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- Doctoral thesis summary -

ADAPTIVE STRATEGIES IN AMPHIBIANS LIVING UNDER ENVIRONMENTAL STRESS

PhD Coordinator
Prof. **DAN COGĂLNICEANU**

PhD Student
FLORINA STĂNESCU

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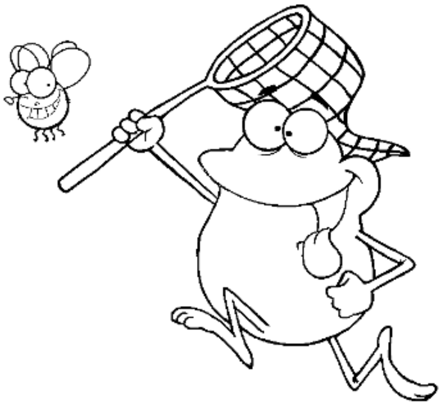

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Key words. Amphibia, adaptive strategies, structure parameters, body condition, osmotic stress, bioacoustics, distribution, conservation.

INTRODUCTION

Amphibians are ectothermic organisms, characterised by complex life cycles that involve the occurrence of some major changes (e.g. morphological, physiological, behavioural changes) throughout their ontogenetic development, usually associated to habitat type transitions (e.g. from aquatic to terrestrial) (Wilbur 1980). Ontogenetic development in amphibians is constrained by environmental humidity, given the high permeability of the egg protective layers (Duellman & Trueb 1994).

Amphibians are suffering a severe worldwide decline, being among the most affected vertebrates with nearly one-third (32.5%) of the species threatened (Houlahan et al. 2000; Stuart et al. 2004; Lannoo 2005; McCallum 2007; Collins & Crump 2009; Baillie et al. 2010). The causes of this phenomenon are complex and yet poorly understood, while the responsible environmental stressors are multiple, interact among them, and often act synergistically (Young et al. 2001); furthermore, their impact may vary regionally, both within and between species (Blaustein & Kiesecker 2002; Gascon et al. 2007; Sodhi et al. 2008; Blaustein et al. 2012). The major responsible factors documented are habitat fragmentation and destruction, climate change, UV-B radiation level increase, chemical pollution, pathogens, alien species, over-exploitation (e.g. Young et al. 2001; Baillie et al. 2010; Vitt & Caldwell 2014). Acting individually or together, environmental stressors may induce a series of direct or indirect changes upon amphibians' phenology, behaviour, physiology, metabolism (also affecting their body condition), and depending on their severity, may cause population declines (e.g. Sodhi et al. 2008; Hoffmann & Sgrò 2011; Blaustein et al. 2012).

As organisms with indeterminate growth, amphibians may adopt a diversity of strategies (or trade-offs) in resource allocation between growth and reproduction to maximize reproductive success and survival, in response to a given environment (e.g. Heino & Kaitala 1999; Fox et al. 2001; Cogălniceanu & Miaud 2003; Morrison & Hero 2003; Iturra-Cid et al. 2010; Hjernquist et al. 2012; Cogălniceanu et al. 2013). I used various approaches and methods and focused my studies upon life history trade-offs in amphibians within the context of global environmental changes. Therefore, I proposed a set of objectives that allowed me to characterize a broad range of life history strategies in the studied populations, throughout different stages of their life cycle (Fig.1).

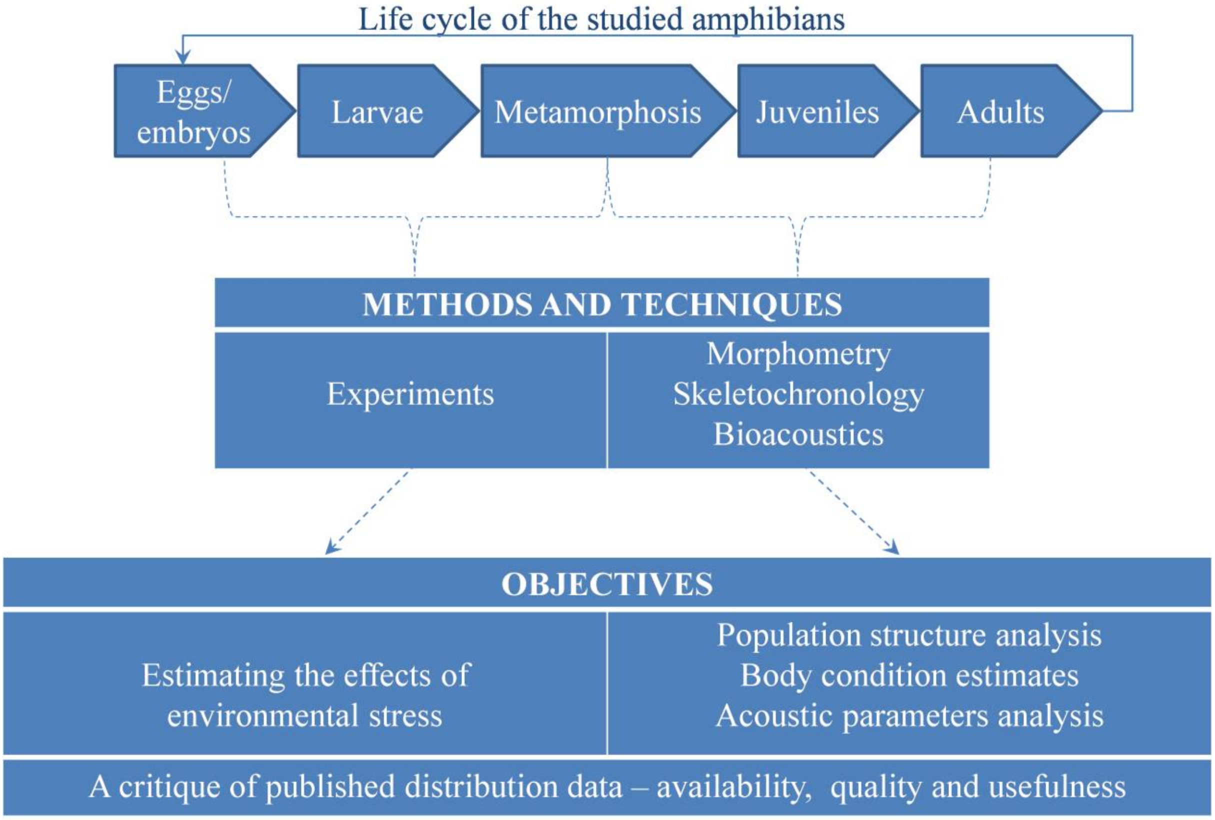

Fig. 1. The thesis work plan related to the life cycle of the studied amphibian species.

I. Population structure parameters in amphibians at the limit of their ranges

Amphibian populations living at the limits of their ranges are vulnerable to even slight variations of the environmental factors and therefore require specific conservation measures. Moreover, the study of these populations provides a window for understanding species' responses to limiting environmental factors and the strategies involved in their survival.

Population structure parameters allow identifying the trade-offs in resource allocation between growth and reproduction, and their variation in time and space, in relation to the environmental factors. I used skeletochronology to estimate age structure and associated parameters and, when possible, reproductive parameters, as an integrative demographic approach in the study of amphibian populations at the limits of their ranges.

I.1. Skeletochronology as a method of age estimation in amphibians.

I tested and compared the applicability and usefulness of the method in a series of amphibian species living in different climatic areas: temperate, subtropical, and tropical. I aimed to highlight the pros and cons of the method applied in various species, depending on their living environment.

I.2. Population structure parameters in *Dermatonotus muelleri* (Boettger 1885) (Anura: Microhylidae).

I investigated a series of life-history traits in a widely distributed but rather poorly studied fossorial anuran, *Dermatonotus muelleri*. Previous research related to this species focused mainly on behavioural aspects, while the present study is the first to explore population structure and associated parameters.

I studied: (i) age-related parameters, (ii) growth patterns, (iii) sexual size dimorphism (SSD), and (iv) female reproductive investment, in a *D. muelleri* population at the southern limit of the species' distribution range, from Arid Chaco, northern Argentina.

I.3. Population structure parameters in *Pelobates fuscus* (Laurenti, 1768) (Anura: Pelobatidae)

I analysed the variation in population structure parameters in *P. fuscus,* a species widely distributed across Europe. I compared: (i) age-related parameters and (ii) growth patterns, in populations at the western (France) and southern (Romania) limits of the species range.

