

Age, size and body condition do not equally reflect population response to habitat change in the common spadefoot toad *Pelobates fuscus*

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ABSTRACT

Urbanization impacts biodiversity both directly through physical expansion over land, and indirectly due to land use conversion and human behaviors associated with urban areas. We assessed the response of a common spadefoot toad population (*Pelobates fuscus*) to habitat loss and fragmentation resulting from urban development by studying changes in size, body condition and age parameters. We compared samples collected in the early 2000s (sample A) and later on during 2012–2014 (sample B). The terrestrial habitats in the study area were severely reduced and fragmented due to the expansion of the human settlement. We found no significant differences in the age parameters between the two sampling periods; the median lifespan shortened from 3.5 (sample A) to 3.0 years (sample B), while the other age parameters were similar in both samples. In contrast, snout-vent length, body mass and body condition experienced a significant decrease over time. Our results suggest that changes in body size and body condition, rather than age parameters, better reflect the response of the common spadefoot toad population to declining habitat quality. Therefore, body measurements can provide reliable estimates of the impact of habitat degradation in amphibian populations.

Subjects Biodiversity, Conservation Biology, Zoology, Environmental Impacts, Population Biology

Keywords Amphibia, Fitness, Growth, Habitat fragmentation, Habitat loss, Life history, Monitoring, Romania, Skeletochronology, Urbanization

INTRODUCTION

Amphibians are suffering a severe worldwide decline, being the most affected group of terrestrial vertebrates, with nearly one-third (32.5%) of the species threatened (*Houlahan et al., 2000; Stuart et al., 2004; McCallum, 2007; Collins & Crump, 2009; Baillie et al., 2010*). The main responsible factors are habitat fragmentation and destruction, climate

Submitted 3 February 2021

Accepted 4 June 2021

Published 15 July 2021

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Academic editor

Laura Brannelly

Additional Information and
Declarations can be found on
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DOI 10.7717/peerj.11678

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change, UV-B radiation level increase, chemical pollution, pathogens, alien species, and over-exploitation (e.g., *Young et al., 2001*; *Baillie et al., 2010*; *Vitt & Caldwell, 2014*; *Nunes et al., 2019*; *Falaschi et al., 2020*). These stressors induce a series of direct or indirect changes upon amphibians' phenology, behaviour, physiology, metabolism, and depending on their severity, may cause population declines and local or regional extinction (e.g., *Sodhi et al., 2008*; *Hoffmann & Sgrò, 2011*; *Blaustein et al., 2012*; *Pimm et al., 2014*; *Catenazzi, 2015*; *Grant et al., 2016*).

Urbanization is a recently recognized key driver of global environmental change that impacts biodiversity, both directly through physical expansion over land, and indirectly due to land use conversion and human behaviors associated with urban areas (*Chace & Walsh, 2006*; *Elmqvist et al., 2013*; *Brice, Pellerin & Poulin, 2017*). Habitat loss and degradation caused by spatial expansion can eliminate organisms outright, but mainly they alter the conditions that a species requires to survive. There is increasing evidence that, along with direct effects in the form of habitat loss and fragmentation, negative impacts of urbanization on biodiversity extend to pollution (chemical, light, noise), microclimate change, introduction of alien species and intensification of traffic (*Elmqvist, Zipperer & Güneralp, 2016*). Urbanization was also found to cause a decline in species richness in several taxonomic groups (*McKinney, 2008*; aquatic macroinvertebrates: *Thornhill et al., 2017*; amphibians and reptiles: *Cordier et al., 2021*), as well as changes in ecological processes (*Miles et al., 2019*, *Fisogni et al., 2020*).

A variety of studies have confirmed that landscape changes have a negative impact on amphibian populations, affecting their abundance (e.g., *Pellet, Guisan & Perrin, 2004*; *Pillsbury & Miller, 2008*), age structure (*Bionda et al., 2018*; *Jennette, Snodgrass & Forester, 2019*), and body condition (*Janin, Léna & Joly, 2011*). Due to reduced habitat quality, amphibian populations under urban pressure show in some cases smaller individual body size and poorer body condition compared to more natural ecosystems (e.g., *Jennette, Snodgrass & Forester, 2019*; *Bókony et al. 2018*; *Lunghi et al., 2018*; *Kärvelo, Laurila & Höglund, 2019*), although the magnitude and even the direction of the response varies according to species resilience (*Iglesias-Carrasco, Martín & Cabido, 2017*; *Murphy et al., 2016*). As organisms with indeterminate growth, amphibians adopt a diversity of life-history strategies, which usually entail trade-offs in resource allocation between growth and reproduction, in order to maximize reproductive success and survival in response to a given environmental variation (e.g., *Heino & Kaitala, 1999*; *Fox, Roff & Fairbairn, 2001*; *Cogălniceanu & Miaud, 2003*; *Morrison & Hero, 2003*; *Iturra-Cid, Ortiz & Ibargüengoytia, 2010*; *Hjernquist et al., 2012*). Temperate amphibians have a complex bi-phasic life-cycle, and the most evident trade-off between the aquatic and terrestrial stages is reflected in body size at metamorphosis, effects of aquatic stressors being carried over into terrestrial growth, survival, and performance (*Székelly et al., 2020*; *Thompson & Popescu, 2021*).

These trade-offs are ultimately reflected in the individual fitness, and affect growth and demographic parameters, like age structure (*Roff, Heibo & Vøllestad, 2006*). Therefore, these parameters can be used to detect population responses to a changing environment (e.g., *Karraker & Gibbs, 2011*; *Zhang & Lu, 2012*; *Amat & Meiri, 2018*). A deteriorated

environment associated to anthropogenic disturbance can determine reductions in body size either because individuals lack the adequate resources necessary for growth, or are confronted with disease and contaminants (Kärverno, Laurila & Höglund, 2019), or because a larger proportion of the population consists of younger individuals due to decreased longevity (Bionda et al., 2018).

Body condition is considered both a measure of individual fitness and an indicator of environmental stress (e.g., Băncilă et al., 2010), since it is related to a series of internal and external factors such as metabolism (MacCracken & Stebbings, 2012), health (Ullman-Culleré & Foltz, 1999), energy reserves (Denoël et al., 2002; Schulte-Hostedde et al., 2005), food availability and habitat quality (Sztatecsny & Schabetsberger, 2005; Janin, Léna & Joly, 2011; Scheele et al., 2014; Unglaub et al., 2018), and climatic factors (Reading, 2007). Thus, changes in body condition can provide valuable information regarding the efficiency of the life strategy adopted by a population in a given environment.

Skeletochronology, alongside capture-mark-recapture studies, is a widely used non-lethal method to estimate age-related parameters in amphibians (Smirina, 1994; Sinsch, 2015). Individual age can be assessed based on the presence of annual lines of arrested growth (LAGs) deposited in the bone tissue during periods of inactivity like hibernation and aestivation (Smirina, 1994; Sinsch et al., 2007). Additionally, changes in the rate of growth as a result of transitions from a life-stage to another (for example reaching sexual maturity) also affect the bone structure, allowing an estimation of the age when an individual becomes adult (Smirina, 1994). Age and size at which transitions occur often change plastically in response to environmental conditions (Roff, 1992), and their study allows us to determine population responses and trends over time. As such, age-related parameters obtained by skeletochronology proved valuable tools when studying population responses to environmental stress like habitat loss and degradation (Middleton & Green, 2015), urbanization (Yetman, Mokonoto & Ferguson, 2012), pollution (Spear et al., 2009; Kaczmarek et al., 2016; Otero et al., 2018), parasitic infestation (Gustafson et al., 2015; Sinsch, Kaschek & Wiebe, 2018), or pathogens (Campbell et al., 2018). They were also successfully used to evaluate performance and well-being of amphibian populations inhabiting differently-managed landscapes (e.g., Orchard, Tessa & Jehle, 2019), or to understand the effects of habitat quality on amphibian life histories (Sinsch et al., 2007).

We assessed the response of a common spadefoot toad population to habitat loss and fragmentation caused by urban development (i.e., increase of associated transport infrastructure and constructed areas) over more than a decade. We assessed changes in age, size, and body condition as proxies of resilience to habitat loss and fragmentation. We hypothesized that the population would respond through (1) decrease in size and body condition, and (2) decrease in average lifespan and longevity. Although the sampling was not continuous during the study timeframe, we expected to find significant changes of life-history parameters, given the relatively short average life-span of the studied species of 5 years (Cogălniceanu et al., 2014).

MATERIALS & METHODS

Study species

The common spadefoot toad *Pelobates fuscus* (Laurenti, 1768) is a widespread species ranging throughout most of Europe (Dufresnes *et al.*, 2019a; 2019b). It is a highly specialized, nocturnal, burrowing anuran, with strong population declines due to habitat loss (Eggert *et al.*, 2006; Temple & Cox, 2009). Breeding occurs in permanent or temporary ponds. The breeding season is short, lasting up to 1–2 weeks in the early spring, followed by a long larval stage (Cogălniceanu *et al.*, 2014). Landscape features have a strong influence on the spadefoot toads' distribution because they have low dispersal abilities, and adults spend most of their terrestrial life close to the breeding ponds (Nielsen & Dige, 1995). During their terrestrial life, suitable habitat for the species is represented by open landscapes with loose, sandy soils, including pasturelands and cultivated fields, forested areas with >75% canopy cover and scrubby areas being avoided, and roads with medium and high traffic loads representing important dispersal barriers in addition to causing high mortality (Eggert, 2002, Nyström *et al.*, 2002).

Study area & habitat changes over time

The study was conducted in northwestern Romania, at the outskirts of Sălicea, Cluj County, Romania (46°40'58.4" N; 23°32'38.8" E, 720 m a.s.l.). Since 2008, the locality is part of one of the 19 territorial units of the metropolitan area of Cluj–Napoca, the fourth most populous city in Romania. The population decreased during 1995–2008 and increased during 2009–2016, but the built-up areas for both primary and secondary residence, as well as entertainment and lodging infrastructure grew constantly since 1995.

The study site consisted of a freshwater permanent pond originally surrounded by steppe grasslands and forests, situated in the vicinity of a medium traffic interprovincial/county road (Fig. 1). This was the only suitable breeding site in the whole area where large numbers of spadefoot toads were observed and thus we can assume that no immigration occurred from nearby areas. Most specimens were caught in and around the pond of approximately 2,700 m², whose area varied over the years depending on the amount of rainfall and snow. Besides *P. fuscus*, the pond was also used as a breeding site by *Lissotriton vulgaris* (Linnaeus, 1758), *Triturus cristatus* (Laurenti, 1768), *Bufo bufo* (Linnaeus, 1758), *Hyla arborea* (Linnaeus, 1758), *Rana temporaria* (Linnaeus, 1758), and *Rana dalmatina* (Fitzinger, 1838).

We evaluated habitat changes within a radius of 300 m from the centre of the breeding pond, in order to quantify the reduction in the availability and quality of surrounding terrestrial habitats. We chose this distance according to the average seasonal/daily movement recorded for this species (Glandt, 1986; Eggert, 2002) and because this species was shown to exhibit a high degree of breeding site fidelity (Hels, 2002). We extracted satellite images available in Google Earth Pro V. 7.3.3.7786 at three different moments in time, relevant for our study timeframe: the first image was taken on March 2003 (Google Earth, 2003), the second on May 2011 (Google Earth, 2011) and the third on August 2014 (Google Earth, 2014). We re-georeferenced the three images in a GIS



Figure 1 Changes of the breeding pond and surrounding area over a decade. Photo credit: Diana Székely. [Full-size !\[\]\(b345a1c4255362eec3746050dd71ccac_img.jpg\) DOI: 10.7717/peerj.11678/fig-1](https://doi.org/10.7717/peerj.11678/fig-1)

environment and digitized the landscape features at an eye altitude of 1.8 km. We extracted the area covered by four landscape features, namely forest, pasture, transport and urban. Forest includes compact patches of deciduous trees dominated by *Quercus* spp. but also fragments of shrubs dominated by *Corylus avellana* and *Rubus* spp. We digitized pasture features as open habitat patches known for seasonal grazing. Transport includes all transport infrastructure (i.e., both paved and unpaved, secondary roads), while urban includes a mosaic of constructed areas (i.e., buildings, yards, construction sites, associated paved surfaces other than roads). The area occupied by each type of landscape feature was expressed as a percentage.

Sampling

Sampling was performed each year in April, during the short breeding season, at two moments in time: between 2000 and 2004 (hereafter moment A, sample A), and between 2012 and 2014 (hereafter moment B, sample B). Sampling and measurements were done by the same two researchers (DS and PS), using the same methods, during both study moments (A and B). They performed Visual Encounter Surveys in and around the pond between 19:00–02:00 and caught the frogs by hand or with a dip net. Only adult individuals were included in our study. Sex was determined based on the presence/absence of the humeral glands which are typical to sexually mature males. Snout-vent length (SVL) was measured with dial calipers at 0.1 mm precision and body mass (BM) with a portable electronic balance at 0.1 g precision, for a total of 279 individuals (120 females, 159 males) in sample A, and 94 individuals (42 females, 52 males) in sample B (Table S1). Females were weighed before egg deposition. The longest toe of the right forearm was clipped and stored in 70% alcohol from 58 individuals (no sex and SVL data assigned) in sample A, and 94 individuals (42 females and 52 males) in sample B. Sex and size data was assigned to each collected toe only in sample B, but not A. At moment A, toe-clipping was only

used for the purpose of individual identification and only a small part of these toes was preserved. Therefore, we could not account for sex when we estimated and compared age parameters between the two sampling moments, nor compare growth based on size-at-age data between the two periods.

