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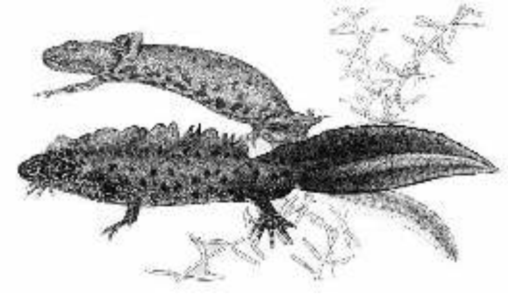
LIFE-HISTORY TRAITS AND BEHAVIOR OF AMPHIBIANS



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CONTENT

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1. INTRODUCTION

1.1 Amphibians in ecological and evolutionary studies

Amphibians are vertebrates that belong to the group of ectothermic tetrapods (their internal temperature varies with the ambient temperature), and they are found worldwide except the polar regions and with a higher species richness in the tropical habitats (AmphibiaWeb, 2023). Amphibians have a complex life cycle which requires in most species both aquatic and terrestrial habitats. This complex life cycle is expressed as morphological and physiological changes (limbs emergence, gills resorption and lungs formation in adults, tail resorption at the time of metamorphosis) but also as ethological modifications (locomotion and feeding behavior) (Duellman and Trueb, 1994). Most species use the aquatic habitats for egg deposition and larval development until metamorphosis, when most move to terrestrial habitats. Other species are fully aquatic, their embryonic, larval, and adult stages occur in water, while other species (e.g., viviparous, and oviparous species) are fully terrestrial even though they need a relatively humid environment for embryonic development because of egg yolk permeability (Duellman and Trueb, 1994). Amphibians have permeable skin which is covered with mucus that not only protects them from infections and parasites but also helps them sense their environment and absorb oxygen (Wells, 2007).

1.2. Amphibian conservation

According to IUCN Red List (2023), amphibians are the most endangered group of terrestrial vertebrates worldwide. More than 35% of species are listed as vulnerable (VU), endangered (EN), critically endangered (CR), extinct in the wild (EW) or extinct (EX). More than 5% are likely to be assessed in a threatened category in the future, being listed as near threatened (NT). The rest of the species are listed as Least Concern (44%) and for more than 15% of species there is insufficient data regarding their geographical distribution and population status to evaluate their threatened risk. Data regarding the geographical distribution of species are essential to conservation actions because it is the main component for estimating the specific diversity, and explaining the ecological patterns of the species (Chapman, 2005). Many species of amphibians are facing serious threats, including habitat loss, climate change, pollution, (Baillie et al., 2010; Beebee and Griffiths, 2005; Blaustein et al., 1994; Jensen and Camp, 2003) and diseases (Berger et al., 1998; Daszak et al., 2000; Kiesecker et al., 2001; Laurance et al., 1996;) leading to severe populations decline. Conservation efforts aim to reduce threats to amphibian populations and protect their habitats (IUCN, 2022). These efforts may include habitat restoration (Pabijan et al., 2020), captive breeding (Silla et al., 2021) and reintroduction programs (Vaissi et al., 2019), and the implementation of laws and policies to protect amphibians and their habitats.

2. ECOTOXICOLOGY

2.1. Ecotoxicology – general aspects

The amplitude, diversity, and long period of conflicts between biodiversity conservation on the hand and human activities on the other hand suggest that Earth already running in the Anthropocene epoch (Lewis and Maslin, 2015). They named two major crises, environmental (which I will focus on it) and societal crises (World Economic Forum, 2023). The fastest deteriorating global risk in the next decade according to the report is "Biodiversity loss and ecosystem collapse" (World Economic Forum, 2023). The intensification of industry and agricultural needs, landscape modification for urbanization and overall global warming and climate change are leading to a strong decline in biodiversity (Vitt and Caldwell, 2014).

Amphibian populations are declining, and main driver are the human activities. At the beginning of 1990, the problem of amphibian declines and how their status are changing rapidly has been extensively recognized (Blaustein, 1990; Vitt et al., 1990). Chemicals production has contributed to the enhancement of human life quality over time, creating useful chemical compounds with high efficiency. However, all of these substances have been developed with little concern about the negative effects on the environment (Zimmerman et al., 2020).

3. AMPHIBIANS BEHAVIOR

3.1. Behavioral adaptations

The domain of behavioral ecology encompasses various aspects of the life history traits of amphibians, such as movement ecology, social interactions, foraging, and predator avoidance. The decisions made by amphibian individuals regarding their behavior affect their fitness because determine their ability to compete with other individuals (intraspecific or interspecific interactions), escape predators, and food acquisition (Vitt and Caldwell, 2014). Individual amphibians strive to balance the advantages of social behavior, which enhance individual fitness, with the costs of obtaining the necessary resources to maintain activity and the potential dangers of predation while engaged in these activities (Vitt and Caldwell, 2014). Another factor that modifies the amphibian behavior is water availability. Changes in water levels during spawning and larval periods can affect the reproductive success of local populations (Wells, 2007), with amphibians skipping reproduction if aquatic environment does not correspond to their ecological needs. The aquatic environment is important because can shape the life-history traits of amphibians starting from eggs and larvae which are vulnerable to desiccation (Kiesecker et al., 2001; Vignoli et al., 2018). The partial or total absence of amphibian reproduction success during the breeding season is the main short-term effect of desiccation (Carey et al., 2003) and extrapolated to the medium term can cause population declines (Stewart, 1995)

3.2. Density effects

Higher densities represent forced events of aggregation caused by several factors. In most cases, these events in adult individuals result from breeding periods and periods of inactivity (hibernation or estivation) where amphibians are overcrowded in shelters as long as environmental factors do not correspond to amphibians demands (Vitt and Caldwell, 2014). In larvae, both aggregation and avoidance occur. Social attraction, coordinated behavior, and limited cooperation have been observed among some anuran tadpoles, which is in contrast to the solitary behavior of most amphibian larvae. These interactions are often facilitated by the recognition of close kin (Pfennig, 1997). For anuran larvae, there are benefits resulting from aggregation and this behavior was observed in 10 anuran families (Wells, 2007). The benefits of aggregation were observed in physiological factors (thermoregulation), feeding, and protection against predators (Wells, 2007). While anuran larvae exhibit aggregation, salamander larvae, are typically solitary and tend to avoid interaction with other individuals. When interactions appear, salamander individuals exhibit aggression and even cannibalism within their population (Wells, 2007). Also, competition for resources such as food and oxygen could be a trigger for salamander larvae to avoid aggregation.

3.3. Larval aggressivity

Most of the amphibian larvae have solitary behavior but this pattern is widespread in Urodela larvae due to their aggressive and cannibalistic behavior (Wells, 2007). The solitary behavior displayed by newt and salamander larvae is likely connected to high competition between larvae, alongside the occurrence of opportunistic cannibalism and the emergence of specific cannibalistic forms (Wells, 2007). Though cannibalistic behavior is present in salamander larvae, individuals prefer to avoid conspecific if another source of food is available (Pfennig et al., 1994). This mechanism is related to the conservation of the overall fitness of the local population (Pfennig et al., 1994). Short-term effects of a high density in early life stage of amphibian larvae include smaller body sizes and growth rates and smaller sizes at metamorphosis (Székely et al., 2017), accelerated metamorphosis (Denver et al., 1998; Kohli et al., 2019) and higher mortality associated with intraspecific and interspecific competition (Rowe and Dunson, 1995; Vaissi and Sharifi, 2016). The long-term effects of density-induced aggressivity in salamander larvae are a smaller growth rate of subadult individuals (Vignoli et al., 2018). Laboratory experiments showed that adults can recover after a bad start caused by high larval densities but it can happen only in favorable environments without predation and diseases and with unlimited food supplies (Vignoli et al., 2018).