II. Population body condition estimates

Body condition is related to a series of internal and external factors (e.g. metabolism, food availability, competition, climatic factors, health). Hence, it can provide important clues about the type and efficiency of the strategies adopted by an organism in a given environment. I estimated body condition in various species and analysed body condition dynamics in time and space. Finally, I discussed the results in relation to their specific environment and life strategies.

II.1. Body condition dynamics in *Pelobates fuscus* (Laurenti, 1768) (Anura: Pelobatidae).

I estimated and analysed body condition variations: (i) within populations (i.e. between sexes and over time), and (ii) between populations, in *P. fuscus* at the western (France) and southern (Romania) limits of the species range.

II.2. Body condition dynamics in *Pelobates syriacus* (Boettger, 1889) (Anura: Pelobatidae).

I estimated and analysed body condition variations between: (i) sexes, (ii) life stages, and (iii) seasons, in a *P. syriacus* population living at the northern limit of the species range.

II.3. Body condition dynamics in *Bufo bufo* (Linnaeus, 1758) (Anura: Bufonidae).

I estimated and analysed body condition variations: (i) within populations (i.e. between sexes), and (ii) between populations, in *Bufo bufo* living under different environmental conditions.

III.Estimating the effects of environmental stress

Osmotic stress

I estimated and compared the effect of osmotic stress in amphibian populations living in habitats exposed to various salinity levels. I focused my studies on early developmental stages (i.e. up to Gosner stage 25), since they correspond to the period of maximum vulnerability in the life cycle of amphibians (i.e. direct exposure, limited mobility).

III.1. The effect of salinity during early developmental stages – a comparative study in *Pelobates fuscus* (Laurenti, 1768) and *P. syriacus* (Boettger, 1889) (Anura: Pelobatidae).

I determined and compared the tolerance threshold to salinity in early developmental stages between the two spadefoots from Dobrogea (southeastern Romania) – *P. fuscus* and *P. syriacus*. I compared these results to those obtained for another three species: *Rana dalmatina*, *Bufo (Bufotes) viridis,* and *Hyla arborea* from the same area (Dobrogea), with different tolerances to salinity.

III.2. The effect of salinity during larval development in *P. fuscus* (Laurenti, 1768) (Anura: Pelobatidae).

I determined the effect of osmotic stress on larval development and survival to metamorphosis in a *P. fuscus* population from Transylvania (western Romania). I analysed: (i) time to metamorphosis, (ii) size at metamorphosis, and (iii) survival, under different salinity levels.

Hydrological stress

III.3. Facultative padeomorphosis in *Lissotriton vulgaris* (Linnaeus, 1758) (Caudata: Salamandridae).

I discussed the occurrence of facultative paedomorphosis in relation to the environmental conditions, in a common newt population monitored for the past four years.

IV. Release calls analysis

Acoustic communication is an important component of the reproductive strategies in anurans. I focused my studies on release calls, since they have received less attention and therefore are less known compared to advertisement calls. Release calls are particularly important in energy conservation during reproductive events, given that they encode and convey information related to the identity and status of the emitter (e.g. species, sex, availability to reproduce). I studied the variations of the acoustic parameters encoded within release calls and analysed their contribution to intra- and interspecific communication.

IV.1. A comparative study of release calls in *Pelobates fuscus* (Laurenti, 1768) and *P. syriacus* (Boettger, 1889) (Anura: Pelobatidae).

I investigated the informative potential of release calls and identified the important acoustic parameters for intra- and interspecific communication, in syntopic populations of the two spadefoots. I tested if the two species exhibit different strategies to minimize competition for the acoustic space.

IV.2. Release calls in *Rhinella schneideri* (Werner, 1894) (Anura: Bufonidae).

I explored release calls in *R. schneideri* from northern Argentina. I compared their acoustic patterns in two populations living in habitats within the two extreme climatic areas of the Great Chaco: Arid Chaco and Wet Chaco.

V. Harmonizing amphibians’ distribution data

I conducted a critical evaluation regarding the quality, availability, and usefulness of published distribution data. I used as a case study the published distribution data regarding amphibians in Romania after 1990. The aim of this evaluation was to promote a set of minimal standards and recommendations for both authors and editors, aiming to facilitate the integration of published data into national/international databases and enhance their usefulness for further research (e.g. meta-analyses, conservation planning).

MATERIAL AND METHODS

The studied amphibians are representatives of three anuran families: Pelobatidae (*Pelobates fuscus* and *P. syriacus*), Bufonidae (*Bufo bufo* and *Rhinella schneideri*) and Microhylidae (*Dermatonotus muelleri*), and one urodelan family: Salamandridae (*Lissotriton vulgaris*).

The studied populations inhabit the following study areas:

(1) Romania (Dobrogea and Transylvania regions):
  - the lagoon system between Sinoe and Vadu (Constanța county) - one population of *Pelobates syriacus, P. fuscus* and *Lissotriton vulgaris*;
  - Luncavița (Tulcea county) - one *Bufo bufo* population;
  - Sighișoara and Saschiz (Mureș county) – two *B. bufo* populations;
  - Sălicea (Cluj county) - one *P. fuscus* population.

(2) France (Saint-Avold) – one *P. fuscus* population. The data set regarding individual age estimated through skeletochronology and morphometric data for this population were kindly provided by Dr. Christophe Eggert. The study area is located in northeastern France, at the western limit of the species' range, and is described in detail by Eggert & Guyétant (1999; 2002; 2003).

(3) Argentina:
  - Misión Nueva Pompeya (Chaco) - one *Dermatonotus muelleri* population and one *Rhinella schneideri* population.
  - Corrientes (Corrientes) - one *R. schneideri* population.

The methodology used throughout the studies is in agreement with all the relevant regulations and received the approval of the Ethics Committee of the Faculty of Natural and Agricultural Sciences, Ovidius University, Constanţa. The collecting permits were granted by the relevant authorities: Danube Delta Biosphere Reserve Administration, Măcin Mountains National Park Administration, Dirección de Fauna y Áreas Naturales Protegidas of the Chaco province.

I performed the statistical analyses using PASW Statistics for Windows, version 18.0 (Chicago: SPSS Inc 2009), and R environment, version 3.0.3 (R Core Team 2014) with the following packages: stats (R Core Team 2014), car (Fox & Weisberg 2011), psych (Revelle 2014), FSA (Ogle 2014) and nlstools (Baty et al. 2014). I tested all data for normality and homoscedasticity using Shapiro-Wilk and Levene tests and chose the subsequent statistic tests accordingly. The significance level was $\alpha = 0.05$.

Skeletochronology. Skeletochronology, alongside capture-mark-recapture studies, has generally proved to be a useful non-lethal method to estimate age and growth-related parameters in amphibians (Halliday & Verrell 1988; Smirina 1994; Sinsch 2015). I adapted the classical protocol proposed by Castanet and Smirina (1990), depending on the particularities of each study species (e.g. the size and density of the bone tissue, storage conditions). I decalcified the bone tissues using 5% nitric acid, rinsed and kept them in distilled water overnight, followed by inclusion in Tissue-Tek® O.C.T. ™ Compound (Sakura Finetek), freezing, and sectioning using a Tehsys 3000 CR cryotome. I cut fine cross-sections (12-14 µm), stained in Ehlrich's haematoxylin and rinsed with distilled water. Cross-sections with the smallest marrow cavity and the thickest cortical bone were permanently mounted on slides using Aquatex® (aqueous mounting agent for microscopy, Merk Milipore) and photographed using an Olympus® E-620 microscope-mounted camera (Olympus® CX 31 microscope with Quick Photo Micro 2.3 software).

Age-related parameters. I computed the following parameters: age of sexual maturity, mean lifespan (i.e. mean of age distribution), longevity (i.e. maximum age), and potential reproductive lifespan (i.e. the time span between the moment of sexual maturity and the maximum age observed in the sample).

Growth patterns. I computed the von Bertalanffy growth model (Bertalanffy 1938) following Beverton & Holt (1957) equation: $SVL_t = SVL_{max} \times (1 - e^{-k \times (t - t0)})$, where SVL_t is the expected or average SVL at time (or age) t, SVL_{max} is the asymptotic average SVL, k is the growth rate coefficient and t_0 is the time or age when the average SVL was zero. I fitted the model and estimated growth parameters (VBGPs) by nonlinear least squares regression. Two estimated VBGPs were significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap.