The methods used in this study received full approval from Ovidius University Constanța through the Ethics Committee of the Faculty of Natural and Agricultural Sciences. Field sampling was approved through Ministerial Order no. 1173/2010.

Skeletochronology and age-related parameters

We estimated age from the collected toes using skeletochronology, based on the protocol of [Castanet & Smirina \(1990\)](#) with slight modifications, as described in [Stănescu et al. \(2016\)](#). The bone tissue was decalcified in 5% nitric acid, rinsed and kept in distilled water overnight, followed by inclusion in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek, Torrance, CA, USA). We cut fine cross-sections (12–14 μm) using a freezing microtome (Tehsys, CA, USA 3,000 CR) and stained them in Ehrlich's haematoxylin. Cross-sections with the narrowest marrow cavity and the widest cortical bone were permanently mounted on slides using Aquatex® (aqueous mounting agent for microscopy, Merck Millipore, Burlington, MA, USA) and photographed using an Olympus® E-620 microscope-mounted camera (CX 31 microscope, Olympus®, Shinjuku City, Tokyo, Japan) and Quick Photo Micro 2.3 software. Three independent observers (FS, DC and ST) counted the LAGs in 3–5 sections per individual. Each LAG was considered to represent 1 year of age.

We computed the following age-related parameters for each of the two samples (A and B): 1. average lifespan (mean and median age); 2. longevity (i.e., maximum age observed); 3. age at sexual maturity, considered as the youngest age class in our sample, either observed or inferred from the bone growth pattern following [Smirina \(1994\)](#); 4. potential reproductive lifespan (i.e., time span between the age at sexual maturity and the maximum age observed in the sample). In addition, we computed: 5. the annual adult survival rate (S) according to Robson and Chapman's formula (see [Miaud, Guyétant & Elmberg, 1999](#)): $S = T / (R + T - 1)$, where $T = N_1 + 2N_2 + 3N_3 + 4N_4 + \dots$, $R = \sum N_i$, N_i = number of individuals in age group i ; 6. adult life expectancy (ESP), the expected total longevity of individuals which have reached maturity, estimated using Seber's formula ([Seber, 1973](#)): $ESP = 0.5 + 1 / (1 - S)$. ESP is the expected average age and differs from the "longevity" value that is simply the highest recorded age, which can be affected by sample size since the probability of encountering older individuals increases with sample size.

Since sex and SVL data were only assigned to the bone samples collected at moment B, comparisons between males and females regarding age distribution and growth were only performed for this sample. We applied a von Bertalanffy's growth model ([von Bertalanffy, 1938](#)) following [Beverton & Holt \(1957\)](#): $SVL_t = SVL_{max} \times (1 - e^{-k \times (t - t_0)})$, 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.

To calibrate the growth model, we used measurements of SVL at metamorphosis (i.e., 21.26 mm) provided by [Stănescu et al. \(2013\)](#). We fitted the von Bertalanffy's growth model and

estimated growth parameters (VBGPs) by nonlinear least squares, and two estimated VBGPs were considered significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap (Stănescu *et al.*, 2016). The growth model and parameters were estimated in R Studio v. 1.1.423 (R Core Team, 2017), with the packages FSA (Ogle, 2016) and nlstools (Baty *et al.*, 2015).

Size-related parameters

We computed the residual body condition index (BCI) based on the linear regression between log-transformed (log10) values of SVL and BM (e.g., Denoël *et al.*, 2002; Băncilă *et al.*, 2010). Since *P. fuscus* shows a significant sexual size dimorphism females being both larger and heavier than males, we computed the BCI separately for males and females. The BCI values had a normal distribution and fulfilled the required assumptions for a true measure of body condition (Blackwell, 2002; Schulte-Hostedde *et al.*, 2005; Băncilă *et al.*, 2010). We considered that positive BCI values indicated a good condition, while negative values indicated a poor condition of the sampled individuals (Jakob, Marshall & Uetz, 1996; Schulte-Hostedde *et al.*, 2005; Blackwell, 2002).

We computed a sexual dimorphism index (SDI) of SVL and BM following Lovich & Gibbons (1992), with the results arbitrarily defined as positive when females are the larger sex and negative in the converse situation.

We performed the statistical analyses using IBM SPSS Statistics for Windows version 20.0 (IBM Corp., Endicott, NY, USA 2011). We tested all data for normality and homoscedasticity using Shapiro–Wilk and Levene tests respectively, and chose the subsequent statistical tests accordingly. The significance level was set at $\alpha = 0.05$.

RESULTS

Habitat change

The favorable habitat (i.e., pasture) decreased from 74.2% to 46% between 2003 and 2014, while transport and urban areas increased from 4.1% to 32.8% (Fig. 2). During a short period of time (2011–2014) urban areas more than doubled. Access to the breeding site was more restricted and involved crossing secondary roads, with a higher mortality risk due to increased traffic in the area.

Morphological parameters

Body size parameters are presented in Table 1. SVL and BM in both sexes were lower in sample B, compared to sample A (females: SVL, $t_{(53.5)} = 2.385$, $p = 0.021$, Cohen's $D = 0.51$; BM, $t_{(52.9)} = 2.647$, $p = 0.011$, Cohen's $D = 0.57$; males: SVL, $t_{(209)} = 2.521$, $p = 0.012$, Cohen's $D = 0.39$; BM, $t_{(72.6)} = 2.918$, $p = 0.005$, Cohen's $D = 0.51$) (Fig. 3). Individuals from sample B had a significantly lower body condition both in males ($t_{(208)} = 2.826$, $p = 0.005$, Cohen's $D = 0.45$), and females ($t_{(160)} = 2.673$, $p = 0.008$, Cohen's $D = 0.47$). Despite the decrease in body size and body condition, the SDI remained unchanged with females being larger (SDI_{SVL} was 1.128 in sample A and 1.124 in sample B) and heavier (SDI_{BM} was 1.492 in sample A and 1.476 in sample B) than males.

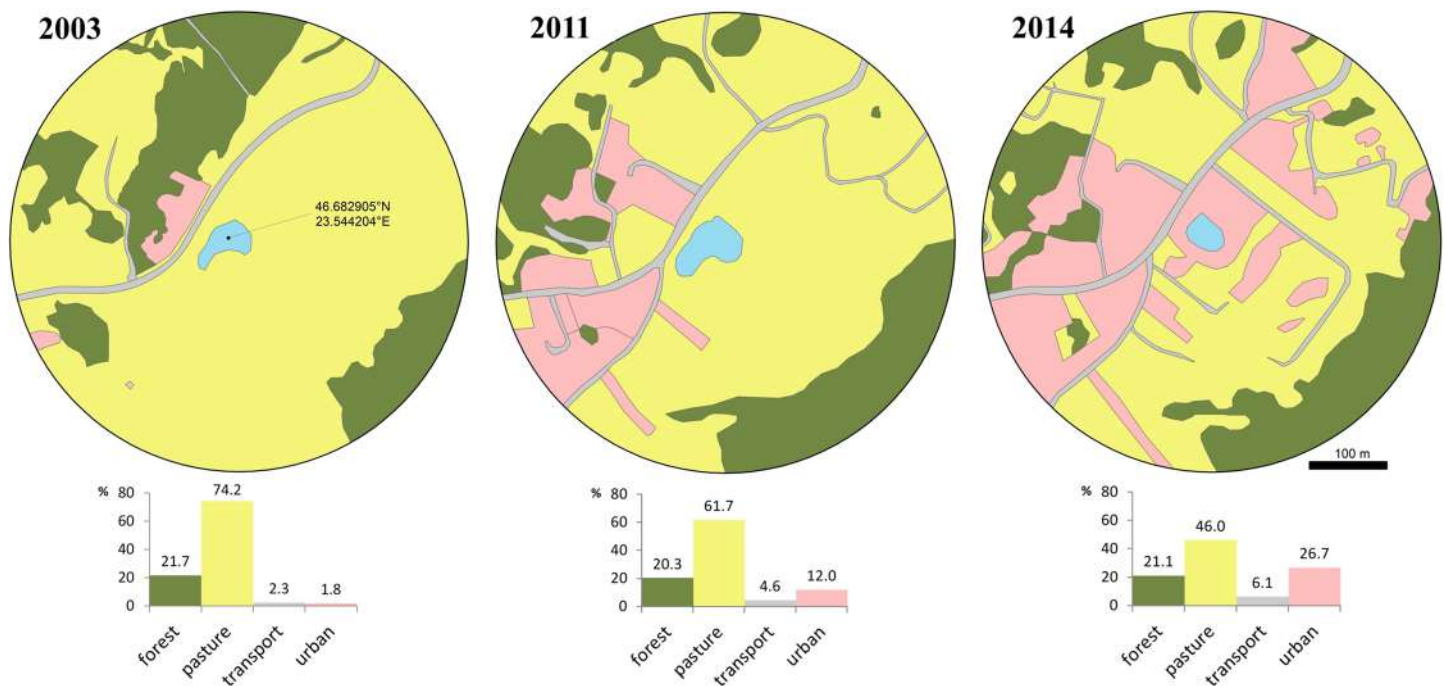


Figure 2 Loss of available terrestrial habitat between 2003–2014, caused by urban development within a 300 m radius from the breeding pond. Habitat cover is expressed as percentage at three moments (2003, 2011, 2014): forest, pasture, transport and urban. Transport includes all transport infrastructure (i.e., both paved and unpaved, secondary roads), urban includes all constructed areas (i.e., buildings, yards, construction sites, associated paved surfaces other than roads). [Full-size DOI: 10.7717/peerj.11678/fig-2](https://doi.org/10.7717/peerj.11678/fig-2)

Table 1 Size-related parameters in the studied *Pelobates fuscus* population, at two sampling moments (A and B).

Sample	Sex	n	SVL (mm)	BM (g)
A (2000–2004)	Males	159	55.2 ± 3.5 45.0–63.0	21.3 ± 3.9 13.0–30.0
A (2000–2004)	Females	120	62.3 ± 3.9 52.0–72.0	31.8 ± 6.0 16.5–45.0
B (2012–2014)	Males	52 (51 in BM)	53.6 ± 4.3 42.8–59.9	19.1 ± 4.8 10.0–28.3
B (2012–2014)	Females	42	59.8 ± 6.2 45.9–75.6	27.6 ± 9.6 12.2–54.4

Note:

SVL = snout-vent length (mm); BM = body mass (g); n = sample size; values are provided as mean ± standard deviation and range (min-max). The sampling interval (years) is provided within parentheses for each sampling moments.

Age-related parameters

Age could be assessed in all 152 individuals (58 in sample A and 94 in sample B) (Table 2 and Table S2). Endosteal resorption was observed in all samples, but did not interfere with LAG count (Fig. 4). There were no significant differences in the age distribution between the two sampling moments (Kolmogorov–Smirnov test: $Z = 0.670$, $p = 0.760$) (Fig. 4 left), nor between males and females from sample B ($Z = 0.756$, $p = 0.634$) (Fig. 4 right). The average lifespan was lower in sample B, but the difference was not significant

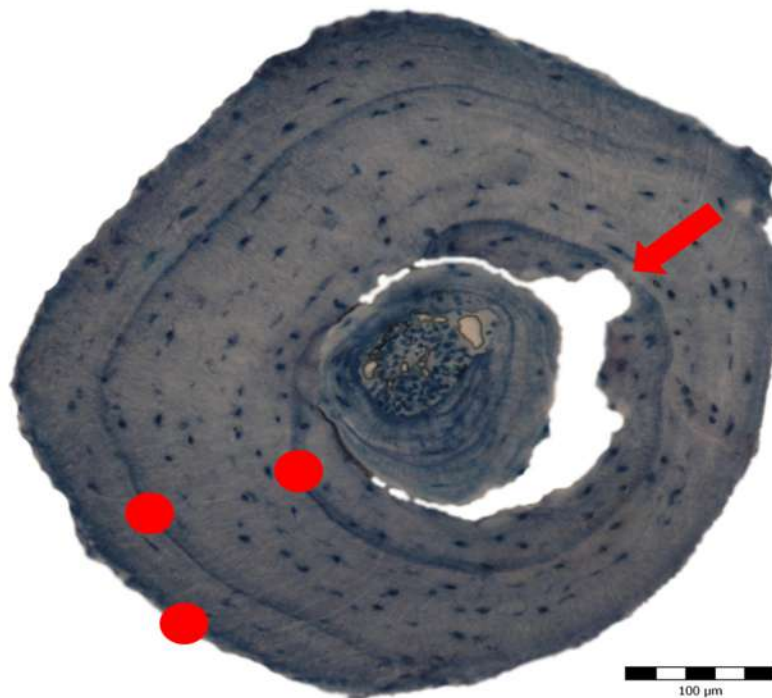


Figure 3 Cross section obtained through skeletochronology from the mid-diaphysis of a phalanx from a 3-year old adult *Pelobates fuscus* sampled in 2004. The red dots mark the lines of arrested growth (LAGs) deposited during hibernation, each corresponding to 1 year of life. The red arrow indicates the beginning of endosteal resorption.