3.4. Predator avoidance

Upon encountering predators, prey species display a range of behavioral reactions, such as relocating themselves immediately or modifying their patterns of movement and activity (Lima and Dill, 1990). The influence of predation on the development of various amphibian life history traits, including the strategies used to escape predators, secretion of toxic substances, the use of camouflage (cryptic color) or warning colors (aposematic color) has played a significant role in amphibian evolution. While it is evident that predation has played a crucial role in evolution, evidence indicates that animals possess the capacity to evaluate and adjust their vulnerability to predation through their behavior within their lifetime (Lima &and Dill, 1990). Furthermore, predation is considered a selective tool that impacts numerous other facets of amphibian behavior and ecology (Tuttle and Ryan, 1981; Wilbur, 1980).

Many species of invertebrates are predators to amphibian species, at every life stage: eggs, larvae, juveniles, and adults. Thus, medicinal leech attack and kill adults of *Lissotriton vulgaris*, *Bufo bufo* and *Rana temporaria* (Merilä and Sterner, 2002). Axelsson et al. (1997) showed that larvae and eggs of *R. temporaria*, *B. bufo*, *Hyla arborea*, *Epidalea calamita* and

Pelophylax esculentus are consumed by singe crayfish (*Pacifastacus leniusculus*) and noble crayfish (*Astacus astacus*) in different laboratory conditions. And nevertheless, tadpoles and juveniles of *Pelobates cultripes* are predated by similar size individuals of *Triops cancriformis* (Phyllopoda) (Knoepffler, 1978).

4. POPULATION STRUCTURE

4.1. Age-related and size-related parameters

Understanding the population dynamics is one of the most important baselines in biodiversity conservation (Tarsi and Tuff, 2012). Demographic studies can provide insights into the factors that influence the size, structure, and distribution of animal populations. This information can be used to develop conservation strategies and management plans for threatened or endangered species (Caughley, 1994). Another advantage of demographic studies represents identifying the declining species, which is critical for conservation efforts. By tracking population trends over time, researchers can detect changes in population size or structure and identify potential threats to the species (Caughley, 1994). One more advantage is represented by the capacity of demographic studies to estimate population growth rates, which can help predict future population sizes and determine the carrying capacity of ecosystems (Sibly and Hone, 2002).

Age estimates are important for understanding the life history and ecology of amphibian populations, which is critical for their conservation. This can be done by several methods but capture-mark-recapture (CMR) and skeletochronology are the most used methods.

Goal and objectives

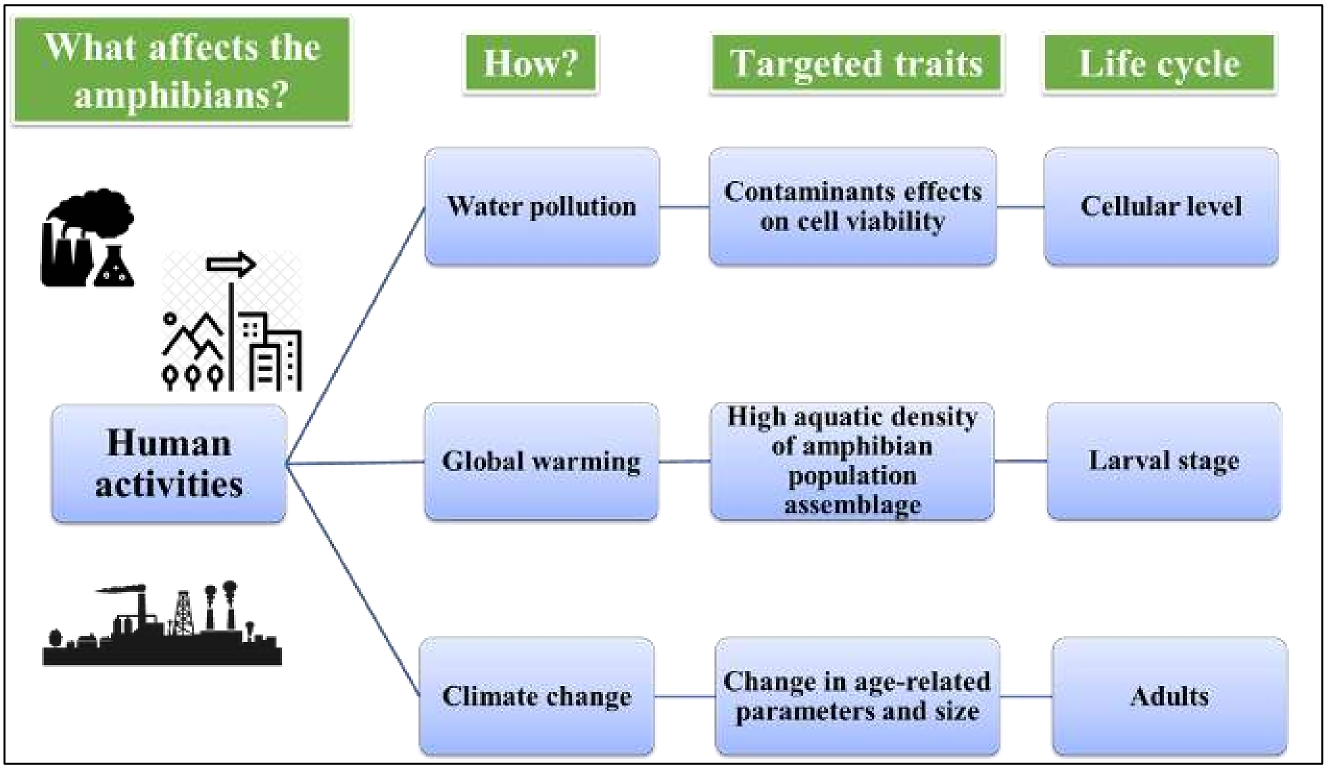
This thesis analyzed and assessed the effects of human activities related threats on different life stages of amphibians. I analyzed effects on the cellular level (viability); larval stage (survival, sizes at metamorphosis and feeding mechanisms); and adults (age structure and growth). Four objectives were established:

1) assessment the lethal concentration of contaminants on amphibians' cell viability;

2) describing the feeding mechanism of *Triturus dobrogicus* larvae and assess the effect of drought on survival and size at metamorphosis;

3) the effect of density on *Triturus dobrogicus* larval stage

4) review the effect of geographical variation and identify long-time population response on age-related and size-related parameters of *Bufo bufo, Pelobates fuscus* and *Rana temporaria* populations.



5. DOES THE NUMBER OF ETHYLENE OXIDE GROUPS INFLUENCE THE TOXICITY OF SULPHATE-BASED SURFACTANTS TO AN AMPHIBIAN CELL LINE?

This chapter is based on the manuscript "**The number of ethylene oxide groups of sulphate-based surfactants influence the cytotoxicity of mixed micelles to an amphibian cell line**" by Sebastian Topliceanu, Monica Almeida, Miguel Oliveira, Dan Cogălniceanu, Isabel Lopes, submitted for publication to Archives of Environmental Contamination and Toxicology. My contribution to this work included: investigation, conceptualization of the manuscript, methodology applied, data analysis, and writing the original draft.

In this chapter I tested of there are differences in LC_{50} of different chemical structures of Sodium lauryl ether sulphate. Based on the results, I can propose whether *in vitro* testing using amphibian cell lines an alternative to classic ecotoxicological testing on live animals.

5.1. Material and methods

Test substance

Sodium lauryl ether sulphate (SLE_nS) is used in commercial pesticides as adjuvants, in personal care and cleaning products but also in industrial excavation processes because of its low cost and being very effective foaming agent. Toxicity of six different anionic surfactants were assessed. The SLE_nS types differentiate in the number of ethylene oxide units presented in their chemical structure. The following SLE_nS stock solutions were provided by BASF SE (BASF GmbH, Dusseldorf) and tested: SLE_0S, SLE_1S, SLE_4S, $SLE_{11}S$, $SLE_{30}S$, $SLE_{50}S$, where the number refers to the ethylene oxide units (EO).

Biological model

The cell line used in this study was the A6 epithelial cell line (ECACC 89072613), isolated from the kidney of an adult male of *Xenopus laevis* (Rafferty Jr., 1969). The cell line was kindly provided by Professor Peter Lorenz from the University of Rostock (Germany).