Body condition estimates. I log-transformed (log10) all data and removed the outliers. I computed the residual body condition index (BCI) based on the linear regression between snout vent length and body mass (e.g. Denoël et al. 2002; Băncilă et al. 2010). BCI values had a normal distribution and were independent of body size.

I considered that BCI > 0 indicated a good condition, while BCI < 0 indicated a poor condition of the tested animals (Jakob et al. 1996, Schulte-Hostedde et al. 2001; Blackwell 2002). I used BCI to analyze body condition dynamics: within populations, between - (i) sexes, (ii) life stages, (iii) seasons (i.e. spring – summer – autumn), (iv) years, and (v) between populations living under different environmental conditions. I used Student's (equal variances) and Welch's (unequal variances) *t* tests, and one-way analyses of variance (ANOVAs) with LSD (i.e. least significant difference) (equal variances) or Tamhane T2 (unequal variances) post-hoc tests, where appropriate.

Experiments. Starting 2013, I determined the salinity of a wide range of aquatic habitats (n = 20) using an Oakton® SalTestr 11 salinity meter. I conducted the survey within and outside the study areas, within the sympatric zone of the two spadefoots, *P. fuscus* and *P. syriacus* (i.e. south and southeastern Romania). The survey period covered the reproduction and larval development periods of the study species. The salinity of the aquatic habitats varied from 0.4 to more than 10‰. The high variability observed in habitat salinity emphasized the need of estimating the effects of osmotic stress on reproductive success. I used two experimental designs with various salinity treatments to test the effects of osmotic stress during developmental stages.

I obtained the desired salinity treatments using deionized or partially dechlorinated tap water (0.0 – 0.4 ‰) and Ocean Fish marine salt for ornamental aquariums. I maintained water parameters (water level, temperature, salinity) at a constant level during the experiments, while photoperiod exposure followed the natural cycle of the animals.

Acoustic analysis. I recorded the animals both in the field and in the laboratory. In the laboratory, I made the recordings inside a semi-anechoic experimental container, and used the artificial amplexus method to obtain the release calls (Leary 1999; Tada et al. 2001; Bowcock et al. 2008). I used a Marantz-PMD660 portable solid-state recorder and a Sennheiser-ME66 directional microphone, at a 44.1 kHz sampling frequency and 16-bit resolution. The distance between the tip of the microphone and the tested animal was constant, at 10 cm. For each recording, I measured air temperature, and weighed and measured each individual. The recording time did not exceed 60 s per individual, in order to reduce the discomfort induced by handling.

I analyzed the sounds using Raven Pro 1.4 (Bioacoustics Research Program 2011). I measured the temporal parameters from oscillograms, and the spectral parameters from spectrograms obtained through short-time Fourier transforms. Spectrograms were configured using Hanning function, at a window size of 256 or 512 samples.

I described the calls using the terminology proposed by Duellman and Trueb (1994).

Evaluation of the published distribution data. I designed and applied a set of criteria to published scientific papers (Table 1) in order to grade and rate the main errors encountered in the published data that diminish their subsequent use. I chose as a case study 80 papers published after 1990 regarding amphibians' distribution in Romania. I evaluated the (i) format, (ii) quality, and (iii) completeness of the provided data and information. The purpose of this assessment was to develop and promote a set of common minimal standards and recommendations for authors and editors, in order to enhance published data integration into national/international databases and facilitate their further use.

Table 1. Criteria used in evaluating the papers published after 1990 regarding amphibians' distribution in Romania. The maximum possible score is 40.

Crt.no.	**Evaluated criteria**	**Possible score**
1	Geographic coordinates	5
	UTM 5 × 5 km grid cell	3
	UTM ≥ 10 × 10 km grid cell	2
	UTM/Google Earth map	1
2	Locality and county/district	1 - 5
3	Occurrence date	1 - 5
4	Material and methods	1 - 5
5	Habitat description	1 - 5
6	Occurrence statistics	1 - 5
7	Altitude	5
8	Supplementary information	5

Evaluation method:

Criterion 1. I assigned the maximum 5-point score when the exact location of the occurrence was provided through geographic coordinates; I assigned a minimum score of 1 point when the location was indicated only by using a UTM grid/ map. When several sub-criteria were met (e.g. geographic coordinates and map), I only took into consideration the highest rated sub-criterion.

Criteria 2-6. I assigned the maximum 5-point score when clear and precise information were provided. Ambiguous expression or providing incomplete information decreased the score.

Criterion 7. Papers that provided the altitude of the exact location of occurrence received 5 points.

Criterion 8. Papers that provided supplementary information also received 5 points: collected specimens and the corresponding museum collection, notes regarding killed animals (e.g. road kills, vandalism, predatorism), abundance, sex, life-stage, human impact (e.g. pollution, habitat destruction), other relevant information for the reported species.

RESULTS AND DISCUSSION

I. Population structure parameters in amphibians at the limit of their ranges

I.1. Skeletochronology as a method of age estimation in amphibians.

I tested and compared the applicability and usefulness of skeletochronology in a series of amphibians from different climate areas (Fig. 2): temperate (*Rana temporaria, Bufo bufo, Pelobates fuscus, P. syriacus, Triturus cristatus, Ichthyosaura alpestris*), subtropical (*Rhinella schneideri, Dermatonotus muelleri, Chacophrys pierotti*, *Melanophryniscus montevidensis),* and tropical (*Smilisca phaeota, Pristimantis achatinus, Trachycephalus jordani, T. typhonius, Ceratophrys stolzmanni*).