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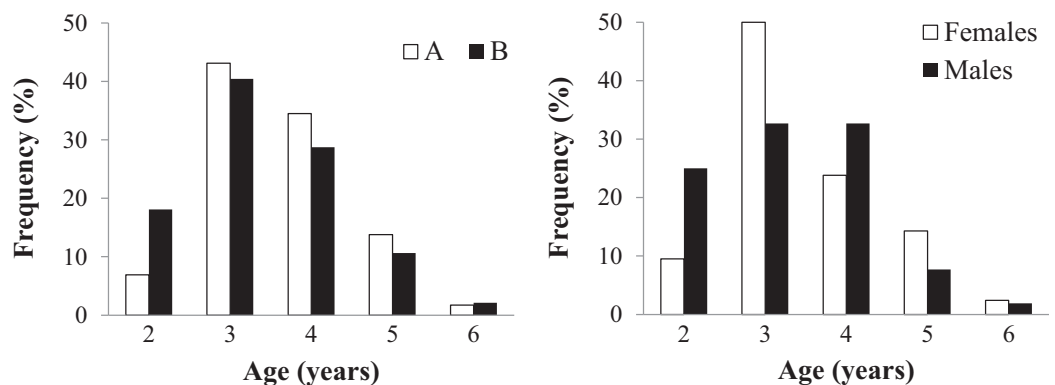


Figure 4 Age distribution of the studied *Pelobates fuscus* population, following skeletochronological assessment. Left - Age distribution for the pooled sample of males and females, at each of two moments in time–2000–2004 (sample A, white bars) and 2012–2014 (sample B, black bars). Right - Age distribution of males (black) and females (white) only within sample B (2012–2014).

Full-size [DOI: 10.7717/peerj.11678/fig-4](https://doi.org/10.7717/peerj.11678/fig-4)

(Mann–Whitney $U = 2359$, $Z = -1.471$, $p = 0.141$); the other age-related parameters were similar in both samples (Table 2).

The relation between age and SVL fitted von Bertalanffy's growth model in both sexes (sample B, Fig. 5). The asymptotic average SVL was significantly higher in females

Table 2 Age-related parameters in the studied *Pelobates fuscus* population, at two sampling moments (A and B).

Age-related parameters	Sample A Males and females	Sample B Males and females	Sample B Males	Sample B Females
Sample size n	58	94	52	42
Median life span	3.5	3.0	3.0	3.0
Average life span $\pm$ SD	3.60 ± 0.88	3.38 ± 0.94	3.28 ± 0.99	3.5 ± 0.94
Age at sexual maturity	2 (85%) 3 (15%)	2 (93.3%) 3 (6.7%)	2 (97.6%) 3 (2.4%)	2 (88%) 3 (12%)
Longevity	6	6	6	6
Potential reproductive lifespan	4	4	4	4
Annual survival rate (S)	0.62	0.58	0.56	0.60
Adult life expectancy (ESP)	3.13	2.89	2.81	3.03

Note:

Sample A: 2000–2004; Sample B: 2012–2014. Values are provided in years. Average lifespan is provided as mean $\pm$ standard deviation.

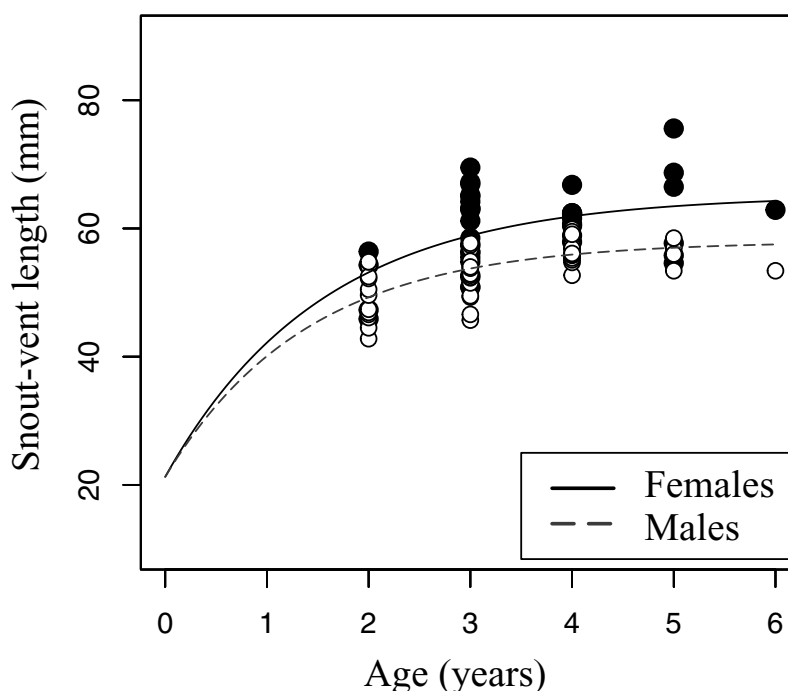


Figure 5 Growth pattern of *Pelobates fuscus* males and females within sample B (2012–2014). The growth pattern was computed following von Bertalanffy's equation, using size-at-age data from sample B. Males ($n = 52$) are represented with white circles and dashed line, and females ($n = 42$) with black circles and continuous line. [Full-size !\[\]\(0f04eac0f44b45c27c855fb13a3dc0b4_img.jpg\) DOI: 10.7717/peerj.11678/fig-5](https://doi.org/10.7717/peerj.11678/fig-5)

(males: $SVL_{max} = 58.0$ mm, CI 95% [55.4–60.5]; females: $SVL_{max} = 65.1$ mm, CI 95% [59.6–70.7]), while the growth rate coefficient was similar in both sexes (males: $k = 0.72$, CI 95% [0.51–0.92]; females: $k = 0.65$, CI 95% [0.34–0.97]).

DISCUSSION

We found that, in the studied common spadefoot toad population, individuals responded to habitat degradation by a significant decrease in size and body condition, and a rather subtle decrease of average lifespan. The less obvious change expected in age-related parameters could be caused by the rather exponential loss of suitable habitat cover (pasture), and might become more evident over the span of several generations after the second sampling period. Thus, we suggest that changes in size parameters better reflect an immediate population response to habitat loss and fragmentation in common spadefoot toad populations and should be integrated into early-warning tools for detecting amphibian population declines, as previously highlighted by other similar research (e.g., [Janin, Léna & Joly, 2011](#)).

We estimate that the documented reduction in body condition will affect reproductive output and success, which could in turn result in decreasing population size. While a lower population density could diminish the competition for food resources and reduce the parasitic and pathogen load in natural, undisturbed ecosystems (e.g., [Dietz, 1988](#)), this might not be the case in ecosystems already disturbed by urbanization ([Cordier et al., 2021](#)). Moreover, local amphibian populations often face multiple threats that act in synergy and are being fueled by anthropogenic development, such as climate change and alien species. Thus, we expect severe consequences that would ultimately lead to the local extinction of the studied population, unless the remaining suitable natural habitat (pasture) is maintained. However, since the study area is not included in a designated natural protected site (e.g., Natura 2000), and given the continuous residential development, we also expect that the remaining habitats will be ultimately engulfed and transformed by the municipality.

Body size is a critical indicator of resource use and is tightly linked to individual fitness, offspring size, annual growth rate and lifespan ([Angilletta, Steury & Sears, 2004](#)). Although many species are resilient to some degree of change in their environment, the often rapid and extensive nature of anthropogenic changes can exceed their adaptive response abilities. For many animals, a change in behavior is very often the first response to human-altered conditions, since it can potentially improve an organism's prospects of surviving and reproducing in a changing world ([Wong & Candolin, 2015](#)). Phenotypic plasticity, i.e., the ability of a particular genotype to express different phenotypes under altered environmental conditions ([Thibert-Plante & Hendry, 2011](#)), also acts as an adaptation in coping with changes. While both behavioral changes and phenotypic plasticity can buffer the impact of major environmental changes, these responses usually involve shifts in life-history traits. For example, in some commercially-exploited fish, intense size-selective harvest leads to slower growth rates, earlier maturation at smaller sizes, and increasing reproductive investment ([Feiner et al., 2015](#)). In turn, the decrease in size often increases mortality due to predation, causing a decline in biomasses and catches ([Audzijonyte et al., 2013](#)).

Life-history theory postulates links between age at sexual maturity, longevity, body size and reproductive investment ([Stearns, 2000](#); [Roff, 2002](#)). There is a trade-off between

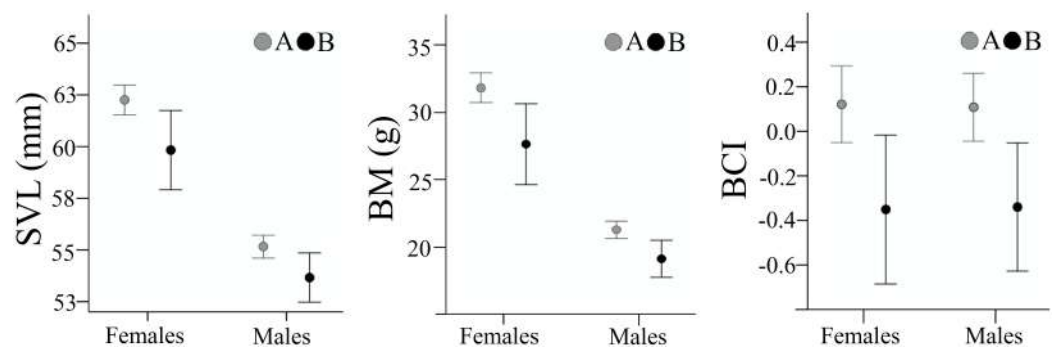


Figure 6 Body size and body condition of male and female *Pelobates fuscus*, at the two sampling moments—A(2000–2004) and B (2012–2014). SVL, snout-vent length (mm), BM, body mass (g), BCI, body condition index. Error bars represent 95% confidence intervals.

Full-size [DOI: 10.7717/peerj.11678/fig-6](https://doi.org/10.7717/peerj.11678/fig-6)

allocating available energy to reproduction, somatic maintenance and growth (Charnov, Turner & Winemiller, 2001; Amat & Meiri, 2018). In some environments, individuals must mature earlier to increase their reproductive success, but they do so at the cost of a smaller body size. For example, in a study of the mountain frog *Nanorana quadranus* (Liu, Hu & Yang, 1960) inhabiting a wide range of habitats with different harshness levels, age structure did not differ between populations, but body size changed significantly (Wang et al., 2019). A study of the geographic variation in average age, body size and reproductive investment linked to variation in temperature and rainfall in the Australian frog *Crinia pseudinsignifera* Main, 1957 showed a variable response, where frogs from harsher environments invested less in their first reproductive event, grew older than their counterparts and achieved a larger body size (Reniers et al., 2015). In a population of Siberian toads *Strauchbufo raddei* (Strauch, 1876), exposed to heavy metal pollution, males increased reproduction investment (i.e., improved advertisement call and secondary sexual characteristics) at the cost of reduced health and longevity, a trade-off that might lead to a population decline (Guo et al., 2018). Jennette, Snodgrass & Forester (2019) found differences in size but not age, among the breeding populations of *Lithobates sylvaticus* (LeConte, 1825) and *Anaxyrus americanus* (Holbrook, 1836) along an urbanization gradient, while in contrast, Sinsch et al. (2007) found that age at maturity in males, and longevity and potential reproductive lifespan in females were significantly correlated to habitat quality in *Bufo viridis* (Laurenti, 1768). These studies, as well as our results, suggest that age-related parameters could be rather discrete, species-specific indicators of life-history trade-offs caused by environmental changes of human origin. While our datasets did not allow for a comparison of growth patterns over time, we still provide baseline data regarding the growth parameters of males and females during the second sampling period, useful for further monitoring studies.

In animals with a complex life cycle like amphibians, changes in both the aquatic and terrestrial habitats have an influence on their body condition, growth and survival (Thompson & Popescu, 2021). The carryover effects of aquatic stressors are often difficult to disentangle from the impact of terrestrial habitat changes and the overall climate-change

induced modifications. In addition, increased anthropic eutrophication of the breeding ponds might further promote amphibian diseases (e.g., [Johnson et al., 2007](#)). Since we did not assess reproductive success and size at metamorphosis (e.g., [Székely et al., 2020](#)), we could not separate the impact of changes in water and land quality and availability in the studied population, but we rather assessed an overall impact of landscape changes, which affected both the aquatic and nearby terrestrial habitats.

As urban environments become increasingly dominant in contemporary landscapes, they cause profound and complex environmental changes that induce fast and pronounced ecological and evolutionary changes in many species ([Brans & De Meester, 2018](#)). The response to urbanization include significant trait shifts: plant species show changes in flowering time, size and seed production (e.g., [Palmat et al., 2016](#)), birds shift egg laying periods and mating behaviour (e.g., [Møller et al., 2015](#)), water fleas (*Daphnia magna*) shift towards a faster pace of life (e.g., [Brans & De Meester, 2018](#)), while invertebrate habitat specialist species shift towards smaller body sizes (e.g., [Magura, Ferrante & Lövei, 2020](#)). These shifts also have an inevitable impact on the trophic resources of amphibians and recent studies have highlighted the rapid and alarming decline in insect biomass and diversity throughout Europe and worldwide during the last decades (e.g., [Sánchez-Bayo & Wyckhuys, 2019](#); [Wagner, 2020](#)). Since insects are a major component of spadefoot toads diet ([Cogălniceanu et al., 1998](#)), we expect that a decrease in insect biomass and availability could affect their body condition and lower winter survival.