MTT and Resazurin assay

To assess the cytotoxicity of the six SLE$_n$S variants to A6 cell lines, two different viability assays were performed, namely the thiazolyl blue tetrazolium bromide (MTT) and Resazurin assay, according to the National Institute of Health (NIH) guidelines (Riss et al., 2016), which were adapted to the A6 cell line.

5.2. Results

MTT Assay

The cell viability showed a significant difference between 24 h and 48 h exposure times for SLE$_1$S and SLE$_{11}$S while there were no significant differences in exposure time for SLE$_0$S, SLE$_4$S, SLE$_{30}$S, and SLE$_{50}$S. Based on the estimated LC$_{50}$ values, both at 24 h and 48 h exposure times, the variants with lower EO units, SLE$_0$S, SLE$_1$S, and SLE$_4$S exhibited slightly higher cytotoxicity than SLE$_{11}$S, SLE$_{30}$S and SLE$_{50}$S (Fig. 1). Though this tendency of SLE$_n$S variants with higher EO units exhibiting lower cytotoxicity, a significant positive association between the number of EO units and the values of LC$_{50}$, after 24 and 48 h of exposure, was not observed.

Resazurin assay

The cell viability showed no significant differences between 24 h and 48 h of exposure, for all six variants of SLE$_n$S. The estimated LC$_{50}$ values increased with the EO units of the SLE$_n$S variants in both 24 h and 48 h (Fig. 1). A significant positive association was found at 24 h and 48 h of exposure, between the number of EO units of the SLE$_n$S variants and the computed LC$_{50}$s.

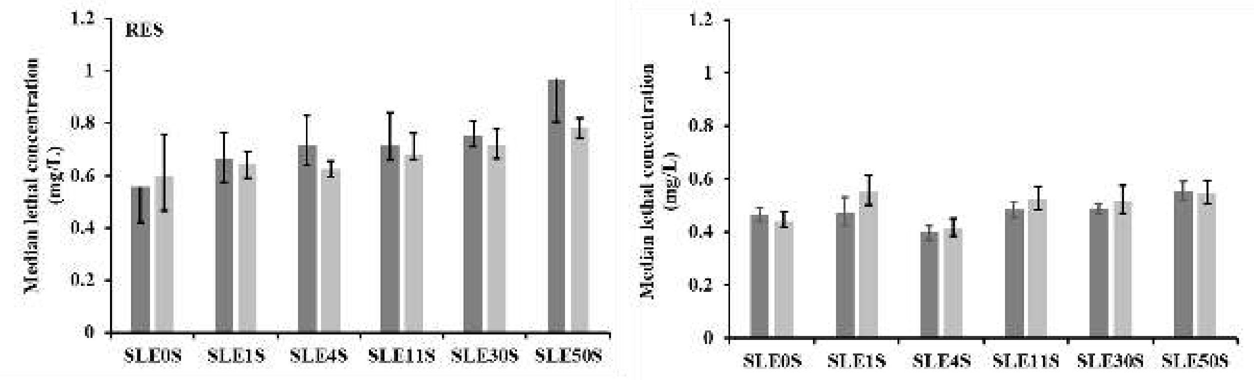


Figure 1. Median lethal concentrations (LC$_{50}$) calculated for the six SLE$_n$S variants, after 24 h (dark grey bars) and 48 h (light grey bars), based on the MTT and resazurin (RES) assays. Error bars represent the 95% confidence limits, missing upper bars means that the upper 95% confidence limit could not be computed.

5.3. Discussion

The results obtained varied according to the assay used. The resazurin assay indicated that SLE_nS variants with a lower number of EO units were the most toxic to A6 cell lines, exhibiting lower values of LC_{50}. Though the results from the MTT assay showed a similar pattern of lower EO units associated with higher toxicity, no significant association was observed between the computed LC_{50} values and the number of EO groups.

6. FEEDING BEHAVIOR AND HOW LARVAL DENSITY AFFECTS THE GROWTH, FEEDING, AND SURVIVAL RATE OF *TRITURUS DOBROGICUS*

This chapter was published in two scientific articles from an international collaboration with the researchers at Shumen University, Shumen Bulgaria and Vienna University, Vienna, Austria.

My contribution to the first article "**Ontogenetic Changes of the Aquatic Food Uptake Mode in the Danube Crested Newt *(Triturus dobrogicus* Kiritzescu 1903)**" by Nikolay Natchev, Kristina Yordanova, **Theodor-Sebastian Topliceanu**, Teodora Koynova, Dimitar Doichev and Dan Cogălniceanu, consists in the experimental design, experiment implementation, data analysis and writing material and methods and results sections for the article. This work was published in 2021 in Frontiers in Ecology and Evolution (see full citation in the reference section). The article can be found here: https://www.frontiersin.org/articles/10.3389/fevo.2021.641657/full

The second article entitled "**Short-term effects of larval density on the body size and behaviour in *Triturus dobrogicus* (Kiritzescu 1903)**" by **Theodor-Sebastian Topliceanu**, Nikolay Natchev, Teodora Koynova and Dan Cogălniceanu, Annals of the Academy of Romanian Scientists, series of Biological Sciences, 12(1), 21 - 28. My contribution to this article included the writing of original draft, data analysis and correspondence with editors. The article can be found here: https://www.aos.ro/wp-content/anale/BVol12Nr1Art.2.pdf

In this chapter, I aimed to describe the feeding mechanism and locomotion of *Triturus dobrogicus* larvae and I tested a series of hypotheses. I estimated if size at metamorphosis and survival rate of larvae are lower at higher densities and I tested if aggressivity is more intense at a higher individual density.

6.1. Studied species

The Danube Crested Newt *Triturus dobrogicus* has a restricted distribution area being present in floodplains of large rivers from eastern Austria to the Danube Delta (Fahrbach and Gerlach, 2018). The species is characterized by its long body and short limbs. The Danube Crested Newt is the most water-related species from its genus and it has the longest larval stage among *Triturus* sp. (Furtula et al., 2009).

6.2. Field work

Animals were collected from the southern part of Danube Delta Biosphere Reserve (Grindul Lupilor: 44°37’15.83” N, 28°48’24.92” E). Seventeen adults (9 males and 8 females) of *Triturus dobrogicus* were caught from a temporary pond before the reproduction period in 2020.

6.3. *Ex-situ* reproduction of *Triturus dobrogicus*

All the laboratory experiments were performed in the spring of 2020. After the experiments, the adults and the juveniles were released back to the capture spot. When the males clearly differentiate the crests and the reproduction started simultaneously, three females and three males were randomly assigned in one box for reproduction. After the larval hatching, we move the freshly hatched larvae to the experimental boxes. These aquaria were filled with 3L of aged tap water, 1/3 of the water from the aquaria was refreshed with aged tap water every 4 days.

6.4. Feeding mechanism

6.4.1. Material and methods

Recordings of feeding behavior started at developmental stage 42 until the developmental stage 50 (pre-metamorphic stage) (after Bernabò and Brunelli, 2019). In pre-metamorphic stage feeding behavior of larvae changed and they start actively searching for food at the water surface. The larvae were fed with 24h before the behavior recordings. The following parameters were noted: **A**, angle between the food particle center of mass and the longitudinal axis of the larvae head (in degrees); **D**, prey distance (cm); **S**, speed of the larvae to the prey during the approach stage (cm/s); **TL**, Length of the larvae (cm); **TT**, length of the trajectory of the tail tip (cm); **ST**, max. speed of the movement of the tail tip during the approach stage (cm/s); **SDi**, distance travelled by the snout tip during the inertial suction (cm); **SS**, speed of the displacement of the snout tip during the inertial suction (cm/s).