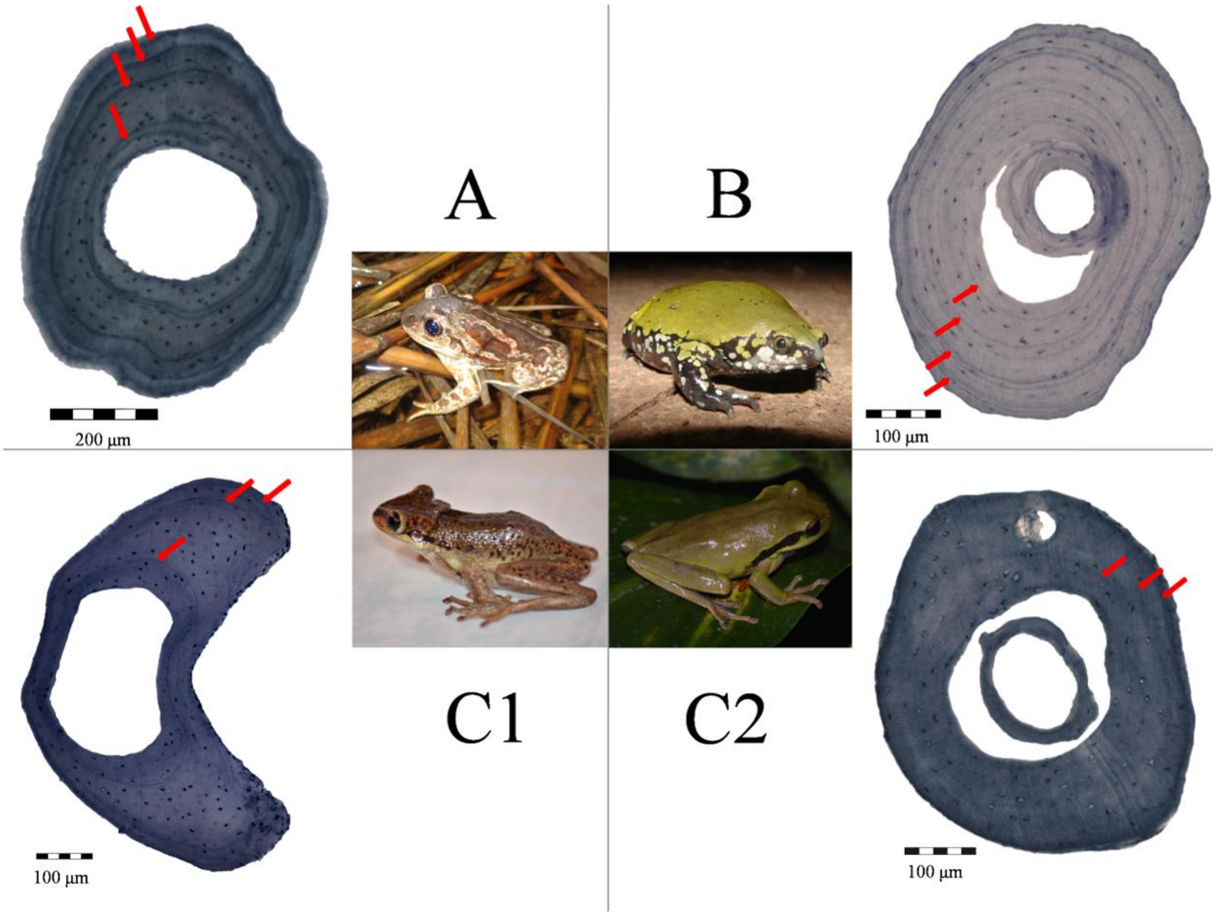

Fig. 2. Cross-sections through phalanges: *Pelobates fuscus* (A – temperate climate), *Dermatonotus muelleri* (B – subtropical climate), *Trachycephalus typhonius* (C1 – tropical climate, dry forest) and *Smilisca phaeota* (C2 – tropical climate, wet forest). Lines of arrested growth are indicated by red arrows.

I assessed the usefulness of skeletochronology for the first time in the five tropical amphibians. Best candidates for studies that involve the use of skeletochronology are species living in habitats that exhibit a pronounced seasonality, such as amphibians from the temperate region. More discrete seasonal variations also allow the use of skeletochronology (see the case of amphibians from tropical wet forests); however, the method still needs validation through capture-mark-recapture studies and requires some technical improvements (e.g. the staining technique). Moreover, skeletochronology should be used with caution in tropical species (as many of them are arboreal) and the effects of phalanx amputation should be tested in advance.

The major constraint of the method relates to estimating the age in long-lived individuals, with a lifespan longer than 8-10 years, or characterized by very short growth periods, as LAGs become difficult to distinguish, tightly packed towards the periosteum's border (Sinsch 2015). This shortcoming can be overcome by using complementary methods, such as capture-mark-recapture.

I.2. Population structure parameters in *Dermatonotus muelleri* (Boettger 1885) (Anura: Microhylidae).

The studied *D. muelleri* population from the Arid Chaco showed a significant female-biased SSD, despite similar age-related parameters (i.e. mean lifespan, age at sexual maturity, reproductive lifespan, and longevity) (Fig. 3) and growth rates (Fig. 4). These results suit best the hypothesis that SSD might stem from differences in the growth pattern before sexual maturity (Halliday & Verell 1988; Shine 1990).

Males and females attained sexual maturity at a similar age (2 years) and both had a low reproductive lifespan (3 years). The most frequent observed age class was represented by 3 years old individuals, indicating that most of the active animals were in their second reproductive year. This might be an indicator of high environmental pressure affecting the survival rates in younger age classes, and explain the high reproductive investment observed in females. Conversely, the high reproductive costs may determine a considerable reduction of lifespan. The high reproductive investment observed in *D. muelleri* can also be explained in the terms of r-selection (Pianka 1970), where in a variable and unpredictable environment with mortality not depending on the population density and at a low intra- and interspecies competition, the selection is directed to the production of larger number of smaller offspring. This study indicates that the short reproductive lifespan justifies the high reproductive investment in *D. muelleri* and this trade-off represents an adaptive response to the specific environmental conditions from the Arid Chaco.

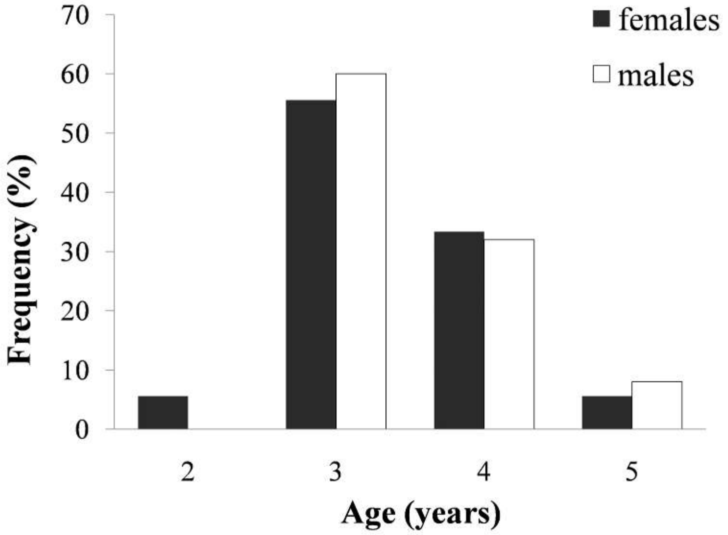

Fig. 3. Age structure of the studied *D. muelleri* population.

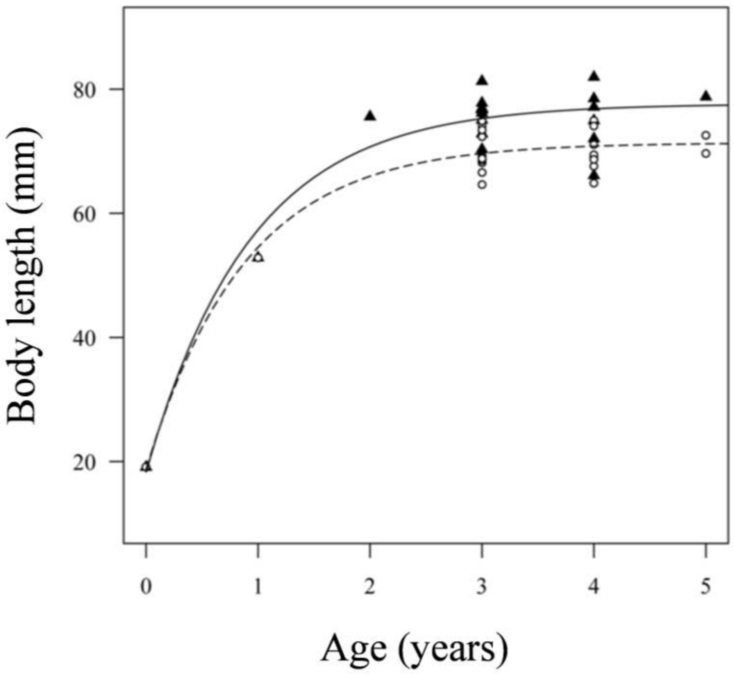

Fig. 4. Growth described by von Bertalanffy's model in *D. muelleri* males (white circles, dashed line) and females (black triangles, solid line).

I.3. Population structure parameters in *Pelobates fuscus* (Laurenti, 1768) (Anura: Pelobatidae).

The compared study in *P. fuscus* populations at the limit of the species' range highlighted some important variations in the age parameters and growth patterns, and provided interesting clues regarding their adaptive strategies. Females from the western (St. Avold) and southeastern (Constanța) peripheral populations were older and had longer reproductive lifespans. In males, longevity and reproductive lifespan were higher in the southern peripheral population. Here, age structure was dominated by 5 and 6-year reproductive adults, compared to 3 and 4 years in the other two populations. Growth patterns varied significantly between the three study populations, with the lowest growth rates observed at the lower limit of the range (Fig. 5). I suggest that interspecific competition and the highly variable environmental conditions at the southern range limit are the main explanatory factors for these results.