In this context, it is important to identify the most suitable monitoring tools that will detect early changes in population health before severe declines occur. Amphibian populations, due to their sensitivity to environmental stress, are often used in biomonitoring, and as such a variety of both invasive and non-invasive tools are available for estimating and measuring the impact of human activities, like fluctuating asymmetry (e.g., [Niemeier, Mueller & Roedel, 2019](#)), micronuclei tests (e.g., [Bosch, Gorla & Aiassa, 2011](#)), immunocompetence (e.g., [Brown, Shilton & Shine, 2011](#)), stress physiology ([Narayan et al., 2019](#)) or measures of heterozygosity ([Eterovick et al., 2016](#)). Our study showed that body measurements that require minimal handling of the animal and can be done on site provide a reliable measure of the effects of habitat loss and fragmentation. Moreover, age assessment showed a decrease in the average lifespan of the common spadefoot toad population in this study. Although changes in age parameters were less obvious in this case, it should be considered that these changes could not be evaluated separately in males and females between the two sampling moments (i.e., reduced and increased urban development), and previous studies showed that at least some age parameters can be sex-dependent in this species ([Eggert & Guyétant, 1999](#); [Cogălniceanu et al., 2014](#)).

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The Romanian National Authority for Scientific Research CNCS–UEFISCDI supported our research through grant PN-II-ID-PCE-2011-3-0173 (DC). Florina Stănescu and

Theodor-Sebastian Topliceanu were supported by the project ANTREPENORDOC, in the framework of Human Resources Development Operational Programme 2014–2020, financed from the European Social Fund under the contract number 36355/23.05.2019 HRD OP /380/6/13–SMIS Code: 123847, and by grant PN-III-1.2-PCCDI-2017-0721 (INTERASPA). There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

Romanian National Authority for Scientific Research CNCS–UEFISCDI: PN-II-ID-PCE-2011-3-0173 (DC).

European Social Fund: 36355/23.05.2019 HRD OP /380/6/13–SMIS Code: 123847.

INTERASPA: PN-III-1.2-PCCDI-2017-0721.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Dan Cogălniceanu conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Florina Stănescu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Diana Székely conceived and designed the experiments, authored or reviewed drafts of the paper, fieldwork and sampling (measurements and tissue collection), and approved the final draft.
- Theodor–Sebastian Topliceanu performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Ruben Iosif performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Paul Székely conceived and designed the experiments, authored or reviewed drafts of the paper, fieldwork and sampling (measurements and tissue collection), and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Ovidius University Constanța provided full approval through the Ethics Committee of the Faculty of Natural and Agricultural Sciences.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Field sampling received approval through Ministry Order No. 1173.2010 (in OM, part I, No. 609/27.VIII.2010).

Data Availability

The following information was supplied regarding data availability:

The raw measurements for body size (snout-vent length and body mass) and age assessments are available in the [Supplemental Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.11678#supplemental-information>.

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Food level and light conditions affect the antipredator behavior in larvae of a stream-breeding amphibian

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Received: 10 August 2020 / Revised: 28 December 2020 / Accepted: 4 January 2021

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Abstract

Understanding how long-term changes in environmental conditions influence the way that individuals cope with threats is essential in the context of behavioral adaptation to a rapidly changing world. However, little is known about the behavioral responses to predation risk for individuals that experienced different environmental conditions for extended periods of time, such as food levels and light conditions. In this experimental study, we tested whether previous long-term exposure to different food levels (low versus high) and light conditions (0-h light versus 8-h light) plays a significant role in shaping the antipredator response (i.e., the probability of emerging from the refuge and the distance moved) to stimuli from caged larval dragonflies, in larvae of the fire salamander (*Salamandra salamandra*). Specifically, we quantified behavioral differences in the response to predation risk in larval salamanders that were reared in the laboratory for 2 months under controlled food and light conditions. The results of this study showed that the interaction between food level and light conditions affected the antipredator behavior of the larvae. Fire salamander larvae maintained at low food levels and in 8-h light conditions emerged from the refuge with a higher probability (i.e., took more risk) than larvae maintained at high food levels and all other combinations of light conditions. Thus, our results highlight the complexity of antipredator responses, pointing attention to the fact that interactions among environmental factors are likely to determine the magnitude of antipredator response.

Significance statement

Few studies have investigated the role of multiple environmental factors on the expression of predator-induced behavioral responses. Specifically, because no study has so far investigated the risk-taking behavior in individuals exposed to long-term, contrasting food levels, and light conditions, we investigated this in amphibian larvae. We showed that environmental conditions interactively determined antipredator behavior. This highlights the importance of considering long-term environmental conditions experienced by an organism and their interactions when experimentally studying behavioral variation to adequately understand its expression in natural conditions.

Keywords Behavioral response · Environmental cues · Food levels · Refuge emergence · Light condition · Salamander

Communicated by A. Taylor Baugh

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Introduction

The antipredator behavior of a prey is determined by the trade-off between predator avoidance and obtaining resources (Sih 1980; Werner and Anholt 1993; Lima 1998). While predator-induced changes in behavior, morphology, and life history increase prey survival, these changes also have fitness costs. Reduced prey foraging activity in the presence of predators has knock-on effects on important fitness components such as growth and investment in reproduction (Cressler et al. 2010). Thus, prey trade-off predation avoidance and feeding activities such that fitness is maximized. Theoretical and empirical studies have been shown that the time allocated to these activities is heavily dependent on the physiological state of individuals and environmental conditions (Lima and Dill 1990; Semlitsch and Reyer 1992). Food availability is an environmental condition which is well known to affect the trade-off between foraging and predator avoidance (Anholt and Werner 1995). For a variety of prey species, it has been demonstrated that the antipredator responses are mediated by food availability and feeding history of individuals (Lima and Dill 1990; Lima 1998). For example, satiated individuals take less risk than food-deprived individuals, with many examples from birds (Heller and Milinski 1979; Tvardíková and Fuchs 2011; Bonter et al. 2013), fish (Godin and Crossman 1994; Sogard and Olla 1996; Poulsen et al. 2010), and insects (Nonacs and Dill 1990). Food level also affects the antipredator behavior of amphibian larvae (Horat and Semlitsch 1994; Anholt et al. 1996; Anholt et al. 2000; Whitham and Mathis 2000; Altwegg 2003; Carlson et al. 2015; Kurali et al. 2018). Although food availability and its effect on behavioral antipredator responses is well documented, there is a lack of studies investigating how other environmental factors may further interact with feeding history to shape threat-sensitive responses of animals.

The light/dark cycle is a major environmental factor that influences the physiology and the behavior of most organisms (Albrecht and Oster 2001). This rhythm is known to have important effects on animal body temperature (Erskine and Hutchison 1982), physiological performance (e.g., tolerance to higher temperatures) (Hutchison et al. 1979), food intake (Zhou et al. 1998), activity patterns (e.g., vertical migration exhibited by salamanders; Anderson and Graham 1967), and a variety of other behaviors (e.g., reproductive, antagonistic and antipredator behavior) (Rand et al. 1997; Wise and Jaeger 1998; da Silva Nunes 1988). Physiological consequences are especially common for amphibians, for which changes in the lighting conditions affect the production of melatonin (Gern et al. 1983), a hormone responsible for many aspects of the photoperiodic physiology and behavior (Vanecek 1998). In salamanders, Wise and Buchanan (2006) suggested that lower levels of nocturnal plasma melatonin caused by a longer photophase increase the metabolic rates, which in turn increase the energy demands.

Thus, we expect that light conditions and food availability interactively determine the behavioral responses of amphibians to predation risk. Previous studies showed that light conditions influence foraging and antipredator behavior in amphibians (Taylor 1984; da Silva Nunes 1988; Ding et al. 2014). Among the various parameters of light that can affect amphibian behavior, light intensity appears to be the most important one. For instance, Panamanian cross-banded tree frogs (*Smilisca sila*) were found to exhibit changes in the antipredator behavior depending on illumination level (da Silva Nunes 1988). Light intensity has also been shown to play a key role in amphibian predator-prey interactions, for example the likelihood to encounter diurnal or nocturnal predators (Taylor 1984). However, day length acts as an environmental cue to which individuals respond by developing behavioral strategies (Ding et al. 2014). Variation of light exposure over a short- and long-term duration may significantly alter the energy demands and the threat-sensitive behavior. Circadian and seasonal patterns of activity are adaptive behavioral traits which allow individuals to effectively exploit available resources.

Light conditions are expected to have marked influence on species with activity patterns regulated by the photoperiod, for instance salamanders (Wise and Buchanan 2006). The European fire salamander (*Salamandra salamandra*) is one of the salamander species which is able to exploit both surface and underground habitats for breeding (Manenti et al. 2011). Fire salamanders present high behavioral plasticity which allows them to colonize underground habitats (Manenti et al. 2013, 2016), a challenging environment due to its unique characteristics, such as low food availability and perpetual darkness. Thus, fire salamanders are ideal organisms for testing the interaction of these environmental variables on antipredator behavior in prey species. The aim of our study was to investigate the interactive effects of the long-term exposure to contrasting food levels and light conditions on the antipredator response in larvae of the fire salamander (*Salamandra salamandra*). We used the probability to emerge from the refuge and the distance moved outside the refuge as antipredator responses describing risk-taking behavior (Sih et al. 1988; Anholt and Werner 1995; Oswald et al. 2020). Specifically, we hypothesized that long-term exposure to low food levels coupled with longer photoperiod would decrease antipredator response in prey (i.e., larvae will emerge from the refuge more frequently and travel longer distances) when exposed to cues from a predator.

Material and methods

Sampling and housing conditions

Fire salamanders give birth to fully aquatic larvae in both surface and underground sites (Manenti et al. 2013). We

collected 96 newborn larvae of *S. salamandra* in April 2017 from three sites in the Romanian Carpathians. Two sites were located underground: inside an artificial subterranean habitat (Buzau, 45.4° N, 26.2° E, 475 m elevation a.s.l., $n = 23$) and inside a cave (Gaura cu Musca Cave, 44.6° N, 21.6° E, 92 m elevation a.s.l., $n = 25$). The third site was above the surface: a small reservoir along a forest stream (Iconie, 44.9° N, 22.7° E, 325 m elevation a.s.l., $n = 48$). We chose three populations to account for potential variation in behavior, while assuring a large sample size to test for multiple experimental factors. In the underground sites, the European cave spider *Meta menardi* is likely to prey on salamander larvae. In surface sites, several dragonfly species (Gomphidae, Anisoptera) coexist with and predate on salamander larvae.

The experiment was conducted in the Laboratory Gallery of Closani Cave (air temperature 13 ± 1 °C), which is the cave-laboratory of “Emil Racoviță” Institute of Speleology, located in Gorj county, southwestern Romania. All collected larvae corresponded to the developmental stage 1 which was recognizable by a well-developed and bluntly rounded tail-fin (Juszczyk and Zakrzewski 1981). The mean $\pm$ standard error of the snout-vent length (SVL) of the salamander larvae at the beginning of the experiment (hereafter SVL initial) was 18.9 ± 0.09 mm.

In the laboratory, we allocated the 96 salamander larvae at random, but we assured a balanced number of larvae having different origins (i.e., surface, $n = 48$; underground, $n = 48$) in each of the four experimental treatments representing a combination of two food levels (low versus high) and two light conditions (0-h light versus 8-h light). Since SVL initial differed significantly among populations ($F_{[2, 90]} = 6.185$, $P = 0.003$; Tables S1 and 2 in the Supplementary Material), we allocated the larvae to the experimental treatments in such a way to assure that larvae did not differ in their SVL initial either between food ($F_{[1, 90]} = 0.141$, $P = 0.708$) or light ($F_{[1, 90]} = 0.166$, $P = 0.685$) treatments (Table S1 in the Supplementary Material). There were 24 larvae per treatment. Larvae were kept individually in plastic containers (length $\times$ width $\times$ height: $21 \times 14 \times 9.5$ cm; 2 cm of water depth). This water depth is within the range of habitats found in the wild and follows previous standardized design (Manenti et al. 2013). Larvae in the 8-h light treatment were reared at a light intensity of 50 lux measured above the rearing containers (the source light was placed on the ceiling of the Laboratory Gallery of Closani Cave). Larvae were fed live worms (*Tubifex* sp.) every third day, and the water was changed the day after feeding. The larvae in high food treatments were fed six live prey items per feeding throughout the first 3 weeks and ten prey items per feeding from the fourth week. The larvae in low food treatments received half the amount of food compared to larvae in the high food treatments. These food levels were established based on those used in similar experiments involving fire salamander larvae (Manenti et al. 2013).

Behavioral experiments

Observations of larval fire salamander behavior were conducted after 2 months of maintaining the larvae in the four treatments described above. The 2-month threshold was chosen because this amount of time was sufficient for the experimental treatments to have a significant effect on the body size of fire salamander larvae (Krause et al. 2011) (Tables S1 and 2 in the Supplementary Material). We also considered 2 months of experimentally manipulated food levels and light conditions to cover more than half of a typical larval phase of fire salamanders (Thiesmeier 2004). Our experimental setup followed a $2 \times 2 \times 2$ repeated-measures design whereby the fire salamander larvae maintained under the two food levels (low versus high) and two light conditions (0-h light versus 8-h light) were subsequently observed during two successive scenarios: no risk (i.e., during the absence of a predatory stimulus), and risk (i.e., during the exposure to a predatory stimulus).