6.4.2. Results

The larvae fed successfully with decreasing of the distance to the food item. In the case of successful feeding, a higher ST represents a lower probability of feeding. The other analyzed variables have no significant differences in feeding probability (fig. 2). The initial distance between newt and prey had a significant effect on the feeding behavior of the larvae of *T. dobrogicus*. When the food particle landed on the bottom of the aquarium at a relatively long distance, the larvae actively swam toward the item. In case the pellet sank at a distance of less than 6 % of the larval body length, it was immediately attacked by the larvae without any activity of the locomotor system. When the particles landed at a distance, which is larger than 74 % of the body length, the larvae reacted in two different manners. In most cases, the animals did not react to the stimulus at all.

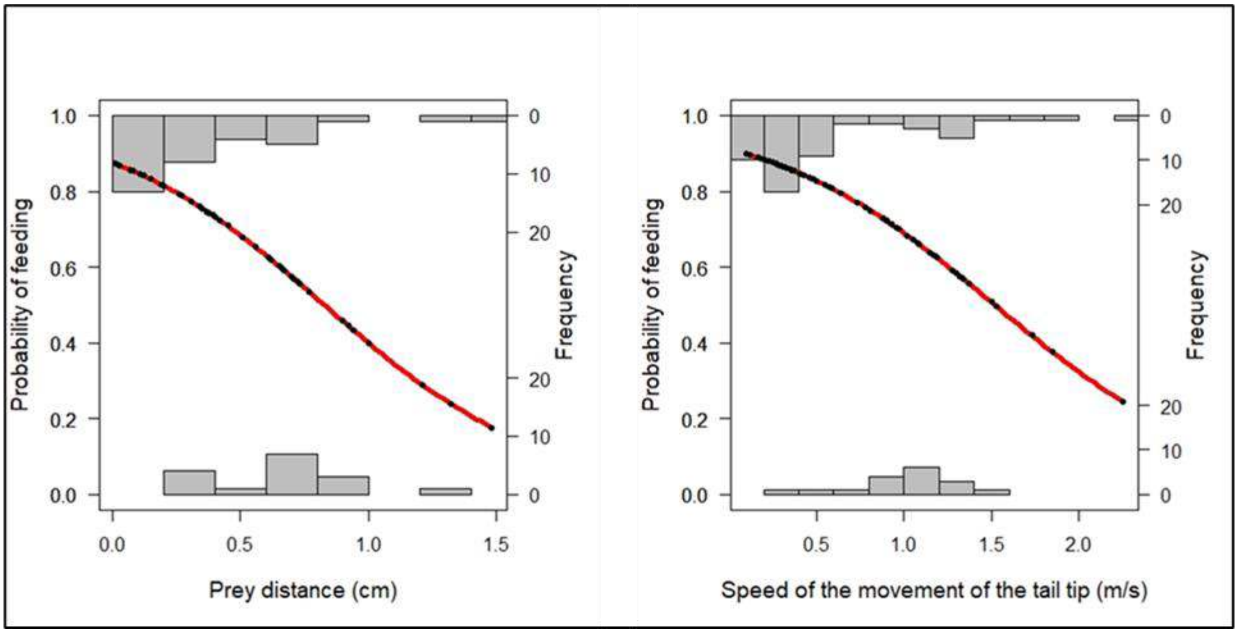


Figure 2. Left: Effects of prey distance on the probability of successful feeding; Right: Effect of speed on the probability of successful feeding

6.4.3. Discussion

The young larvae in *T. dobrogicus* are spending time predominantly on the bottom and have more or less a two-dimensional hunting field. Only after the development of operational limbs in the late pre-metamorphose phases, the larvae start to exploit the whole volume of the water basin and they are able to attack prey from multiple directions. This shift may allow the larvae for hunting of larger and more agile prey, which in turn could contribute to the feeding efficiency and may result in improvement of the general fitness of the specimens.

6.5. Effect of density on body size and aggressivity

6.5.1. Material and methods

Larvae were moved to 3L experimental boxes after the hatching. The experimental boxes consist in 3L of aged tap water. The experimental design consists in three different densities: 1 ind/L (low density); 2 ind/L (medium density) and 3 ind/L (high density) with 3 replicates each.

Growth rate, survival rate, aggressivity and cannibalism

The larvae were photographed on the hatching day and every 8 days of the experiment, until the 88th experiment day and at metamorphosis. The larvae were measured and the following variables were noted: total length (**TL**); snout-vent length (**SVL**) – starting with the 48th day of the experiment when the cloaca was visible.

6.5.2. Results

Growth rate and metamorphosis

I found significant differences in size at metamorphosis between densities. Individuals from lower densities have bigger sizes at metamorphosis compared with individuals from higher densities. Mean SVL showed no significant differences between densities during the larval stage. The youngest larvae metamorphosed at 84 days after hatching (low density) while the oldest larvae metamorphosed at 159 days after hatching (high density) (table 1).

Table 1. Minimum, maximum, and mean values of SVL at metamorphosis and duration of larval stage.

	Low density (n=5)	**Medium density (n=11)**	**High density (n=18)**
	mean ± SD (min – max)	**mean ± SD (min – max)**	**mean ± SD (min – max)**
Metamorphosis SVL (mm)	37.76 ± 1.48 (35.26 - 38.91)	34.96 ± 3.30 (29.86 – 39.28)	33.31 ± 3.47 (28.6 – 42.14)
Larval stage (days)	105 ± 20.32 (84 - 138)	113.82 ± 17.04 (89 - 158)	108.06 ± 16.05 (92 - 159)

Survival rate, aggressivity and cannibalism

Survival rate was higher at low density (0.67) and similar survival rates have been found in medium and high density (0.56) (fig. 3). The level of aggressivity showed significant differences between densities. The highest level of aggressivity was found in high density (1.25) followed by medium density (1.17) and low density (1.15). Cannibalism was noted twice in low and medium density while in the high density, we observed 5 cases of cannibalism.

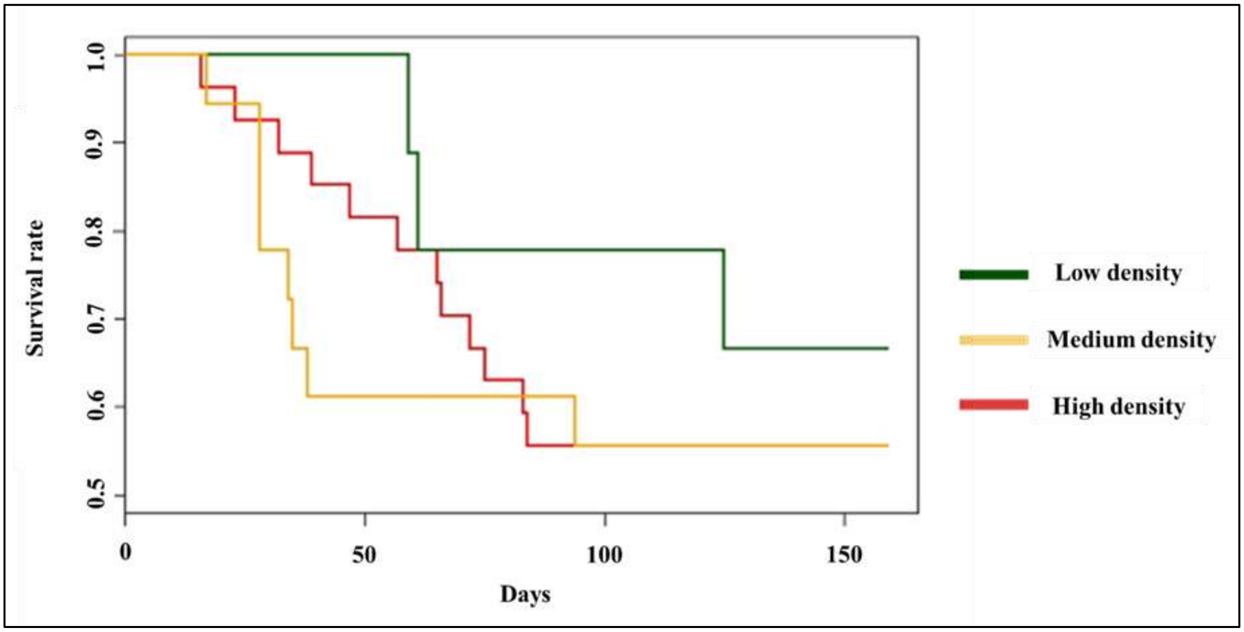


Figure 3. Survival rate of *T. dobrogicus* larve in low, medium, and high density.