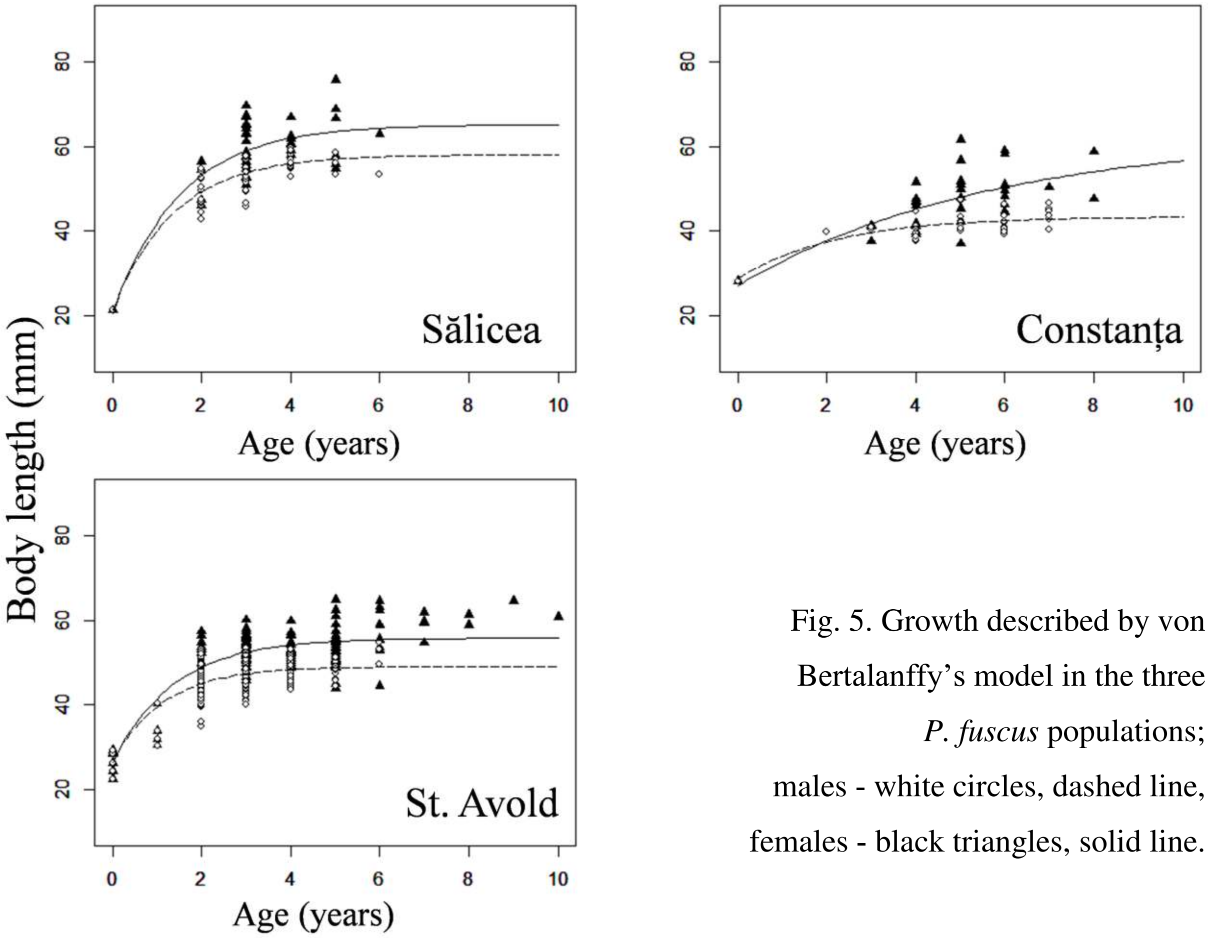

Fig. 5. Growth described by von Bertalanffy’s model in the three *P. fuscus* populations; males - white circles, dashed line, females - black triangles, solid line.

Species with a broad distribution range, such as *P. fuscus*, occupy habitats characterized by varying environmental conditions, and the effects of these variations are reflected upon population structure parameters and the selected adaptive trade-offs. Hence, the compared analysis of age and growth parameters in populations distributed across a wide range allows identifying and explaining the selected life strategies. Understanding the relationships between the intrinsic (i.e. specific) and extrinsic (i.e. biotic and abiotic) factors that led to certain adaptive strategies, is of major interest for evolutionary biology and ecology studies, as well as for conservation science.

II. Population body condition estimates

II.1. Body condition dynamics in *Pelobates fuscus* (Laurenti, 1768) (Anura: Pelobatidae).

The results showed that the western population (St. Avold) was in a significantly better condition compared to those living at the lower range limit (Sălicea and Constanța) (Fig. 6).

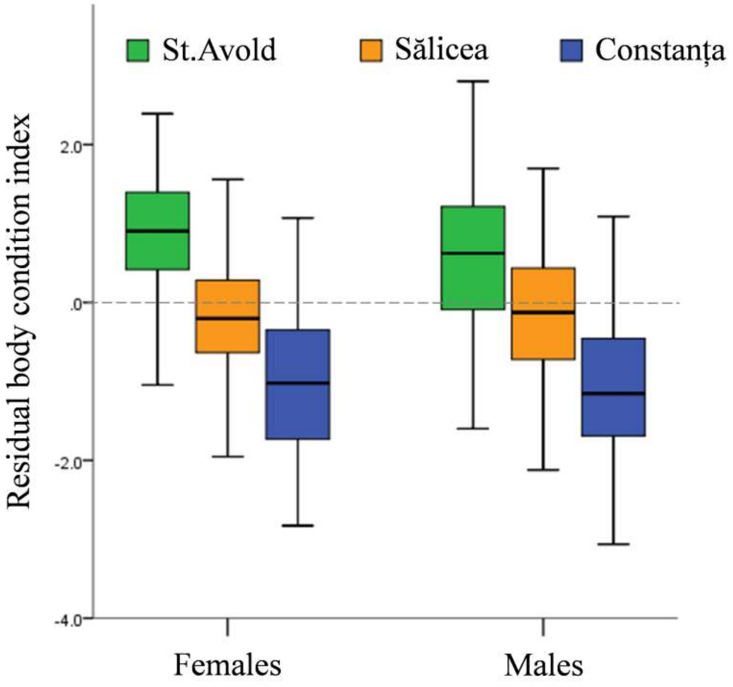

Fig. 6. Body condition in males and females of the three *P. fuscus* populations: Saint-Avold, Sălicea and Constanța.

Sălicea population experienced significant variations in body condition over time, between 2000-2004 and 2012-2014. The habitat changes and contractions occurred through the continuous urbanization of the study area during the study period could explain these variations.

The southeastern peripheral population (i.e. Constanța) exhibited the lowest body condition. Iosif et al. (2014) showed that the distribution pattern is not at equilibrium with climate at the southern limit in *P. fuscus*, and at the northern limit in *P. syriacus*. Since these areas of their distribution ranges overlap with the species' sympatric zone, I suggest interspecific competition as one of the major selective forces acting upon Constanța population. Competition and predatorism by larger species (i.e. *P. syriacus*, *Pelophylax* kl. *esculentus*), the specific harsh environment of the Black Sea coastal area (i.e. highly variable water level, frequent strong winds, aridity and the partly salinized habitats) might also explain the observed pattern.

II.2. Body condition dynamics in *Pelobates syriacus* (Boettger, 1889) (Anura: Pelobatidae).

I showed that seasonal dynamics affected equally the body condition of males and females. Even though the reproductive investment during spring is significantly higher in females than in males (Cogălniceanu et al. 2013), this pattern was not reflected in the estimated BCI.

Freshly metamorphosed animals had a poor body condition, which might be explained by stress factors occurring in the aquatic habitat, such as high larval density, competition, predatorism. Furthermore, the massive migration of the metamorphs indicates that the larvae metamorphosed synchronously (Fig. 7). Synchronous metamorphosis might therefore be another explanatory factor of the observed low body condition in juveniles, since not all individuals had equal odds to gain sufficient energetic stores prior to their transition to the terrestrial phase. I suggest that the low body condition resulted as a trade-off experienced by larvae between early metamorphosis in a poorer condition and the risk of being trapped and experience high mortality rates in an unsuitable aquatic environment (e.g. competition, predatorism, desiccation risk, high temperature).

Fig. 7. Freshly metamorphosed *P. syriacus* during a massive migration event, towards the surrounding terrestrial habitats, June 2013, Grindul Lupilor.

Adult individuals had a lower body condition during spring and autumn (Fig. 8) which may be explained by the specific climate of the study area (i.e. Black Sea coast). Hot and dry summer conditions reduce the ability of the animals to store energetic resources. Hibernating with a low energetic supply can have negative consequences on both future reproductive success, and survival (Reading & Clarke 1995; Reading 2007). In turn, warm winters result in increasing the metabolic rate and implicitly in resource supply depletion during hibernation, thus affecting the amount of energy available for future reproduction (Ryser 1989).