The behavioral observations were conducted in white opaque plastic containers ($l \times w \times h$: $30.5 \times 21.5 \times 14.8$ cm, 2 cm of water depth). We used opaque containers to visually isolate the salamander larvae. Each container was divided in two distinct zones: a refuge ($6.5 \times 21.5 \times 14.8$ cm) and an open arena ($25 \times 21.5 \times 14.8$ cm). The refuge was located at one end of the container and had a chamber-like construction, being separated from the arena by an opaque plastic sliding door, while the top of the refuge partition was covered with a removable opaque plastic lid (Fig. 1). The arena was represented by the remaining open space in the container and served for the observation of the behavior (Figs. 1 and 2). Prior to the onset of each behavioral observation, in the no risk scenario, we placed an empty white, plastic-framed cage ($h \times$ diameter: 6.4×3.5 cm) covered by transparent mesh (mesh size = 0.2 cm), in one corner of the arena (opposed to the refuge). In the risk scenario, we placed the framed mesh cage in the same way but with two live dragonfly larvae inside. We used in total eight Gomphidae (Anisoptera) dragonfly larvae of similar sizes (mean length $\pm$ SD: 18.4 ± 2.7 mm). We placed the same two dragonfly larvae in one cage but the caged dragonfly pairs were randomly assigned during the behavioral observations. We collected the dragonfly larvae from Bulba River, which is located in proximity to the surface population of salamanders. The dragonfly larvae were collected the night before we started the behavioral experiment. The dragonfly larvae were not fed before or among behavioral observations, and after the experiment, they were released at the collecting site. Previous studies indicated that salamanders rely on chemical cues for predator recognition (Cupp 1994) and that chemical alarm cues obtained from dragonfly larvae of Anisoptera elicited strong antipredator response in larval fire salamanders (Manenti et al. 2016). In both risk scenarios, a transparent mesh bag ($l \times w$: 8.5×7 cm) with 10 live *Tubifex* sp. was placed in the other corner of the arena. The position of

Fig. 1 The experimental setup: A refuge, B arena, C mesh bag with live *Tubifex*, and D cage with live dragonfly larvae. The size of organisms is increased for a better readability of the figure

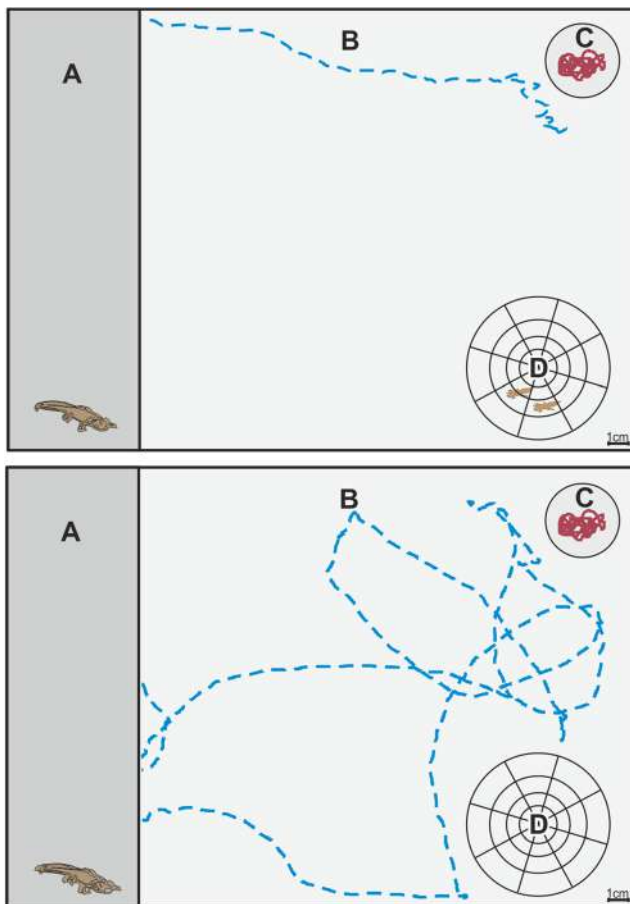
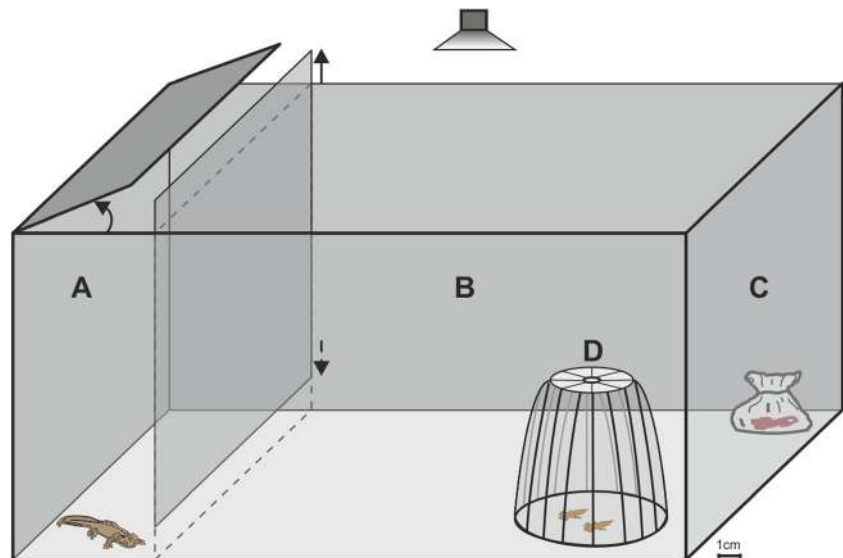


Fig. 2 Examples of tracks of movement during 15-min trials in a fire salamander larva, obtained from video-tracking software (Ethovision). Upper image—the larva was tested in the presence of an experimental stimulus, i.e., caged live dragonfly larvae, and lower image—the larva was tested in the absence of an experimental stimulus; A refuge, B arena, C mesh bag with live *Tubifex*, and D cage for predators

the cage and food bag in the left or right corner of the arena was randomized, to prevent cognition bias. The antipredator response of each fire salamander larva was tested three times in each risk scenario; we repeated the trials every third day, just before feeding, which allowed us to obtain a similar satiation level in salamander larvae among trials. We started with the no risk scenario trial and then alternated between risk and no risk scenarios. The order of individual larvae tested in each trial was randomized.

During each behavioral observation, a larva was placed in the refuge and allowed to acclimate for 5 min; then, the sliding door separating the arena from the refuge was lifted and the larva was allowed to move freely throughout the container (i.e., refuge and arena) during a 15-min time period. To prevent disturbing the focal salamander larva during recording, the experimental setup was placed behind a wall. Behavioral observations were recorded (25 images/s) from above, in the dark (average light intensity 0.01 lux), using an HD infrared camera (IP Hikvision DS-2CD2610F-1). Recording was done in the dark to mimic the natural conditions of foraging activity, since salamander larvae are largely nocturnal or crepuscular in feeding activities (Manenti et al. 2016). The water in the experimental containers was changed after each behavioral observation. At the end of a trial, the salamander larvae were returned to the rearing containers, and fed.

The video files were analyzed using Noldus Ethovision XT13 video-tracking software (Noldus Information Technology, Wageningen, the Netherlands). This software converted the images of the salamander larvae into pixels in each image and processed them across time to provide detailed quantitative data of the movement behavior (see Delcourt et al. 2013 for a review). The videos were processed at the original frame rate of 25 images per second (720×1280 pixels). The behavior of salamander larvae was quantified by

measuring (i) the refuge emergence, a binary variable scored as “1” if the larva left the refuge and “0” if the larva did not leave the refuge within a 15-min time period after acclimation; and (ii) distance moved, a continuous variable measured as the total distance moved in the arena from the time the larva left the refuge during the 15-min time period. As both the emergence from the refuge and the distance moved were captured automatically with the same settings by the behavioral software, there were no observer biases. After the behavioral observations, larvae were photographed on millimeter paper which was glued on the bottom of the rearing containers. Snout-vent length to the nearest 0.01 mm was measured for each salamander larva from the digital photographs using the software ImageJ v. 1.50i (Schneider et al. 2012). Measurements in ImageJ were conducted by a single person (IN).

Data analysis

We fitted a generalized linear mixed model (GLMM) with refuge emergence as the dependent variable, and a linear mixed-effects model (LMM) with the total distance moved as the dependent variable, respectively. The distance moved was log transformed. In the models, we included the risk scenario, food and light conditions, and population as fixed effects, and the identity of the larva as a random factor. The manipulation of both food and light prior to the behavioral trials resulted in differences in the size of salamander larvae (see Table S1 and 2 in the Supplementary Material), which could potentially influence their antipredator responses. Thus, we included in the models the SVL of salamander larvae after 2 months of exposure to different food and light conditions (hereafter SVL final) and population origin as covariates in the models. However, because neither population nor SVL final had significant effects and did not affect the statistical significance of other variables (see Table S3 in the Supplementary Material), we removed them from the subsequent analyses. We assumed a binomial error distribution and logit-link function for the GLMM, and a Gaussian distribution and identity link function for the LMM. As five larvae metamorphosed (two larvae in 0-h light, and high food treatment, two larvae in 8-h light and high food treatment, and a larva in the 8-h light, and low food treatment) before carrying out the third trials in both risk scenarios, the study of emergence from the refuge was based on 566 behavioral observations instead of 576 (96 larvae * three trials * two risk scenarios). The models were fitted using the glmer (GLMM) and lmer (LMM) functions in R (R Core Team 2020). In both cases, predictions and standard errors were generated with the bootMer function in the lme4 R package (Bates et al. 2015). To assess the significance of model predictors, we used Wald and likelihood-ratio chi-square tests (type II analysis of deviance) in R package “car” (Fox and Weisberg 2011). To examine differences

among the predictors’ levels, we used least square means (LS-means) for multiple comparisons and calculated the *P* values adjusted using the Tukey method.

To minimize observer bias, blinded methods were used when all behavioral data were recorded and analyzed.

Results

From a total of 566 behavioral observations, salamander larvae emerged from the refuge in total 316 times: 71 times in the high food 0-h light treatment, 65 times in the high food 8-h light treatment, 78 times in the low food 0-h light treatment, and 102 times in the low food 8-h light treatment.

The GLMM revealed that both risk scenario and food level had a significant effect on the probability that salamander larvae emerged from the refuge ($P < 0.01$ for both, Table 1). When exposed to the presence of dragonfly larvae (i.e., risk scenario), salamander larvae were significantly less likely to leave the refuge than in their absence (i.e., no risk scenario) (odds ratio = 0.581 ± 0.105 , z ratio = -3.008 , $P < 0.01$, Fig. 3a). Salamander larvae at high food level had a significantly lower probability to emerge from the refuge than those at low food level (odds ratio = 0.512 ± 0.109 , z ratio = -3.147 , $P = 0.01$, Fig. 3b). The main effect of light on the salamander larvae probability to emerge from the refuge was not significant ($P = 0.146$, Table 1).

The GLMM also revealed a significant interaction effect between food level and light conditions on the probability that salamander larvae emerged from the refuge ($P < 0.05$, Table 1). LS-means test showed that salamander larvae at low food level maintained in 8-h light conditions were significantly more likely to emerge from the refuge compared to those at high food level maintained both in 8-h light (odds ratio = 0.308 ± 0.095 , z ratio = -3.821 , $P < 0.001$, Fig. 3c) and 0-h light conditions (odds ratio = 0.370 ± 0.114 , z ratio = -3.240 , $P < 0.01$, Fig. 3c). Salamander larvae at low food level maintained in 8-h light conditions showed significantly

Table 1 Results of the mixed models assessing the effects of risk scenario (presence versus absence of stimuli from caged larval dragonflies), food level (low versus high), and light conditions (0-h light versus 8-h light) on the refuge emergence and distance moved by fire salamander larvae

	χ^2	df	<i>P</i>
Probability of refuge emergence			
Risk scenario	9.046	1	0.003
Food	9.148	1	0.002
Light	2.113	1	0.146
Food:light	5.742	1	0.017
Log (total distance moved)			
Risk scenario	1.939	1	0.164
Food	2.106	1	0.147
Light	2.662	1	0.103
Food:light	1.716	1	0.190