6.5.3. Discussion

The results of this study showed that metamorphs from higher density have smaller sizes at metamorphosis, longer aquatic periods and show higher levels of aggressivity. Time at metamorphosis in our study is considerably faster (84 – 159 days). These results suggest that the competition during the larval stage accelerates the development of larvae to move out of the water to avoid larval intraspecific competition. A higher survival rate was found in low density, though survival rate did not show significant differences between densities. Moreover, the level of aggressivity and even cannibalism increased in higher individual densities. Overall, the present study showed how increased larval densities affect the reproductive success of the newt population by increasing density.

7. VARIATION IN LIFE HISTORY TRAITS OF AMPHIBIANS

Chapter 7.3. was published with title: “Age, size and body condition do not equally reflect population response to habitat change in the common spadefoot toad *Pelobates fuscus*”, in PeerJ (2021) (see full citation at the reference section). My contribution to this work included performing the analyses, analyzing the data, authoring and reviewing drafts of the paper, and approving the final draft. The article can be found here: https://peerj.com/articles/11678/

Chapter 7.4. is subject to a collaboration with the researchers from University of Belgrade, Institute for Biological Research “Siniša Stanković”– National Institute of Republic of Serbia, Department of Evolutionary Biology, Belgrade, Serbia and the University of Niš, Faculty of Sciences and Mathematics, Department of Biology and Ecology, Serbia. This chapter was drafted and currently, the authors perform the final revision before submitting. My contribution to this manuscript included fieldwork in 2019, laboratory work, authoring and reviewing drafts of the paper, and approving the final draft.

Chapter 7.5. consist of a literature review, including the data from the two manuscripts mentioned above.

This chapter consists in three work directions. Thus, the objectives of the chapters were assessed as follows: Assessment of changes in age and growth-related parameters induced by habitat modification in *Pelobates fucus* (Chapter 7.3). Assessment of changes in age-related parameters and body size over time in two populations of *Bufo bufo* (Chapter 7.4). Literature review of changes in age and growth-related parameters in *Rana temporaria* (Chapter 7.5). In the last chapter I tested two hypotheses: (1) if longevity increases with altitude and (2) if body size (SVL and BM) increases with altitudes.

7.1. Study species

7.1.1. *Pelobates fuscus*

The common spadefoot (*Pelobates fuscus*) is found in mostly lowlands of continental Europe. It is spread from the western part of Belgium and the Netherlands to the eastern part of Russia and it is absent in the Iberic Peninsula and in most parts of Scandinavia. In Romania, the species reach the southern part of its range (Székely et al., 2013) and it is widespread from 0 up to 800m a.s.l.

7.1.2. *Bufo bufo*

The common toad (*Bufo bufo*) is a widespread species in Europe. In Europe it is spread in the entire continent excepting Iceland, Ireland and the extreme north of Scandinavia and Iberic Peninsula (AmphibiaWeb, 2023). In Romania, the species inhabiting different types of habitats (including forests, grasslands, and wetlands) in the entire country and it is spread from 0m a.s.l. up to 2000m a.s.l (Cogălniceanu et al., 2013).

7.1.3. *Rana temporaria*

The common frog (*Rana temporaria*) is spread throughout Europe and a small part in the west of Asia. In Europe, it is observed in all countries, except Portugal and Iceland. This species inhabits many types of forest and aquatic habitats across Europe, recorded from sea level up to 2700m a.s.l. in the Pyrenees (IUCN, 2023). In Romania, the species is present in the Carpathian Mountains, Transylvania and the North-West part of Moldova, from 100m up to alpine regions above 2000m a.s.l (Cogălniceanu et al., 2013).

7.2. Skeletochronology protocol

I adapted the classical protocol for skeletochronology proposed by Castanet și Smirina (1990). The phalanx used for laboratory analysis were entered into different steps to transform the raw biological material to proper model for sectioning during skeletochronology.

7.3. RESPONSE OF *PELOBATES FUSCUS* TO HABITAT MODIFICATION

7.3.1 Material and methods

Study area

The study site is located in north-western Romania, in a hilly region from Cluj county, Romania (46.6829° N; 23.5441° E; 720 m a.s.l.). The study area encompassed a freshwater permanent pond that was initially bordered by steppe grasslands and forests. It is located near a medium-traffic interprovincial/county road.

Fieldwork

Sampling of adult individual was performed every year in April, during the breeding season, at two moments in time: between 2000 and 2004 (sample A, moment A), and between 2012 and 2014 (sample B, moment B). All animals were caught during night surveys. Sex was determined based on the presence of the humeral glands present only in sexually mature males and Snout-vent length (SVL) and Body mass (BM) were measured for each individual.

Age-related and size-related parameters

Following age-related parameters were computed: 1. average lifespan, as the mean age of breeding individuals, 2. longevity (i.e. maximum age observed), 3. age at sexual maturity, 4. potential reproductive lifespan. SVL and BM data was log-transformed and the residual body condition index (BCI) was calculated based on the linear regression between SVL and BM (e.g. Denoël et al., 2002; Băncilă et al., 2010). Positive BCI values indicated a good condition, while negative values indicated a poor condition of the sampled individuals (Jakob et al., 1996, Schulte-Hostedde et al., 2005; Blackwell, 2002).

7.3.2. Results

There were no significant differences in the age distribution between the two sampling moments, A and B. The median lifespan was only slightly lower in B sample, while the other age-related parameters were similar in both samples (fig. 4).

In sample B, both sexes had a median lifespan of 3 years old and matured sexually at the age of 2 or 3 years old. Longevity and potential reproductive lifespan were also similar, of 6 and 4 years respectively. Results showed no significant differences in the age distribution between males and females (fig. 4).

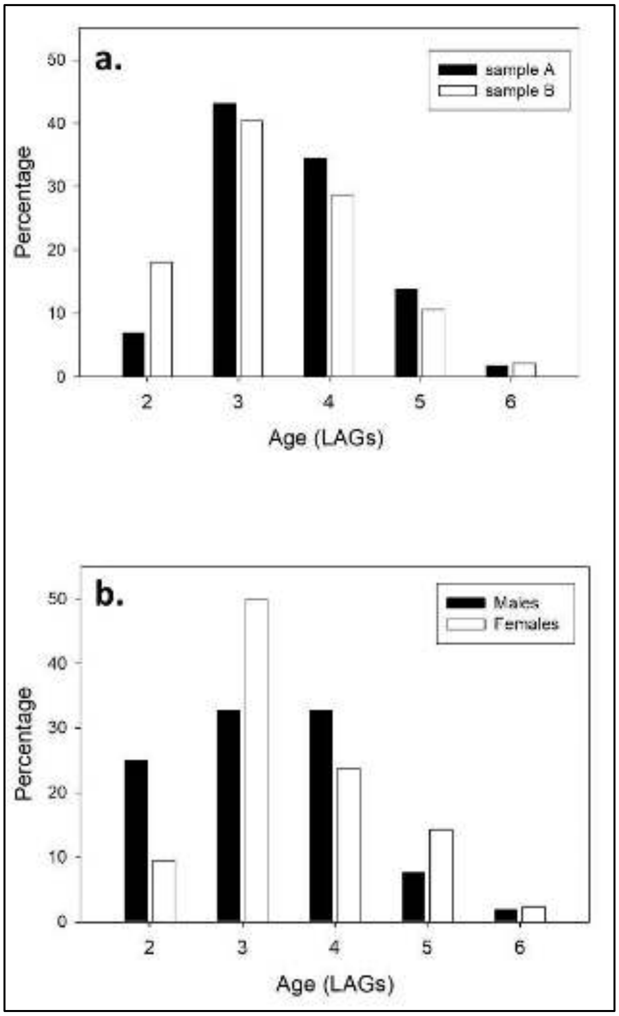


Figure 4. (a) Age distribution of *P. fuscus* populations, at two moments – 2000 - 2004 (sample A, black) and 2012 - 2014 (sample B, white); (b) Age distribution of males (black) and females (white) of the *P. fuscus* population within sample B (2012 - 2014).