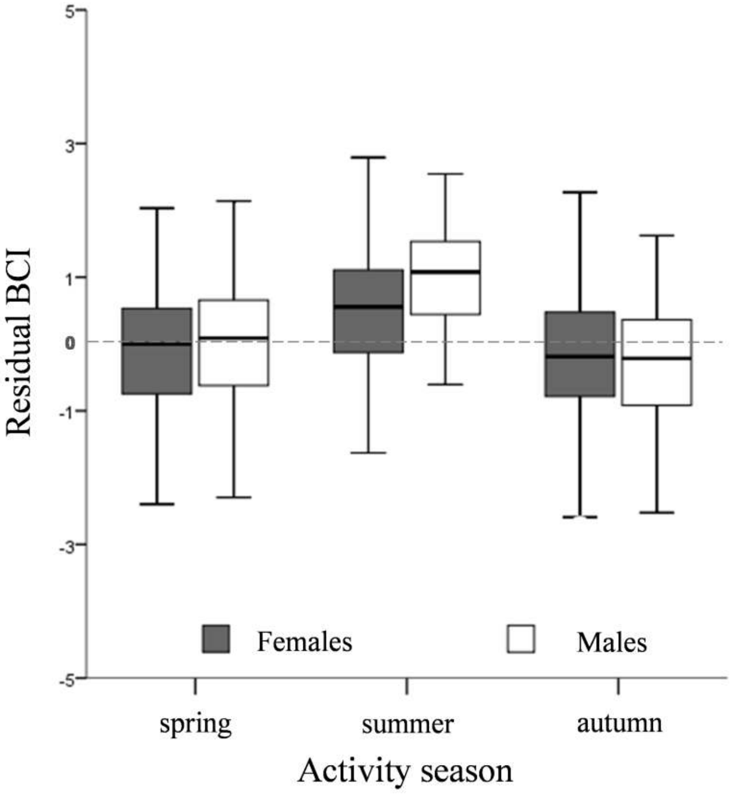

Fig. 8. Seasonal dynamics in *P. syriacus* body condition, males and females from Grindul Lupilor population.

This study suggests that *P. syriacus* from Grindul Lupilor are experiencing unfavourable climate conditions. Given the actual context of global climate change, and the forecasted increases in temperature and rainfall extremes, *P. syriacus* populations at the northern limit of the species' range might be under threat.

II.3. Body condition dynamics in *Bufo bufo* (Linnaeus, 1758) (Anura: Bufonidae).

The present study indicated a sex-differentiated impact of specific environmental stressors on body condition in *B. bufo*. Females' body condition was similar in the three study populations, while males were in a significantly better condition in northern Dobrogea (Luncavița) than Transylvania (Saschiz and Șercheș) (Fig. 9).

The poor condition observed in Transylvanian males could be the result of competitive behaviour during reproduction, but this is not sufficient to explain the significant difference compared to Luncavița males. I suggest another explanation, related to differences in the observed sex ratio. All three populations were characterized by a male-biased sex ratio: Luncavița – 3.76♂♂:1♀, Șercheș - 4.45♂♂:1♀, Saschiz – 8.33♂♂:1♀. I hypothesize that the highly male-biased sex ratios in Șercheș and Saschiz have resulted in higher energetic costs in males engaged in reproductive events, since competition for females was harsher in these populations. Davies and Halliday (1979) showed that only a low percent of *B. bufo* males successfully acquired a mate during reproductive events (20.5%), and 38.5% of the successful males managed to get a partner through competitive behaviour (i.e. dislocation of other males from amplexus, male-male fight for female possession). Thus, females appear to be a limiting resource in male reproductive success in natural *B. bufo* populations (Davies & Halliday 1979; Höglund 1989).

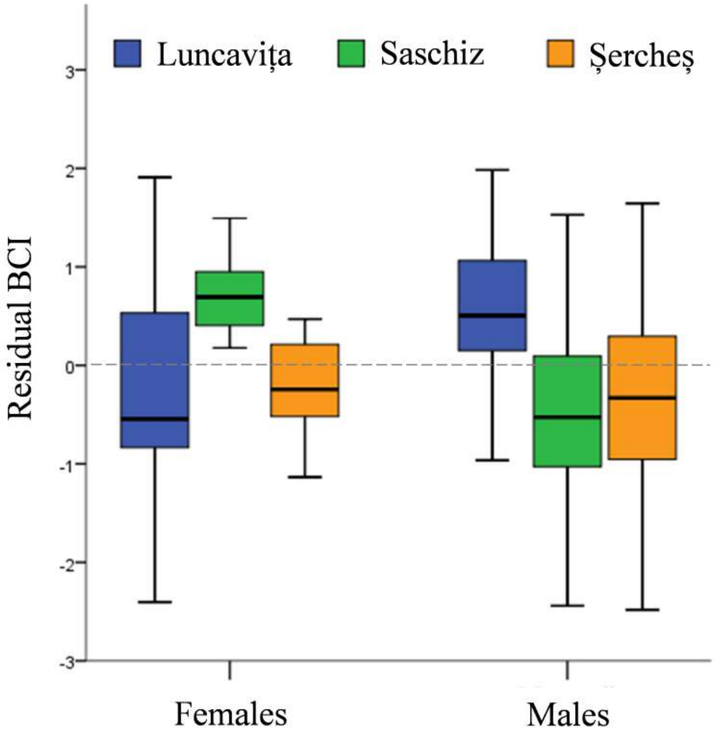

Fig. 9. Body condition in males and females of the three *B. bufo* populations: Luncavița (Dobrogea), Saschiz and Șercheș (Transylvania).

Sex ratio observed in this species is highly variable, ranging from 1.9 up to 8.5♂♂:1♀ (Arntzen 1999). The main proposed causal factors are - earlier sexual maturation of males, compared to females (Hemelaar 1988; Reading 1991); females do not reproduce every year (Kuhn 1994; Schmidt & Anholt 1998); females change spawning habitats more often than males (Hodrova 1985); females are subjected to higher mortality rates than males (Gittins 1983; Hodrova 1985). The present study indicates that high male-biased sex ratios may become an environmental stressor during reproduction and negatively influence males' body condition.

III. Estimating the effects of environmental stress

Osmotic stress

III.1. The effect of salinity during early developmental stages – a comparative study in *Pelobates fuscus* (Laurenti, 1768) and *P. syriacus* (Boettger, 1889) (Anura: Pelobatidae).

For most species in the experiment, embryos could not develop under extreme salinity conditions (i.e. 9 ‰), except in *Bufo (Bufotes) viridis* (Table 2). I arbitrarily characterized the salinity tolerance threshold (STT) of a species as weak, moderate or high, depending on the survival rates recorded in the high salinity treatment (i.e. 6‰):

- weak STT: < 10%
- moderate STT: 10% - 50%
- high STT: > 50%

Table 2. Survival (%) to Gosner 25 stage in the two spadefoots, compared to other amphibians in Dobrogea and their salinity tolerance threshold (STT). N = number of replicates per treatment.

Species/	**Treatment (‰)**				
Site	**0**	**3**	**6**	**9**	**STT**
P. fuscus/ Viile, N=4	82.1±10.2	9.3±0.8	0.0	0.0	weak
R. dalmatina/ Viile, N=4	86.1±9.6	69.1±4.7	0.0	0.0	weak
P. fuscus/ Vadu, N=5	98.0±1.5	89.7±17.3	3.2±2.2	0.0	weak
H. arborea/ Lupilor, N=3	91.8±2.8	96.1±2.6	14.2±5.7	0.0	moderate
P. syriacus/ Lupilor, N=9	92.0±3.5	90.3±4.1	43.8±17.2	0.0	moderate
B. viridis/ Ceamurlia, N=5	94.8±4.1	94.7±1.9	93.1±4.4	0.9±0.7	high

The higher STT recorded in *P. syriacus* may be one of the major explanatory factors for the species' higher abundance in the coastal areas compared to *P. fuscus* (Fig. 10). Furthermore, Székely et al. (2010) showed that *P. syriacus* larvae are characterized by phenotypic plasticity that allows early metamorphosis in order to avoid mass mortality due to habitat desiccation. Together, these results indicate that *P. syriacus* is characterized by a set of more advantageous physiological adaptations compared to *P. fuscus*, which allow colonizing harsher environments, where for instance, interspecific competition is lower.