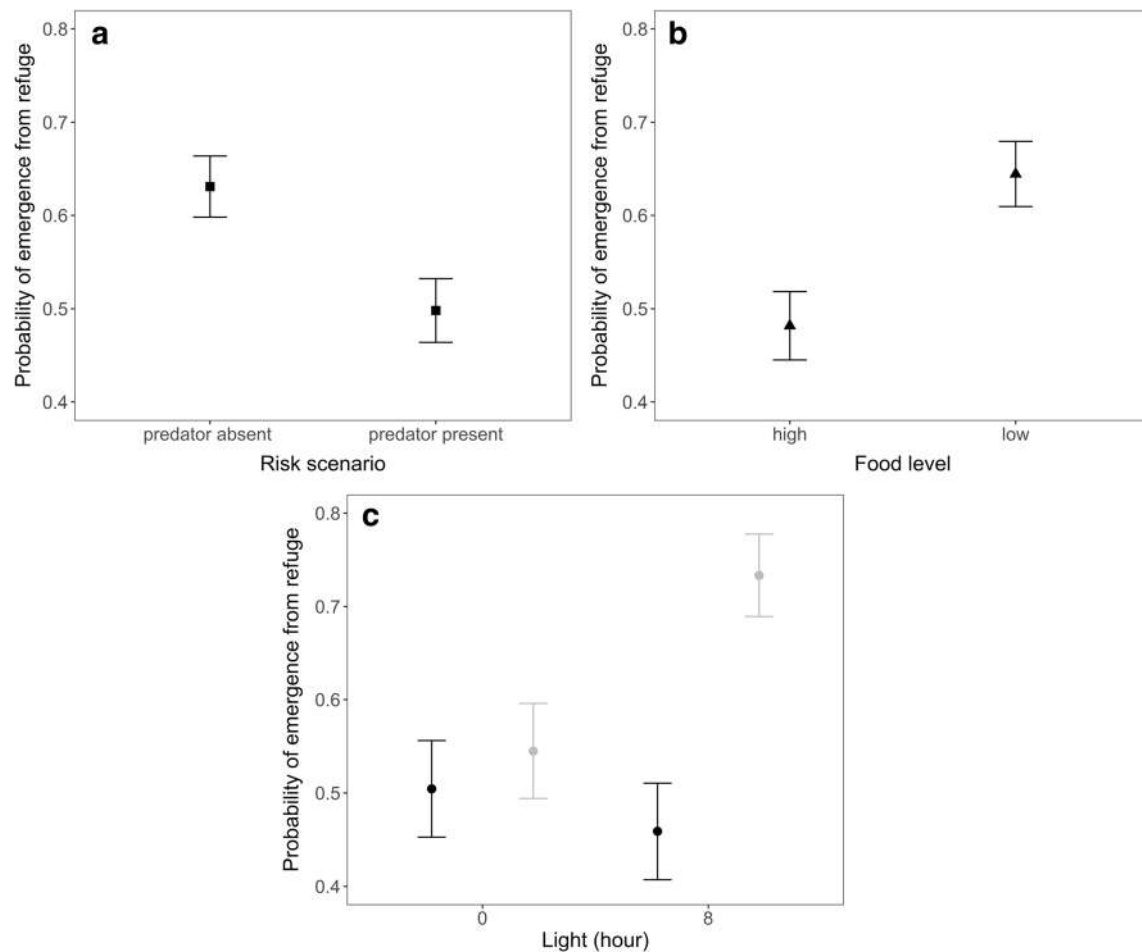


Fig. 3 The probability of refuge emergence in relation to risk scenario (presence versus absence of stimulus from caged dragonfly larvae), food level (low versus high), and light conditions (0-h light versus 8-h light) in the fire salamander larvae: a main effect of the risk scenario, b main effect of the food level, and c the two-way interaction effect between food level and light conditions (see Table 1 and text for statistical tests). In a and b,

squares and triangles indicate the mean values of the probability to emerge from the refuge and bars represent standard errors (SE). In c, grey dots and lines represent the mean value and SE of refuge emergence of salamander larvae in the low food level, and black dots and lines those in the high food level, respectively

lower refuge emergence than larvae in the same food treatment but maintained in 0-h light conditions (odds ratio = 0.435 ± 0.132 , z ratio = -2.734 , $P = 0.05$, Fig. 3c).

The LMM revealed that neither the risk scenario, food level, nor light conditions had a significant effect on the distance moved by salamander larvae which emerged from the refuge ($P = 0.164$, $P = 0.147$, and $P = 0.103$, respectively, Table 1). The interaction effect between food level and light conditions on the distance moved by salamander larvae was also not significant ($P = 0.190$, Table 1).

Discussion

Food availability and light conditions are known to play a major role in the behavioral responses of ectotherms to predators, including those of amphibians (Lienart et al. 2014). However, whether the interaction of these environmental

factors affects the antipredator behavior of amphibians remains poorly understood. The results of our study showed that food level and light conditions interact to shape the antipredator behavior of *S. salamandra*. Fire salamander larvae maintained for 2 months on low food levels and in 8-h light conditions took more risk (i.e., displayed significantly higher probability to leave the refuge) when exposed to larval dragonflies, compared to salamander larvae maintained in high food level and other light conditions. These results indicate that fire salamander larvae had the ability to modulate their behavior depending on immediate predation risk and past variation of environmental conditions. While Oswald et al. (2020) showed that risk-taking behavior of salamander larvae depends on the predation risk in the natal habitat, the results presented in this study show that risk-taking behavior is complex and depends on multiple environmental conditions. More generally, while it is well understood that the growth/predation risk trade-off is determined by an interaction

between food level and risk, our results indicate that the interactions with environmental factors may modulate the trade-off.

Our findings contribute to a growing body of literature on antipredator behavior in amphibians. Antipredator responses such as shelter-seeking behavior, an increased refuge use, spatial avoidance, and reduced activity have been all reported for amphibian species exposed to a predation risk (Holomuzki 1986; Semlitsch 1987; Sih et al. 1988, 1992; Lawler 1989; Alford 1999; Kats and Dill 1998; Van Buskirk and Schmidt 2000; Teplitsky et al. 2003; Winandy et al. 2016). In this study, we provided evidence that fire salamander larvae responded to a risky situation by significantly reducing the emergence from refuges. However, salamander larvae did not display a significant decrease of movement in the presence of a predation risk. The availability and use of a refuge has been associated with positive effects for the prey. For instance, the presence of a refuge contributes to a reduction of prey-predator encounter rates by increasing the habitat complexity or impairing visual abilities and movements of the predator (Sredl and Collins 1992; Babbitt and Tanner 1997). The refuge use implies a reduction of the time when prey is exposed to predators (Sih et al. 1988; Lawler 1989), which in turn increases prey survival. However, negative effects associated with prey refuge use have also been documented, such as a reduction of foraging opportunities with possible negative impacts on prey state (Sih 1987). Thus, our results suggest that salamander larvae evolved flexible strategies to use the refuge, in order to minimize the negative effects associated with the periods of time spent in the refuge (Martín and López 1999). This means that after adopting a less secure but needed response in a risky situation (i.e., emergence from the refuge), salamander larvae behaved in a similar way (i.e., moved similar distances) regardless of predation risk.

Food level is an important intrinsic determinant of activity levels in animals (McNamara and Houston 1986). In the present study, food level significantly affected the decision of fire salamander larvae to emerge from the refuge both as main factor and in interaction with light conditions. Poorly fed salamander larvae maintained in day/night cycle were significantly more likely to emerge from the refuge compared to those maintained in other food levels and light conditions. The foraging models of McNamara and Houston (1986) and Mangel and Clark (1986) predict that the decision of actions taken by an animal depends on its energetic requirements. Although many factors induce changes in the energetic requirements, it has been suggested that food availability and light conditions are key factors. Thus, the expected increase of energetic requirements in salamander larvae maintained for 2 months under low food and a day/night cycle requires them to forage more often and thus, to leave the refuge to achieve food intake.

As this study highlights, the risk-taking behavior is determined by environmental conditions. We found that predation risk, food level, and light conditions interactively determined the refuge-emergence probability of fire salamander larvae. However, light conditions alone did not significantly affect the risk-taking behavior of salamander larvae. These results emphasize the importance of understanding how the interactive effects of environmental conditions determine behavioral decisions. Moreover, whereas most experiments focused on the effect of short-term changes in the environment, our study mimicked the natural long-term exposure to an environment which differed in two crucial environmental parameters (i.e., the level of food resources and the regular interval of daylight and darkness). Short-term changes in environmental parameters play an important role in predator-prey dynamics, with implications for population dynamics (Lienart et al. 2014). However, understanding how longer-term environmental changes mediate behavior will certainly improve our ability to disentangle the contribution of behavioral plasticity to species persistence under environmental change. We therefore stress the need to consider multiple environmental effects and their interactions when studying behavioral variation in animal populations and the importance of assessing the long-term changes in the environment on behavioral responses.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00265-021-02966-w>.

Acknowledgments We are grateful to the following master, PhD students, and volunteers for their invaluable help in rearing the fire salamander larvae: Sabina Vlad (Ochiană), Elena Șușter, Alexandra Telea, Anca Soare, Sebastian Topliceanu, Roberto Festuccia, Ștefan Cătălin Baba, Augustin Nae, Ionuț Popa, Valerică Toma, Marius Robu, and Dragos Bălășoiu. We highly appreciate the invaluable advice offered by Diana Székely and Dan Cogălniceanu, which greatly improved our experimental design. We thank both reviewers for their constructive comments on the manuscript.

Authors' contributions RIB, FS, and MD conceived the idea of the study. RIB, FS, and BRS analyzed the data. RIB and MD wrote the manuscript with input from FS and BRS. MD supervised the work. RP and IN contributed to the design and carrying out the behavioral experiments.

Funding The collaboration between Ovidius University Constanța, Institute of Speleology of the Romanian Academy and University of Liège was supported by a grant from Wallonie-Bruxelles International (W.B.I.) and Executive Unit for Financing Higher Education, Research, Development and Innovation CCCDI-UEFISCDI (ANCS), project number 105BM/2017. RIB was additionally supported by UEFISCDI through grant PN-II-RU-TE-2014-4-1536, and grant PN-III-P1-1.1-TE-2019-1233. MD is a Research Director at Fonds de la Recherche Scientifique – FNRS (Belgium). FS was partly supported by the project ANTREPRENORDOC, in the framework of Human Resources Development Operational Programme 2014-2020, financed from the European Social Fund under the contract number 36355/23.05.2019 HRD OP /380/6/13 – SMIS Code: 123847, and by grant PN-III-1.2-PCCDI-2017-0721 (INTERASPA).

Data availability The dataset analyzed during the current study is available in the KNB repository: <https://knb.ecoinformatics.org/view/urn%3Auid%3A7d01db76-0c1a-429c-a442-a8788e85f419>.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval Because this study was only observational and not experimental with regards of laboratory research on animals, it did not need specific ethical approval. The collection, captive maintenance, and observation procedure of the fire salamander larvae complied with all relevant guidelines, notably the Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Romanian Speleological Heritage Commission issued environmental permit no 78/10.02.2016. Both 8-h and 0-h light experimental conditions used to rear the fire salamander larvae mimicked natural situations, as this species is naturally present in these two situations in the wild as they can be present in caves (Manenti et al. 2011). No larva died during the experiment.

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Review Article

Alien Vertebrates in Romania – A Review

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Abstract: We provide an updated review regarding the presence of alien vertebrates in Romania (i.e., fish, reptiles, birds, and mammals), taking into consideration both published data and a selection of international, regional and national public online databases. The literature review resulted in a preliminary checklist of 51 alien vertebrates in Romania, of which 41 were validated. The review of international and regional databases resulted in a preliminary checklist of 116 alien vertebrates, of which only 44 were validated after a quality check. The review of the national online databases resulted in a list of seven alien bird species in Romania, all of them valid. The final list contains 46 alien vertebrate species for Romania, of which 17 are established, and 29 are considered casual. Fish are the most abundant group (29 species) representing 63% of all vertebrate species, followed by mammals (eight species), birds (seven species), and reptiles (two species).

Key words: Checklist, alien species, fish, reptiles, birds, mammals.

Introduction

Human-driven biotic invasions are one of the most important direct drivers of biodiversity loss and ecosystem service changes worldwide (BELLARD et al. 2016, DOHERTY et al. 2016, WALSH et al. 2016), second only to the direct destruction of habitats. There are currently more than 14,000 alien species recorded in Europe (EASIN, <https://easin.jrc.ec.europa.eu/>) with more than half originating from outside of the EU territories (ROY et al. 2019), and the number of occurrences is rapidly increasing due to new invasions and also the intensification of research on this topic (SEEBENS et al. 2017). Invasive alien species cause annual damages in the European Union that amount to at least €12 billion (KETTUNEN et al. 2009). While for other human-mediated

impacts, there are clear and widely accepted indicators, the development and application of a set of internationally agreed indicators to assess the status of biological invasions is still ongoing (WILSON et al. 2018).

Invasion science, as a new discipline, is riddled with uncertainties, which is why national- and regional-scale measures to prevent or control alien species have done little to slow the increase in the extent of invasions and the magnitude of impacts (LATOMBE et al. 2019). It is assumed that the first uncertainty that any management action faces is the confirmation of the presence of an alien species in a region or country. Therefore, the updated national/regional checklists provide useful information both for policymakers and the general public (NENTWIG et al. 2018).

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A first preliminary assessment of the presence of alien species in Romania has identified 982 alien species, of which 65 are freshwater, marine, and terrestrial vertebrates (ANASTASIU et al. 2016). However, the assessment does not include online databases, nor do the authors provide a validated checklist of species and their status. To fill a knowledge gap and contribute to the implementation of the EU regulations on invasive alien species in Romania, we provide here an updated and comprehensive checklist of alien vertebrates reported in Romania as a result of an extensive review of scientific literature and the most relevant public online databases.

Materials and Methods

Literature review

We used Web of Science and Google Scholar databases (accessed on 20th June 2019) and created search queries with combinations of keywords ('alien species', 'invasive species', 'Romania', 'vertebrate', 'fish', 'amphibia', 'reptile', 'bird', 'mammal'), both in English and Romanian, with no restriction for timespan. Furthermore, we reviewed publications not indexed by the scientific search engines (grey literature). We searched published abstracts, scientific articles, books, book chapters, conference proceedings, doctoral theses, and scientific/official reports. We also reviewed published literature available as hard copies only, which was mostly the case for old publications in Romanian.

Review of online databases

We reviewed the available data on alien vertebrate species in Romania from seven public online databases (accessed on 20th June 2019): three international databases – Invasive Species Compendium (CABI), Global Invasive Species Database (IUCN GSID), and A Global Information System on Fishes (FishBase), as well as two regional – European Alien Species Information Network (EASIN) and Delivering Alien Invasive Species Inventories for Europe (DAISIE), and two national databases – ORNITODATA and Birding Romania. The international and regional databases were considered relevant for the purpose of our study since they also include data from smaller online national repositories, citizen science projects, and smartphone applications (e.g., iNaturalist, Invasive Alien Species Europe app). The Romanian online databases are, to the best of our knowledge, the only public databases available for Romanian vertebrates

at the time of this review. We performed specific search queries within each of these databases, using filters that allowed us to extract only datasets regarding alien vertebrates recorded in Romania.

