strong correlation between amphibian body size and abundance, but not environmental temperature (Green and Middleton 2013). Although amphibians may respond with accelerated ontogeny during periods of high environmental temperatures and faster drying of breeding pools (Newman 1992; Urban *et al.* 2014), amphibians reach smaller sizes at metamorphosis.

B. variegata is a semi-aquatic species commonly found in marshes, ponds and wet meadows in various regions of Europe. The presence of sunlight in the habitat plays a key role in the toad's life cycle and overall prosperity. Sunlight enables the growth of algae and plankton, an important food source for the yellow-bellied toad. The penetration of sunlight into the water column influences the distribution and behaviour of prey species, which in turn impacts the toad's foraging patterns and success in capturing food (Martof 1962; Lillywhite *et al.* 1973; Barandun and Reyer 1997). Additionally, sunlight is essential for maintaining ideal water temperatures in aquatic habitats, thereby facilitating the toad's reproductive activities and metabolic processes (Barandun and Reyer 1997; Rafińska 1991). Sunlight stimulates toad breeding activity and courtship displays, which are important aspects of their mating behaviour (Christein and Taylor 1978).

Studying the divergence of habitat-related phenotypic and morphological variation in frogs at the interspecific and species level can provide valuable insights into the ecological and evolutionary factors shaping their populations. Studying multiple factors, such as genetics, behaviour, and ecology, helps to fully understand the mechanisms acting on these variations (Visser *et al.* 2019). The spatial variation in ventral patch patterns and body size observed in *B. variegata* populations in our study suggest the influence of environmental and genetic factors.

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