A significant decrease was found in SVL and BM in both sexes between the sampling moments A and B (fig. 5). Body condition significantly decreased between the two sampling moments both in males and females (fig. 5).

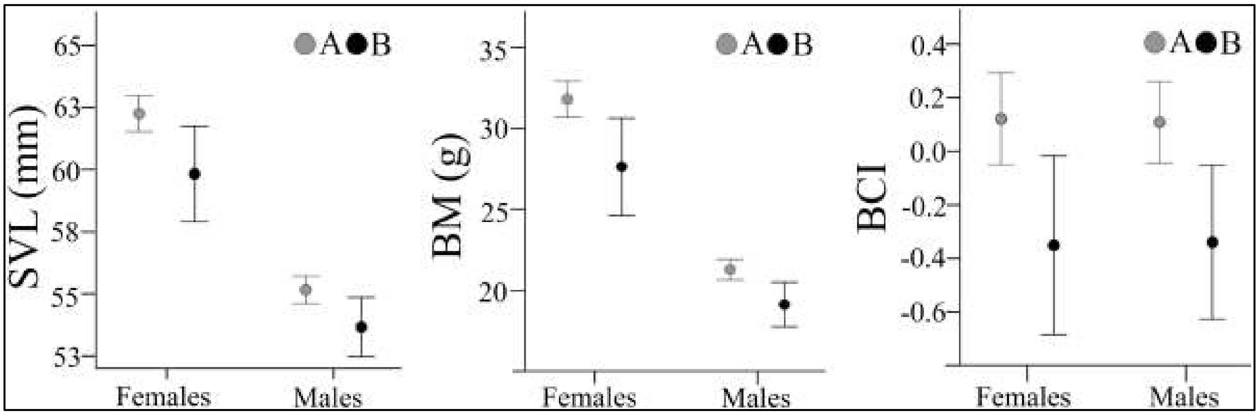


Figure 5. Box plot of SVL, BM and BCI of *P. fuscus* males and females, at the two sampling moments, A and B.

7.3.3. Discussion

The studied common spadefoot toad population responded to habitat degradation by a decrease in size and body mass, indicating that changes in size and body condition, rather than age structure, better reflect the rapid population response to declining habitat quality and can be used as an early-warning tool for amphibians' population decline.

7.4. CHANGES IN AGE AND BODY SIZE IN TWO POPULATIONS OF *BUFO BUFO* OVER TIME

7.4.1. Material and methods

Field study

I studied two populations, one in Romania and one in Serbia. The first population is located around a permanent pond within Macin Mountains National Park in the northern Dobrogea Region, Romania (N: 45.232118°; E: 28.307371°; 69 m altitude). The second population of *B. bufo* is located in Central Serbia, near villages Zuce and Belgrade (N: 44.682167°; E: 20.552000°, 240 m altitude).

7.4.2. Results

Age-related parameters

The *B. bufo* population from Romania showed no difference in mean age during the 10 years study period. The mean age of males ranged between 4.07 yrs in 2012 and 4.77 yrs in 2016. The mean age of females ranged between 4.11 yrs in 2012 and 4.67 yrs in 2009. Contrarily, the *B. bufo* population from Serbia showed significant differences in mean age over time. The mean age of males ranged between 4.54 yrs in 2021 and 5.7 yrs in 2015 while the lowest mean age of females was 3.92 yrs in 2018 and the highest mean age was 5.43 yrs in 2015.

Size-related parameters

Both SVL and BM varied significantly in both studied populations during the study period, for both sexes (fig. 6; fig. 7).

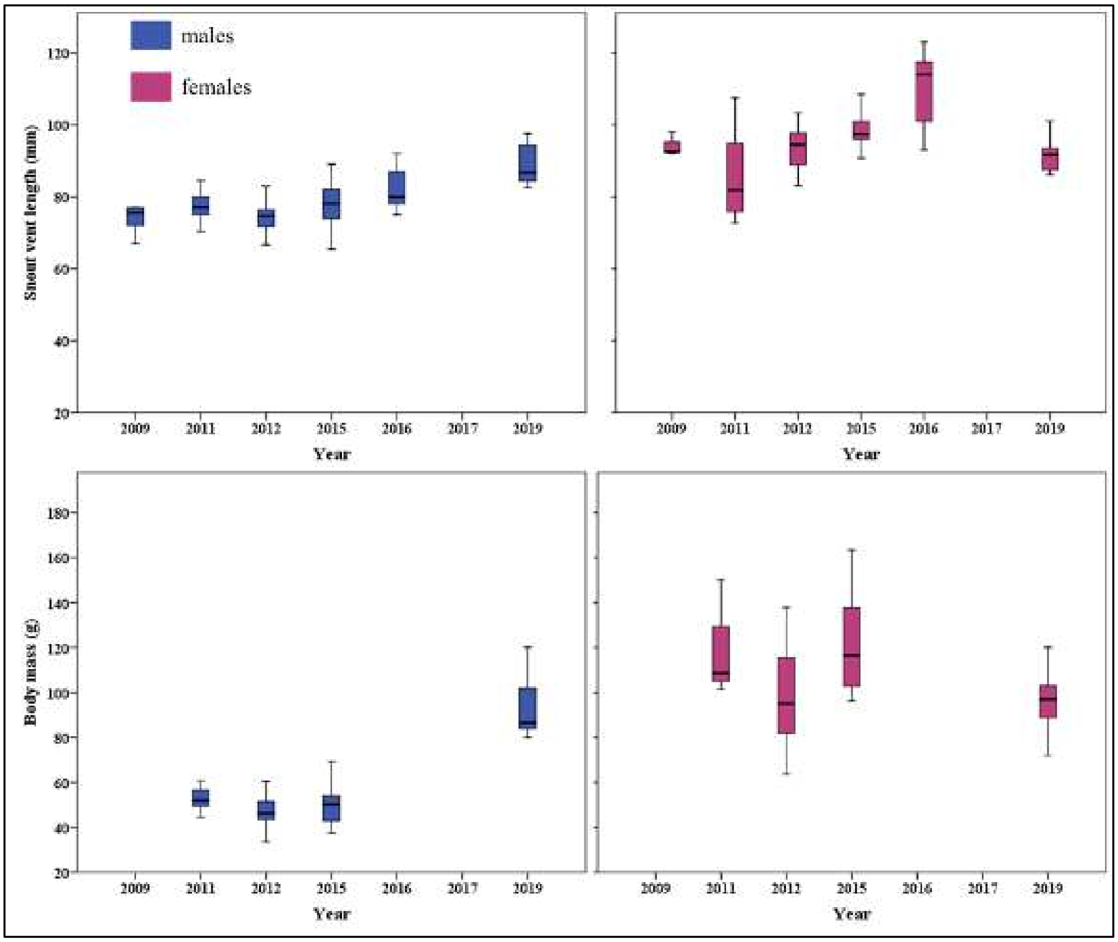


Figure 6. SVL and BM in males and females over time in *B. bufo* population from Romania