Data validation

The quality check of the resulted data was performed based on the authors' knowledge. We first verified if the reported species were indeed alien to Romania. We did not consider hybrids or subspecies of native species in our study. We excluded: (i) species present only in captivity and domesticated species, (ii) species reported based only on fossil records, (iii) species with uncertain origin (or cryptogenic, *sensu* CARLTON 1996), (iv) data with untraceable source, and (v) species with improbable presence in Romania. Since there is a lack of consensus in the scientific literature regarding the terminology relevant to invasion ecology that includes qualities open to subjective interpretation (COLAUTTI & MACISAAC 2004), we used the classification and terminology following BLACKBURN et al. (2011). Thus, we referred to casual as introduced species that are surviving in the new habitat but are not spreading, and to established as alien species that are reproducing and spreading, with some becoming invasive.

Results

The literature search (Web of Science, Google Scholar, and hardcopy literature) resulted in a list of 153 relevant publications which covered the period 1947–2018, as follows: 122 scientific articles, 17 books, two book chapters, six conference proceedings, three doctoral theses, and three reports (Annex 1). The literature review resulted in a preliminary checklist of 51 alien vertebrates in Romania, of which 41 were validated. The review of international and regional online databases resulted in a preliminary checklist of 116 alien vertebrates, of which only 44 were validated after a quality check. The review of the national online databases resulted in a list of seven alien bird species in Romania. These records were considered valid since the data reported in these databases are already curated by professional ornithologists with vast expertise in their field.

The international and regional databases included most erroneous records, with 42% of the records referring to species native in Romania. Apart from these, we discarded records of species present only in some form of captivity (zoos, aquaria) or domesticated, doubtful records (e.g., species with improbable presence in Romania,

Table 1. Checklist of alien vertebrates in Romania based on review of scientific literature and online databases. Species recorded from citizen science are marked with an asterisk (*). Alien species assessed among the worst in Europe by NENTWIG et al. (2018) are marked with (#) followed by the corresponding ranking. The presence of species of European Union Concern is checked following the Commission Implementing Regulation (EU) 2019/1262 amending Implementing Regulation (EU) 2016/1141 to update the list of invasive alien species of Union concern.

No	Species name	Group	Alien status	Species of EU concern (EU 2019)
1	<i>Aix galericulata</i> *	Birds	casual	
2	<i>Aix sponsa</i> *	Birds	casual	
3	<i>Alopochen aegyptiaca</i>	Birds	casual	yes
4	<i>Anser indicus</i>	Birds	casual	
5	<i>Branta canadensis</i> (#1)	Birds	casual	
6	<i>Phasianus colchicus</i>	Birds	established	
7	<i>Psittacula krameri</i> * (#67)	Birds	casual	
8	<i>Ameiurus melas</i>	Fish	established	
9	<i>Ameiurus nebulosus</i>	Fish	established	
10	<i>Carassius auratus</i> (#20)	Fish	casual	
11	<i>Carassius gibelio</i>	Fish	established	
12	<i>Clarias gariepinus</i>	Fish	casual	
13	<i>Clarias ngamensis</i>	Fish	casual	
14	<i>Coregonus albula</i>	Fish	casual	
15	<i>Coregonus lavaretus</i>	Fish	casual	
16	<i>Coregonus peled</i>	Fish	casual	
17	<i>Ctenopharyngodon idella</i> (#65)	Fish	established	
18	<i>Gambusia holbrooki</i> (#80)	Fish	established	
19	<i>Hypophthalmichthys molitrix</i>	Fish	established	
20	<i>Hypophthalmichthys nobilis</i>	Fish	established	
21	<i>Ictalurus punctatus</i>	Fish	casual	
22	<i>Ictiobus bubalus</i>	Fish	casual	
23	<i>Ictiobus cyprinellus</i>	Fish	casual	
24	<i>Ictiobus niger</i>	Fish	casual	
25	<i>Lepomis gibbosus</i>	Fish	established	yes
26	<i>Megalobrama terminalis</i>	Fish	casual	
27	<i>Micropterus salmoides</i> *	Fish	casual	
28	<i>Mylopharyngodon piceus</i>	Fish	casual	
29	<i>Oncorhynchus mykiss</i> (#60)	Fish	casual	
30	<i>Parabramis pekinensis</i>	Fish	casual	
31	<i>Perccottus glenii</i>	Fish	established	yes
32	<i>Planiliza haematocheila</i>	Fish	casual	
33	<i>Poecilia reticulata</i> (#76)	Fish	casual	
34	<i>Polyodon spathula</i>	Fish	casual	
35	<i>Pseudorasbora parva</i> (#59)	Fish	established	yes
36	<i>Salvelinus fontinalis</i>	Fish	established	
37	<i>Dama dama</i>	Mammals	established	
38	<i>Myocastor coypus</i> (#17)	Mammals	casual	yes
39	<i>Neovison vison</i> (#19)	Mammals	casual	
40	<i>Nyctereutes procyonoides</i> (#66)	Mammals	established	yes
41	<i>Ondatra zibethicus</i> (#4)	Mammals	established	yes
42	<i>Oryctolagus cuniculus</i>	Mammals	casual	
43	<i>Rattus norvegicus</i> (#2)	Mammals	established	
44	<i>Rattus rattus</i>	Mammals	established	
45	<i>Podarcis siculus</i> *	Reptiles	casual	
46	<i>Trachemys scripta</i>	Reptiles	casual	yes

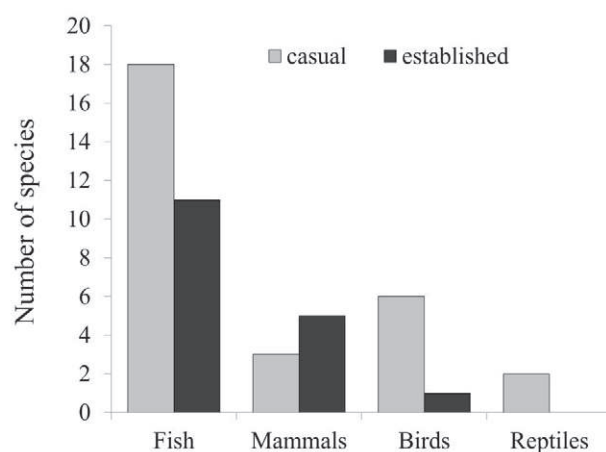


Fig. 1. Alien vertebrates in Romania according to their establishment status.

erroneous identification/ taxonomic confusion, and source not indicated), cryptogenic species, and fossil records. Exotic fish reported both in the literature and international databases, which can survive only in thermal water bodies, were also excluded. We provide in Annex 2 a list of the species and subspecies excluded after the quality check and a justification for their exclusion from the final checklist of alien vertebrates in Romania.

The checklist of alien vertebrates in Romania after data validation resulted in 46 alien species (Table 1). Of these, 41 species were recorded both in the scientific literature and online databases, while five species were recorded only in online databases, through citizen science. Based on the available published information, 17 of the alien species were considered established, with self-sustaining populations in the wild, while the rest of the species (29) were considered casual aliens since no evidence regarding their reproductive success in the wild was found in the published literature (Fig. 1).

Discussion

We report 46 alien vertebrate species for Romania, of which 17 are established, and 29 are considered casual. Fish are the most abundant group representing 63% of all vertebrate species. There are only two reptile species recorded in the country and no alien amphibian species. Among the 149 alien species ranked as the worst aliens in Europe by NENTWIG et al. (2018), 40 (27%) are vertebrates, and our checklist includes 13 of them (representing 28%). Of the 66 species of European Union concern, eight are present in Romania, of which five (three fish and two mammals) have already become established in natural ecosystems (Table 1).

Our study showed that scientific literature provides more reliable data regarding the alien vertebrates in Romania, while international online databases contain numerous errors and require supplementary efforts for data validation. For example, 53 native species and subspecies were erroneously recorded as aliens to Romania. Also, some domestic species were included in the list as aliens, although there are no established wild populations (e.g., sheep, cattle, water buffalo). A special category is represented by tropical aquarium fish species that are sometimes released in the wild, but they can only survive in thermal water bodies. This is a common situation in urban areas, with many exotic species surviving in thermal water bodies, but not presenting any risk of spread (WEIPERTH et al. 2015). While public databases provide multiple applications for decision-makers, scientists, and the general public, stricter quality check and control of the data is required to avoid these biases.

The revised checklist of alien species has some limitations in the sense that it does not include citizen science data from other potential sources, such as social media and personal blogs. Citizen science is in its incipient stages in Romania, and while some efficient tools are available (e.g., iNaturalist, GBIF, IAS Europe app), a significant amount of biodiversity records, including alien species, from well-intended citizen scientists may remain inaccessible or difficult to access for conservation managers, scientists and policymakers. Besides data accessibility, data validation is essential in data management of online databases. Not all online databases allow public access to readily available datasets and/or provide information regarding the validation process of the input data. We decided not to consider these sources of information (i.e., social media, personal blogs) since their verification and validation by our group of experts would have been time consuming and well beyond the scope of this paper.

The alien vertebrate species present in Romania were mainly introduced intentionally either for fur farming (e.g., the American mink *N. vison*), hunting (e.g., the fallow deer *D. dama* and the pheasant *P. colchicus*), fishing (e.g., Asian carp, the rainbow trout *O. mykiss*), or pet trade (e.g., the rose-ringed parakeet *P. krameri*, the pond slider *T. scripta*). Only a few of them were introduced accidentally, e.g., the two species of rats (*R. norvegicus* and *R. rattus*) introduced in historical times, which are now widespread and have a high impact on native species, economy and human health.

Since the number of alien species worldwide is rapidly increasing with no sign of saturation

(SEEBENS et al. 2017), and in some cases so is their rate of spread (ROQUES et al. 2016) and impacts (TITTENSOR et al. 2014), our list of alien vertebrate species in Romania will require constant updating and impact evaluation.

Acknowledgments: This work was supported by the project East and South European Network for Invasive Alien Species – A tool to support the management of alien species in Bulgaria (ESENIAS-TOOLS), by a grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, project number PN-III-1.2-PCCDI-2017-0721 (INTERASPA), and partly by the project CNFIS-FDI-2020-0523. FS was partially supported by a STSM grant within the COST Action CA17122 – Increasing understanding of alien species through citizen science (Alien CSI) and by the project ANTREPRENORDOC, in the framework of Human Resources Development Operational Programme 2014–2020, financed from the European Social Fund under the contract number 36355/23.05.2019 HRD OP /380/6/13 – SMIS Code: 123847.

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Annex 1. List of reviewed literature and online databases.

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Annex 2. Records of species and subspecies reported in the reviewed literature and online databases, which were not considered as valid, following data quality check.

No	Species name	Taxonomic group	Justification for exclusion
1	<i>Hyla arborea</i>	amphibians	native
2	<i>Lissotriton vulgaris meridionalis</i>	amphibians	subspecies of a native species with the source not found; improbable presence, doubtful record
3	<i>Anas platyrhynchos</i>	birds	native
4	<i>Anser anser</i>	birds	native
5	<i>Aquila chrysaetos</i>	birds	native
6	<i>Athene noctua</i>	birds	native
7	<i>Columba livia</i>	birds	native
8	<i>Cygnus olor</i>	birds	native
9	<i>Passer hispaniolensis</i>	birds	native
10	<i>Streptopelia decaocto</i>	birds	native
11	<i>Tadorna ferruginea</i>	birds	native
12	<i>Tyto alba</i>	birds	native
13	<i>Alburnus alburnus</i>	fish	native
14	<i>Aspius aspius</i>	fish	native
15	<i>Barbus meridionalis</i>	fish	refers to preserved specimens which later proved to be <i>B. petenyi</i> , which is a native species; see BĂNĂRESCU (1964) and KOTLIK et al. (2002)
16	<i>Barbus peloponnesius</i>	fish	refers to preserved specimens which later proved to be <i>B. petenyi</i> , which is a native species; see BĂNĂRESCU (1964) and KOTLIK et al. (2002)
17	<i>Benthophilus stellatus</i>	fish	native
18	<i>Betta splendens</i>	fish	can survive only in thermal waterbodies
19	<i>Carassius carassius</i>	fish	native
20	<i>Clupeonella cultriventris</i>	fish	native
21	<i>Coregonus maraena</i>	fish	source not found; improbable presence, doubtful record. Possibly confusion with <i>C. lavaretus maraenoides</i>
22	<i>Cyprinus carpio</i>	fish	native; see ZHOU et al. 2003 and XU et al. 2014
23	<i>Cyprinus carpio haematopterus</i>	fish	subspecies of a native species
24	<i>Cyprinus carpio var koi</i>	fish	ornamental variety of a native species
25	<i>Esox lucius</i>	fish	native
26	<i>Gambusia affinis</i>	fish	error; <i>G. holbrooki</i> was introduced in Romania; see BĂNĂRESCU 1964
27	<i>Gymnocephalus cernuus</i>	fish	native
28	<i>Huso huso</i>	fish	native
29	<i>Huso huso x Acipenser ruthenus</i>	fish	native
30	<i>Leucaspis delineatus</i>	fish	native
31	<i>Leuciscus idus</i>	fish	native
32	<i>Leuciscus leuciscus</i>	fish	native
33	<i>Macropodus opercularis</i>	fish	can survive only in thermal waterbodies
34	<i>Neogobius fluviatilis</i>	fish	native
35	<i>Neogobius gymnotrachelus</i>	fish	native
36	<i>Neogobius kessleri</i>	fish	native
37	<i>Neogobius melanostomus</i>	fish	native
38	<i>Oreochromis mossambicus</i>	fish	source not found; improbable presence, doubtful record
39	<i>Perca fluviatilis</i>	fish	native
40	<i>Percarina demidoffii</i>	fish	native
41	<i>Pethia conchoni</i>	fish	can survive only in thermal waterbodies
42	<i>Pethia ticto</i>	fish	can survive only in thermal waterbodies