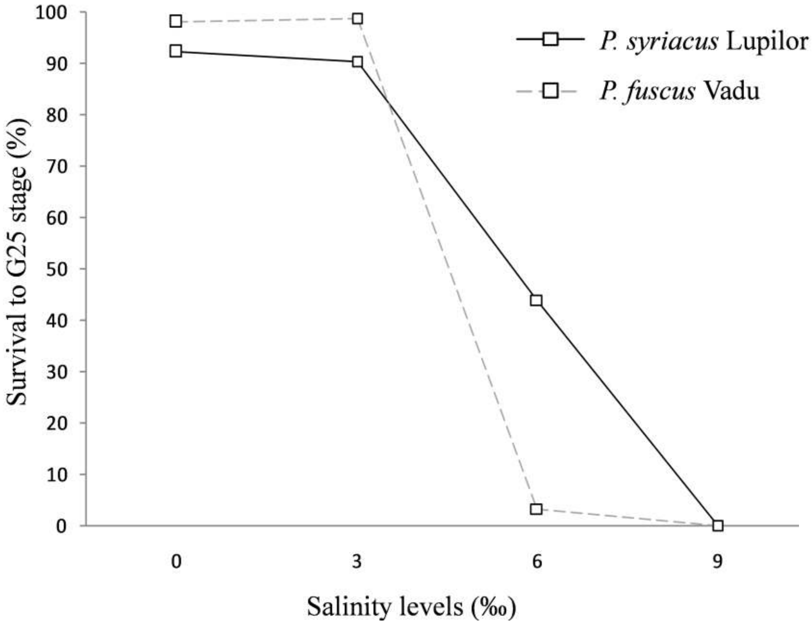

Fig. 10. Survival rates under different salinity treatments, during early developmental stages of the two spadefoots from the Black Sea coast.

III.2. The effect of salinity during larval development in *Pelobates fuscus* (Laurenti, 1768) (Anura: Pelobatidae).

The results of this study showed that *P. fuscus* tadpoles have a tolerance threshold for brackish waters up to 4‰, above which survival is impaired. I found no significant variations in the time to metamorphosis (Kruskal-Wallis χ = 0.283, p = 0.868), body mass (Kruskal-Wallis χ = 3,332, p = 0,190) and body length of the metamorphs (Kruskal-Wallis χ = 2,010, p = 0,366), between treatments. Tadpole survival varied across the treatments, with the sharpest decline recorded in the 8‰ salinity treatment (i.e. 100% mortality).

There are only few studies related to osmotic stress in genus *Pelobates*, focused on post-metamorphosis stages, during the terrestrial phase of the animals. In a study using adult *P. syriacus*, the individuals could be acclimated to solutions up to 450 mOsm/L NaCl (Shpun et al. 1993). This is a moderate threshold compared to *Bufo (Bufotes) viridis* individuals that acclimated to solutions up to 800 mOsm/L NaCl within the same experiment. Compared to these results, I found a relatively low osmoregulatory capacity in *P. fuscus* tadpoles, corresponding to only 136.8 mOsm/L NaCl. Thus, I emphasise the need of monitoring and conservation of adequate spawning sites in order to enhance the survival of this species, especially in those regions where important population declines have already been reported.

Hydrological stress

III.3. Facultative padeomorphosis in *Lissotriton vulgaris* (Linnaeus, 1758).

Facultative paedomorphosis resulted as a trade-off faced by larvae between metamorphosis and transition to an arid terrestrial environment and remaining into water as paedomorphs and risk desiccation but having abundant food resources. I observed a rather balanced metamorph-to-paedomorph ratio in the studied population. Although small, the paedomorphic sample was female-biased, suggesting that the response of the population to this trade-off may be sex-dependent.

Facultative paedomorphosis was previously reported in Romania from smooth newt populations inhabiting both shallow temporary and permanent aquatic habitats with abundant vegetation. Facultative paedomorphosis was expressed at different extents within each of these populations. Most of the records (66.6%) are from Danube Delta, a 5500-km^2 wetland characterized by an unpredictable and variable hydrological regime. Aquatic predators (mainly fish) were present in 83.3% of the sites.

Although it has been proven that the presence of aquatic predators, mainly fish, negatively affects newt populations and implicitly paedomorphic individuals (e.g. Denoël et al. 2005a; b), the presence of dense vegetation cover seems to mitigate their impact by providing favourable microhabitats (Hartel et al. 2007).

Facultative paedomorphosis was observed in a low number of populations and only in few areas in the country. Since long periods of selection against paedomorphosis might lead to the loss of this phenotype (Semlitsch & Wilbur 1989; Denoël et al. 2005a), I emphasize the importance of long-term monitoring and further research of their habitats in order to better understand and protect these populations.

IV. Release calls analysis

IV.1. A comparative study of release calls in *Pelobates fuscus* (Laurenti, 1768) and *P. syriacus* (Boettger, 1889) (Anura: Pelobatidae).

I provided for the first time a quantitative description of release calls in two syntopic spadefoots, *Pelobates fuscus* and *P. syriacus*. Release calls had a species-specific structure, but there was no clear structural differentiation among sexes (Fig. 11). Temporal acoustic parameters encoded species-specific information in both species, while in *P. fuscus* they were also sex-specific. Dominant frequency was similar in both species and sexes, and was independent of body size, which suggests that the structural and temporal particularities of the acoustic parameters are sufficient for the acoustic space partitioning, in syntopic populations.

Release calls in *P. fuscus* and *P. syriacus* play a significant role in both intra- and interspecific communication. The quantitative description of the major acoustic parameters will provide the necessary basis, important in further comparative studies.

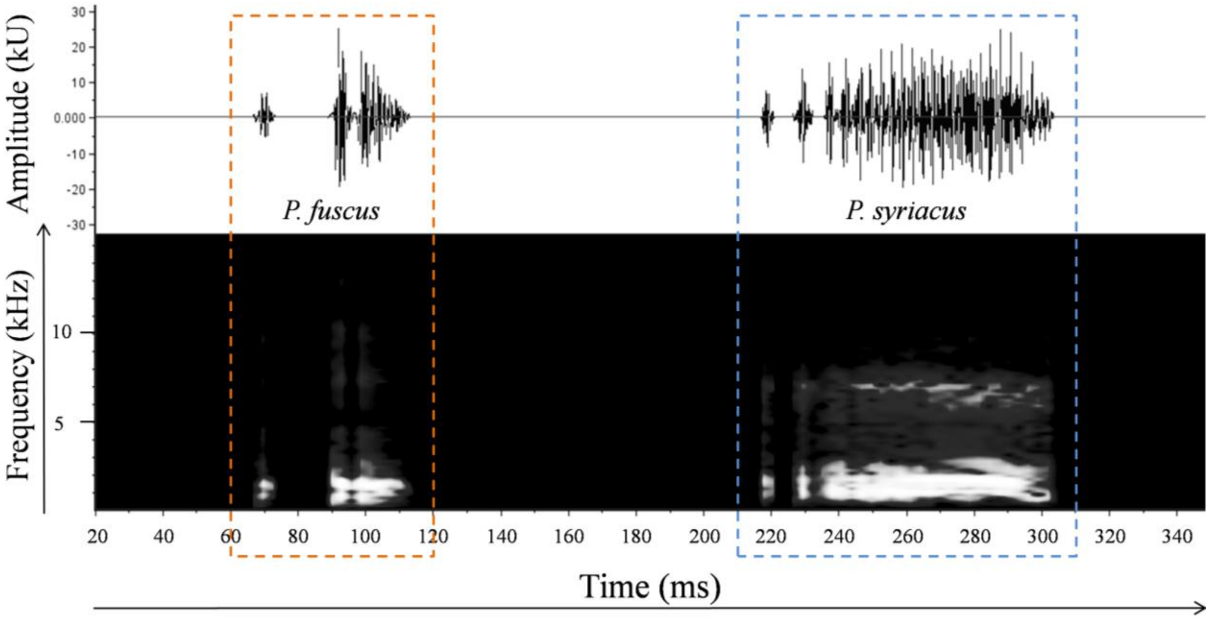

Fig. 11. Oscillogram (up) and spectrogram (down) of an acoustic unit in a series of release calls. *P. fuscus* – orange frame, *P. syriacus* – blue frame.