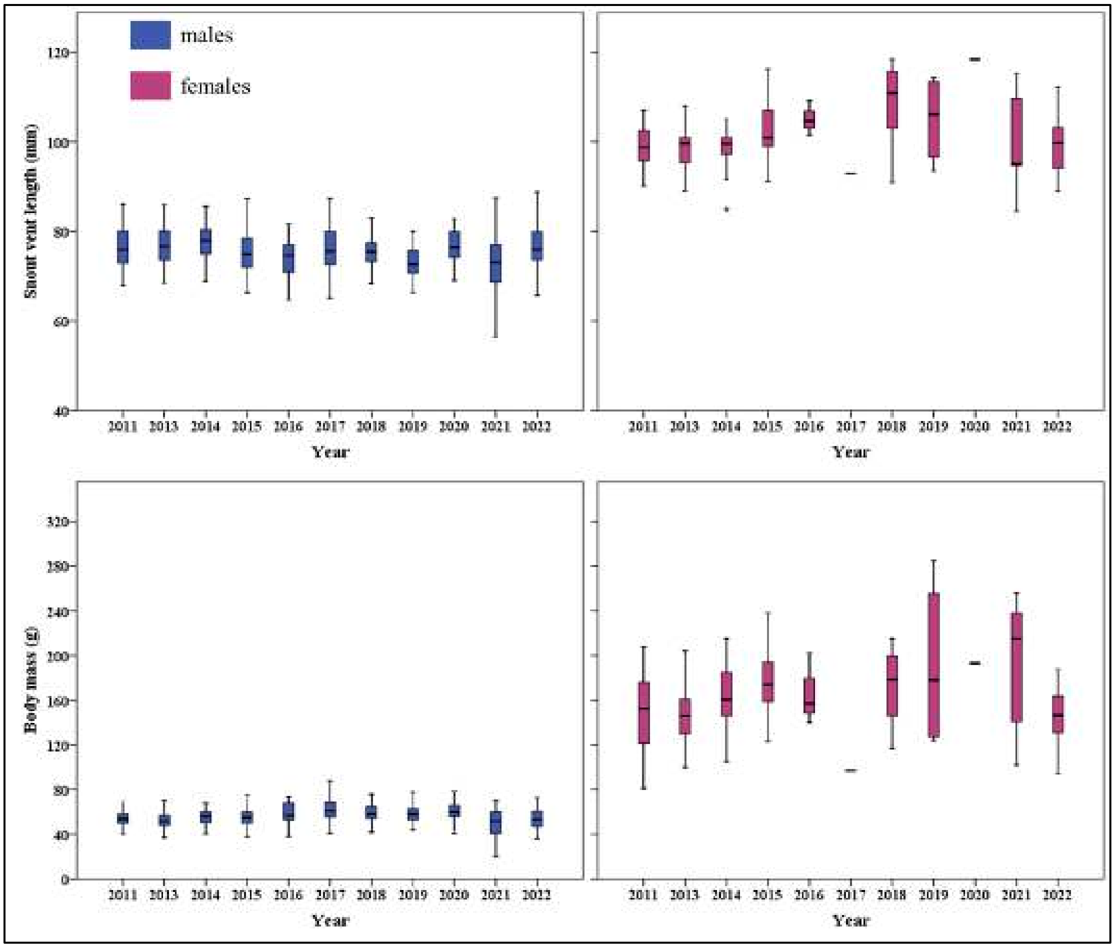


Figure 7. SVL and BM in males and females over time in *B. bufo* population from Serbia

Body Condition Index (BCI)

I found significant differences in BCI in all years of study between males and females of *B. bufo* population from Romania. BCI was higher for females with positive values (0.348), compared with negative values found for males (-0.132). Significant differences were observed also in *B. bufo* population from Serbia. BCI was higher for females and showed positive values (0.49), compared with negative values calculated for males (-0.14).

7.4.3. Discussion

While the population from Romania showed constant mean age, the population from Serbia has a significant variation over time. The age, growth rate and body size of individuals is influenced by a variety of environmental factors (Tomašević et al., 2008). Annual variation in temperature and precipitation impacts the reproductive success and the availability and quality of the trophic resource. This requires trade-offs in allocation of energy resources among survival, growth and reproduction (Cogălniceanu & Miaud, 2003; Amat & Meiri, 2018). During the study period, BCI of females in both populations was positive in almost all years of study. Contrary, BCI of males was mostly negative in both *B. bufo* populations studied. The BCI values of individuals may be diminished due to limited food availability or drought and unfavorable temperatures during hibernation or migration (Reading & Clarke, 1995). A lower BCI leads to reduced investment in reproduction, resulting in fewer offspring and a decrease in population size (Cogălniceanu et al., 2021).

7.5. LITERATURE REVIEW OF VARIATION IN AGE AND BODY SIZE OF THREE DIFFERENT SPECIES OF AMPHIBIANS ACROSS ALTITUDINAL GRADIENT

7.5.1. Material and methods

Literature analysis

Data of age-related and size-related parameters of *Bufo bufo, Rana temporaria* and *Pelobates fuscus* were extracted from available literature in online database (Web of Science, Google Scholar). I run an advanced search with the following keywords: “skeletochronology”, “age structure”, “amphibian”, “amphibian population structure”, “body size” and their combination. All the information available regarding the age-related parameters and body size were extracted and unified in a database.

7.5.2. Results

Literature analysis

I found 43 different references which focused on the age-related parameters and body size: 22 for *B. bufo*, 16 for *R. temporaria* and 5 for *P. fuscus.* Thirty-three different populations from 11 countries throughout Europe were studied in *B. bufo*. The oldest population studied was from 1975 and the newest population studied was in 2017. In *R. temporaria*, I identified 37 different populations from 11 countries from Europe. The oldest population studied was from 1958 and the newest population studied was in 2013. In *P. fuscus*, I identified 6 different populations from 3 countries (Romania, Italy, and France).

First hypothesis tested showed that altitude has a significant effect on longevity. *B. bufo* individuals from populations located at higher altitudes have a higher longevity for both sexes compared with individuals at lower altitudes (fig. 8) while in *R. temporaria* and *P. fuscus* altitude has no effect on the longevity.

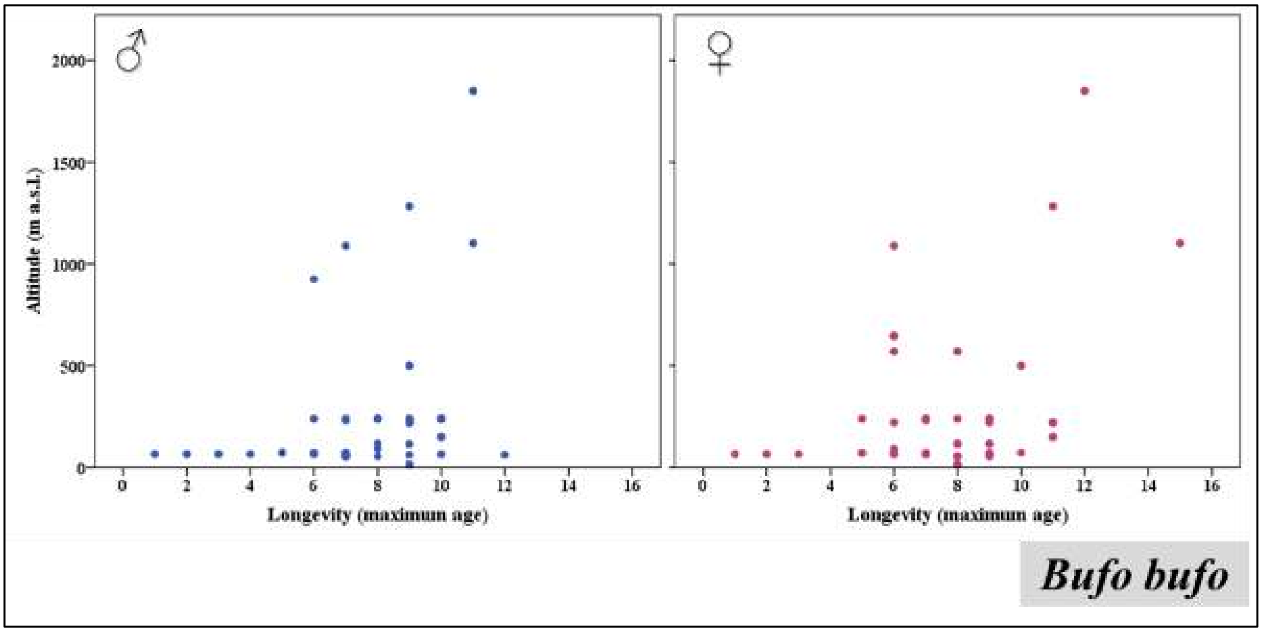


Figure 8. Regression between altitude and longevity in *B. bufo*

The second hypothesis tested showed that altitude has a significant effect on individuals' body size and body mass in *B. bufo* and *R. temporaria* (fig. 9). Thus, individuals from higher altitudes have a higher body size and body mass. Contrary, altitude has no significant effect on either body size or body mass in individuals of *P. fuscus.*

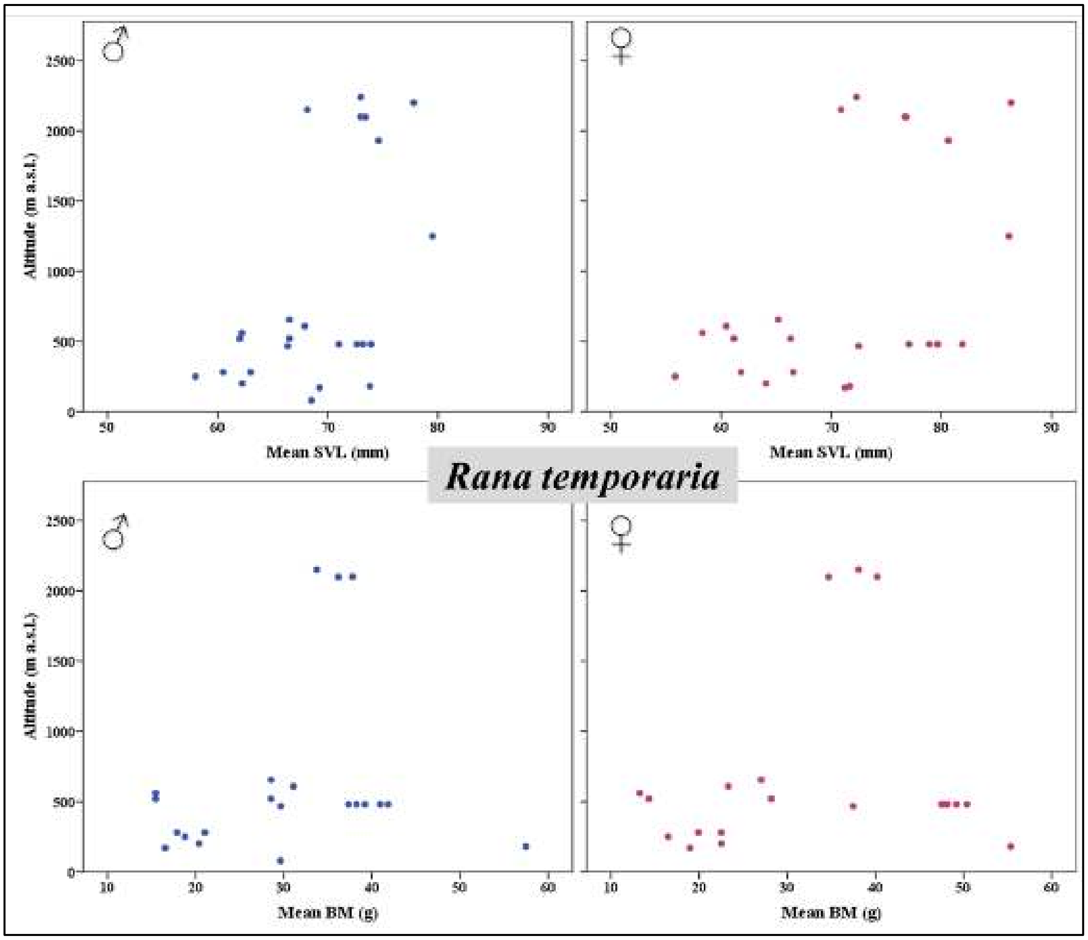


Figure 9. Regression between altitude, SVL and BM in *R. temporaria*

7.5.3. Discussion

The review performed in this chapter showed that different species of amphibians have different strategies for coping with the variation in environment, mainly related to altitude. Many amphibian species tend to be larger at high altitudes compared with the species at low and mid altitudes, as a response to cold climate (Ryser 1996; Miaud et al., 1999), but not all species follow this rule. There are exceptions where the low and mid altitude species are larger than those at high altitudes (see Howard & Wallace, 1985), or species when the climate has no influence on the body size (Ryser, 1996; Morrison & Hero, 2002).

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PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS

Scientific publication with Impact Factor (ISI).

1. Cogălniceanu, D., Stănescu, F., Székely, D., **Topliceanu, T. S.**, Iosif, R., & Székely, P. (2021). Age, size and body condition do not equally reflect population response to habitat change in the common spadefoot toad *Pelobates fuscus*. PeerJ, 9, e11678.
2. Natchev, N., Yordanova, K., **Topliceanu, T. S.**, Koynova, T., Doichev, D., & Cogălniceanu, D. (2021). Ontogenetic Changes of the Aquatic Food Uptake Mode in the Danube Crested Newt (*Triturus dobrogicus* Kiritzescu 1903). Frontiers in Ecology and Evolution, 9, 641657.

Scientific publication without Impact Factor (BDI).

1. **Topliceanu, T. S.,** Natchev, N., Koynova, T., & Cogălniceanu, D. (2023). Short-term effects of larval density on the body size and behaviour in *Triturus dobrogicus* (Kiritzescu 1903). Annals of the Academy of Romanian Scientists, series of Biological Sciences. 12(1), 21-28. https://doi.org/10.56082/annalsarscibio.2023.1.21

Manuscripts submitted and in preparation

Topliceanu, T. S., Almeida, M., Oliveira, M., Cogălniceanu, D., & Lopes, I., (submitted). The number of ethylene oxide groups of sulphate-based surfactants influence the cytotoxicity of mixed micelles to an amphibian cell line. Archives of Environmental Contamination and Toxicology.

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Stănescu, F., Băncila, R. I., Plăiașu, R., **Topliceanu, T. S.,** Vlad, S., Cogălniceanu, D. (in preparation). Variation in age and body size in *Rana temporaria* across altitudinal and latitudinal gradient

Scientific communications

1. **Topliceanu, T. S.**, Jovanović, B., Stănescu, F., Ćorović, J., Aleksić, I., Vlad, S., ... & Crnobrnja-Isailović, J. (2022). Life history changes observed over 17 years in a Common Toad population from Serbia. In Program and Book of Abstracts: the 21st European Congress of Herpetology; 2022 Sep 5-9; Belgrade, Serbia (p. 264). Belgrade: Institute for Biological Research" Siniša Stanković"–National Institute of Republic of Serbia, University of Belgrade.
2. Vizireanu, M., Telea, A., **Topliceanu, T. S.,** Cogalniceanu, D., (2021). Behavioral changes induced by predators in spadefoot toad juveniles (genus *Pelobates*). DELTAS AND WETLANDS, the 28th Scientific Symposium.
3. Stănescu, F., Vlad, S. E., Telea, A., Szekely, D., Marangoni, F., **Topliceanu, T. S.,** Cogălniceanu, D. (2021) Skeletochronology, an underrated tool for amphibian conservation in the Neotropics. SCDS-UDJG 2021, 9 th Edition, Galaţi, 10th-11th Of June 2021
4. **Topliceanu, T. S.,** Băncilă, R.I., Buhaciuc, E., Plăiașu, R., Roșioru, D., Stănescu, F., Balázs, V., Kovács, T., Cogălniceanu, D. (2015). Influența factorilor de mediu asupra parametrilor structurali populaționali la *Rana temporaria* (Amphibia, Anura). BIOTA Symposium. Biodiversitate: Tradiţii şi Actualitate, Cluj-Napoca, România.
5. **Topliceanu, T. S.**, Stănescu, F., Székely D., Székely P., Cogălniceanu, D. (2014). Age-related parameters in a *Pelobates fuscus* (Laurenti, 1768) (Anura: Pelobatidae) population from NW Romania over a decade. Abstracts, 6th Annual Zoological Congress of "Grigore Antipa" Museum (CZGA), Bucharest, Romania.