IV.2. Release calls in *Rhinella schneideri* (Werner, 1894) (Anura: Bufonidae).

I provided for the first time a quantitative description of release calls in *R. schneideri* males. Release calls consisted of various combinations of pulsed notes, referred to as simple notes. I considered such a simple note as the basic acoustic unit of release calls in this species (Fig. 12). Simple notes were typically emitted:

- **Grouped** - two, three consecutive simple notes; I considered these groups as **compound notes**. These compound notes were in turn emitted in various combinations;
- **Series** – simple notes emitted successively, at rather equal, well defined time intervals;
- **Trill** - simple notes emitted successively, at significantly shorter time intervals than within a series.

Release calls elicited by the males from the two study populations varied in their structure. Males from Corrientes emitted mainly simple notes as series or trills, rarely interposed with compound notes, while males from Chaco emitted mainly compound notes. Always, release calls were accompanied by vibrations of the body. Nevertheless, release calls were characterized by a high structural variability, and in some cases, a single male could elicit all the observed structural types (i.e. single simple notes, compound notes, series, trill), during one recording session (i.e. 60 seconds).

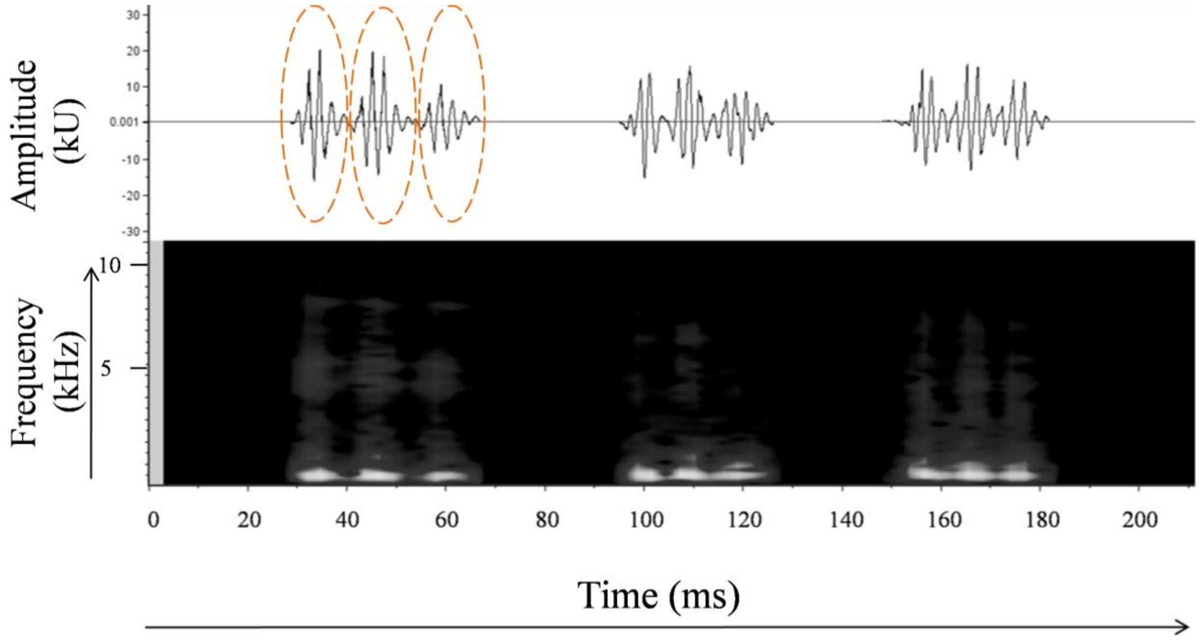

Fig. 12. Oscillogram (up) and spectrogram (down) of three simple, pulsed notes - basic acoustic units of the release calls in *Rhinella schneideri*. Pulses are indicated by the orange frames, within the oscillogram.

The results indicate the existence of different dialects for the two distinct climate areas (i.e. Arid Chaco, Wet Chaco). I suggest that the differences in morphometric parameters associated to acoustic production might explain the observed interpopulation variability. Thus, the environmental conditions that acted as selective forces upon morphometry also reflected into the pattern of the acoustic signals.

V. Harmonizing amphibians’ distribution data

The assessment study highlighted a series of difficulties in collecting, use, and exploitation of published distribution data. The main shortcomings were related to the format and completeness of the data and associated metadata (e.g. ambiguous, incomplete, and scattered throughout text, inconsistent format).

Following the proposed set of criteria, the 80 evaluated papers had an average score of 24.8 ± 6.3 points (mean ± SD); 51.3% of these papers had scores below average. Overall, the evaluated papers had higher degrees of fulfilment of the following criteria 4. Material and methods (95%), 3. Occurrence date (85%) and 2. Locality and county/district (79%); conversely, the graded papers achieved the lowest scores for criteria 1 and 7 (Table 3).

Table 3. Overall degree of fulfilment of the assessed criteria (n = 80 published papers), expressed as percentage of the global maximum possible score for each criterion.

Crt.no.	Evaluated criteria	Degree of fulfilment (%)
1	Geographic coordinates	18.75
	UTM 5 × 5 km grid cell	2.50
	UTM ≥ 10 ×10 km grid cell	6.25
	UTM/Google Earth map	25.00
2	Locality and county/district	78.75
3	Occurrence date	85.00
4	Material and methods	94.75
5	Habitat description	59.00
6	Occurrence statistics	69.75
7	Altitude	22.50
8	Supplementary information	60.00

I proposed two sets of standards and recommendations for publishing faunistical and occurrence data. The proposed guidelines address both authors and editors, and aim to enhance published data further integration and exploitation to their full potential.

GENERAL GUIDELINES (faunistical data)

- original data and information should be easily and clearly delineated from those already published;
- where possible, the associated metadata regarding habitat, environmental conditions at the moment of the study, life stage of the observed individuals, number of individuals, etc., should be exhaustively provided;
- It is highly desirable to provide supplementary information, such as killed animals (e.g. road kills, intentional killing, vandalism, predatorism/cannibalism), the presence

of predatory species, syntopic species, human impact, collected specimens (if applicable) – the name of the institution and the collection where the specimens were deposited and specimen code, photographs, video/audio recordings.

- Raw distribution data and associated metadata should be made available online, as annexes in an accessible format (Wieczorek et al. 2012; Costello & Wieczorek 2014).

SPECIFIC GUIDELINES (occurrence data)

- occurrence site should be indicated by precise geographic coordinates;
- it is recommended to provide information regarding the instrument used to determine the geographic coordinates of the occurrence site, the brand and type (e.g. GPS, Google Earth, Google Maps, etc.);
- where, for various reasons, the author does not want to provide the exact site of occurrence using geographic coordinates (e.g. to protect a study population), it is recommended to use UTM grids (e.g. 5×5 km) indicating: the geographic coordinates of the corresponding UTM cell centroid, the code of the UTM cell, the source for the UTM grid (e.g. Lehrer & Lehrer 1990), the name of the nearest locality and the corresponding county/district; in such cases it is also recommended to provide the altitude of the exact occurrence site, and not the altitude of the UTM cell centroid;
- for transects up to 500 m within a narrow altitudinal range, the author may provide the geographic coordinates corresponding to the centroid of the transect;
- it is recommended to provide the geographic coordinates in a consistent format; WGS 1984 datum with decimal degrees is preferred, as it is the most commonly used by GPS units around the globe;
- altitude should be provided for the exact occurrence site;
- it is desirable to provide supplementary information regarding the occurrence site: locality, county, toponymy (where applicable), and avoid using exclusively local toponymy, which are difficult to locate for instance, by non-native researchers;
- occurrence date should be accurately provided (e.g. dd-mm-yyyy);
- it is recommended to provide occurrence data for each species (where applicable), in a centralized format, most desirable as a table, at least for the essential data such as occurrence site, altitude, date.

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Anfibios y Reptiles de Rumania, su Diversidad y Conservación. Visiting researcher (Romania - Argentina bilateral cooperation project PN II CAPACITĂŢI) - 19 November 2013, National University of Misiones: Faculty of Exact Sciences, Chemistry, and Natural Sciences, Posadas (Argentina) and 11 December 2013, National University of North-East: Faculty of Exact, Natural and Agricultural Sciences, Corrientes (Argentina).