No	Species name	Taxonomic group	Justification for exclusion
43	<i>Phoxinus phoxinus</i>	fish	native
44	<i>Poecilia sphenops</i>	fish	can survive only in thermal waterbodies
45	<i>Proterorhinus marmoratus</i>	fish	native
46	<i>Proterorhinus semilunaris</i>	fish	native
47	<i>Rhodeus sericeus</i>	fish	native
48	<i>Rutilus rutilus</i>	fish	native
49	<i>Salmo trutta</i>	fish	native
50	<i>Sander lucioperca</i>	fish	native
51	<i>Scardinius erythrophthalmus</i>	fish	native
52	<i>Silurus glanis</i>	fish	native
53	<i>Sparus aurata</i>	fish	native
54	<i>Squalius cephalus</i>	fish	native
55	<i>Telestes souffia</i>	fish	native
56	<i>Tinca tinca</i>	fish	native
57	<i>Trichopodus trichopterus</i>	fish	can survive only in thermal waterbodies
58	<i>Xiphophorus hellerii</i>	fish	can survive only in thermal waterbodies
59	<i>Xiphophorus maculatus</i>	fish	can survive only in thermal waterbodies
60	<i>Bos taurus</i>	mammals	domestic/captivity
61	<i>Bubalus bubalis</i>	mammals	domestic/captivity
62	<i>Capra ibex</i>	mammals	cannot be considered alien since fossil remains were found in Peștera cu Oase (cave), Romania. See QUILÈS et al. (2006)
63	<i>Cervus elaphus</i>	mammals	native
64	<i>Crocifera suaveolens</i>	mammals	native
65	<i>Delphinapterus leucas</i>	mammals	error; possibly part of a museum collection; source not found
66	<i>Erinaceus concolor</i>	mammals	probably refers to <i>E. romanicus</i> ; confusion due to taxonomic re-evaluations
67	<i>Erinaceus europaeus</i>	mammals	probably refers to <i>E. romanicus</i> ; confusion due to taxonomic re-evaluations
68	<i>Lepus europaeus</i>	mammals	native
69	<i>Lepus timidus</i>	mammals	source not found; improbable presence, doubtful record
70	<i>Macaca sylvanus</i>	mammals	probably recorded from captivity; source not found
71	<i>Microtus oeconomus</i>	mammals	source not found; probably recorded from Pleistocene fossils found in Romania; see PETCULESCU & ȘTIUCĂ (2008)
72	<i>Mus musculus</i>	mammals	cryptogenic
73	<i>Mustela erminea</i>	mammals	native
74	<i>Ovis aries</i>	mammals	domestic
75	<i>Ovis aries musimon</i>	mammals	subspecies of a domesticated species; uncertain origin; see ANTONE (2013)
76	<i>Procyon lotor</i>	mammals	source not found; probably recorded from captivity
77	<i>Rangifer tarandus</i>	mammals	source not found; probably recorded from captivity or Pleistocene fossils found in Romania; see PETCULESCU & ȘTIUCĂ (2008)
78	<i>Rupicapra rupicapra</i>	mammals	native
79	<i>Sus scrofa</i>	mammals	native
80	<i>Vulpes lagopus</i>	mammals	fossil record
81	<i>Podarcis muralis maculiventris</i>	reptiles	subspecies of a native species
82	<i>Zamenis situla</i>	reptiles	source not found; probably recorded from captivity or museum collection; might be in reference to FUHN & VANCEA (1961) citing KIRITZESCU (1930), regarding a misidentified specimen from an old collection of Natural History Museum

Testing the hybrid superiority hypothesis in crested and marbled newts

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Funding information

Core Fulbright Visiting Scholar Program, Grant/Award Number: 632

Abstract

We tested the hybrid superiority hypothesis in the zone of overlap and hybridization of the newts *Triturus cristatus* and *T. marmoratus*. To do so, we compared size, age, and growth-related parameters in F_1 hybrids and both parental species in Mayenne, France. We found significant differences in snout-vent length (SVL), body mass and average lifespan between the parental species and hybrids, increasing from *T. cristatus* – *T. marmoratus* – hybrids. The relation between age and SVL fitted von Bertalanffy's growth model and showed that SVL_{max} was significantly larger in hybrids than in the parental species, while the growth coefficient was lower in hybrids and *T. marmoratus* than in *T. cristatus*. *Triturus cristatus* appears to be the better competitor, since it attains sexual maturity faster and thus achieves more annual breeding opportunities. At the evolutionary level, the observed heterosis appears not to have further consequences as the hybrids are largely infertile. Our results support the hypothesis raised for the genus *Triturus*, that infertile hybrids allocate resources to growth.

KEYWORDS

age, growth, heterosis, *Triturus cristatus*, *Triturus marmoratus*

1 | INTRODUCTION

Previously isolated populations may come into contact, with a mixed population arising that incorporates genes from one genetically distinct population into another (Futuyama, 1998). This secondary contact between taxa is known as natural hybridization. Consequently, natural hybridization may be an important process in the shaping of the evolutionary trajectories of many species (Genovart, 2009). Natural hybridization can either promote evolutionary divergence between taxa, for example by reinforcement, or prevent it, and involves many ecological factors (Genovart, 2009). The outputs of hybridization may be the establishment of a stable and localized hybrid zone that does not cause the disappearance of the original species, the disappearance of one of the two original species, or even the appearance of a new species, resulting from hybrid speciation (Mallet,

2007). Hybrid phenotypes are often intermediate between those of parental species (Holzman & Hulsey, 2017), but hybridization can generate novel phenotypes when traits are complex, much faster than evolutionary processes relying on mutation and selection (Sefc et al., 2017; Stelkens & Seehausen, 2009).

Hybridization may result in overall heterosis (also known as hybrid vigor): a higher fitness of hybrids, expressed either as higher biomass production or faster growth rate, as compared to their parents (e.g., Bar-Zvi, Lupo, Levy, & Barkai, 2017; Bernardes, Stelkens, & Greig, 2017). Heterosis can stabilize polymorphisms by increasing fitness in hybrids and thereby favor the maintenance of hybridization either through the improved exploitation of novel ecological niches or the masking of recessive deleterious mutations (Arnold, 1997). Therefore, two opposing mechanisms are in place: hybrid incompatibility acts to avert ongoing hybridization while heterosis favors the maintenance of hybrids. Incompatible alleles at different loci, inherited from the parental species, may not be harmful in F_1 but can be deleterious when homozygous, a condition which should

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be more frequent in higher generation hybrids, potentially causing hybrid breakdown in F_2 (Stelkens, Schmid, & Seehausen, 2015). Since hybrid zones are dynamic, interspecific variation in life-history traits is an important parameter in understanding them (e.g., Scribner, 1993). While hybridization was reported in all vertebrate groups (e.g., in birds, Grant & Grant, 1992), it is more common in fish and amphibians, being facilitated by frequent external fertilization and incomplete reproductive isolation mechanisms (Scribner, Page, & Bartron, 2000; Wells, 2010). Most studies of interspecific hybridization in amphibians have addressed variation in life-history traits related to reproductive output, including clutch size, offspring size, or the viability of offspring (Arntzen & Hedlund, 1990; Kruuk et al., 1999). Few studies have investigated age-related parameters such as rates of survival, age at maturity, and age-specific fecundity in hybrid and parental populations (e.g., Francillon-Vieillot, Arntzen, & Géraudie, 1990; Peter, 2001; Scroggie, 2012).

In European newts, natural hybridization has been reported between *Triturus cristatus* Laurenti, 1768, and *T. marmoratus* Latreille, 1800 (Caudata: Salamandridae) (Arntzen & Wallis, 1991; Vallée, 1959), *T. marmoratus* and *T. pygmaeus* Wolterstorff, 1905 (Espregueira Themudo, Nieman, & Arntzen, 2012), *Lissotriton vulgaris* Linnaeus, 1758 and *L. montandoni* Boulenger, 1880 (Babik, Szymura, & Rafiński, 2003), *L. vulgaris* and *L. helveticus* Razoumovsky, 1789 (Arntzen, de Wijer, Jehle, Smit, & Smit, 1998; Griffiths, Roberts, & Sims, 1987), and also among species in *T. cristatus* species group (Wielstra & Arntzen, 2011; Wielstra, McCartney-Melstad, Arntzen, Butlin, & Shaffer, 2019). In captivity, hybridization is more frequent (Cogălniceanu, 1994) and may even involve intergeneric reproduction (Macgregor, Sessions, & Arntzen, 1990; Mancino, Raggianti, & Bucci-Innocenti, 1979). Newts of the genus *Triturus* provide an excellent case study for evolutionary biology and the study of inter-specific hybridization, since species ranges are largely contiguous, with pairs representing different levels of relatedness (Arntzen, Üzü, Ajduković, Ivanović, & Wielstra, 2018; Arntzen, Wielstra, & Wallis, 2014).

One out of many hybrid zones of secondary contact formed after the last glaciation is between *T. cristatus* (crested newts) and *T. marmoratus* (marbled newts) (Harrison, 1990; Hewitt, 1988). The two species differentiated as distinct lineages about 27.6 Ma (Wielstra & Arntzen, 2011), but still share many morpho-anatomical characters (Mancino, 1990). Their ranges are mostly allopatric, with the exception of a relatively narrow zone in Central and Western France where they overlap (Lescure & de Massary, 2013). Here, some form of ecological isolation was expressed since typically *T. marmoratus* prefers hilly and wooded areas, while *T. cristatus* prefers flat and open areas (Schoorl & Zuiderwijk, 1980). The F_1 hybrids (henceforth "hybrids") between the two species are easily distinguished by their intermediate morphologies (Vallée, 1959). Within the hybrid zone, hybrids are infrequent (at ca. 4% of the total population) and introgression is rare (<0.1%) (Arntzen, Jehle, Bardakci, Burke, & Wallis, 2009; Arntzen & Wallis, 1991). There is a strong asymmetry in the direction of the cross, with one class (*cristatus*-mothered) making up about 90% of hybrids. Since both classes of hybrids are present

at similar frequencies at the embryo stage, the asymmetry appears to be due to differential selection on the two hybrid classes after hatching, rather than prezygotic effects (Arntzen et al., 2009). The hybrid zone appears to be shifting, with *cristatus* spreading into the *marmoratus* range, possibly triggered or accelerated by anthropogenic change (Visser, de Leeuw, Zuiderwijk, & Arntzen, 2017).

Size and age in *T. cristatus*, *T. marmoratus* and their hybrids were previously studied by Francillon-Vieillot et al. (1990). The authors reported that size and age of hybrids had intermediate values between those of the parental species. Later it became apparent that the study may have been biased, since the pond that was the source of most of the hybrids was created in 1970 and sampled in 1981, opening the possibility that older hybrids were underrepresented. In the present study, we test the hybrid superiority hypothesis by comparing size, age, survival, and growth-related parameters in hybrids and their parental species: *T. cristatus* and *T. marmoratus*.

2 | MATERIALS AND METHODS

2.1 | Sampling

The location of the hybrid zone in Mayenne (France) was previously described by Schoorl and Zuiderwijk (1980) and Visser et al. (2017). The material for this study was collected opportunistically from 2006 to 2013 in mature ponds documented to widely exceed the reported longevity of the species. Hybrid newts were sampled from 10 ponds, *T. marmoratus* from two ponds and *T. cristatus* from four ponds. Upon capture by dip-netting, the snout-vent length (SVL) of the newts was measured up to and including the insertion of the hind legs, without the cloaca; this measurement was preferred for consistency with the previous study in the area by Francillon-Vieillot et al. (1990). Body mass (BM) was measured using a portable electronic balance. One toe was cut and stored in 70% ethanol. All newts were released immediately afterwards at the pond where they were found. We collected toes from 120 adult newts and, in addition, toes from 11 juveniles of *T. cristatus* were used for calibration. Not all individuals that were toe-clipped were measured and weighed (missing data $n = 14$).

2.2 | Age estimation through skeletochronology

We followed the method described by Castanet and Smirina (1990) with small modifications. Briefly, in each preserved toe we removed the soft tissues, then decalcified in 5% nitric acid for 2 to 20 min, depending on the size of the bone, and washed it in tap water overnight. The bones were subsequently embedded in TissueTek O.C.T Compound (Sakura Finetek), and 14 μ m thick cross sections were cut with a Tehsys CR 3000 cryotome. Sections with the smallest marrow cavity and the thickest periosteal bone were stained with Ehrlich's hematoxylin for 15 min and washed in distilled water for another 15 min. The slides were made permanent using an aqueous mounting agent for microscopy (Aquatex, Merck Milipore). Informative sections were photographed using an Olympus E-620

TABLE 1 Snout-vent length (SVL) and body mass in *T. cristatus*, *T. marmoratus*, and hybrids

Species	Sex	N _{SVL}	SVL (mm)	SSD _{SVL}	N _{BM}	Body mass (g)	SSD _{BM}
<i>T. cristatus</i>	F	24	65.8 ± 6.6 (55.0–84.0)	1.12*	23	9.3 ± 1.7 (6.5–14.9)	1.12*
	M	14	59.0 ± 4.2 (51.0–65.0)		14	8.3 ± 1.5 (6.3–13)	
<i>T. marmoratus</i>	F	13	66.5 ± 3.3 (62.0–72.0)	1.12*	13	14.4 ± 1.2 (12.3–16.9)	1.32*
	M	19	59.6 ± 2.9 (53.0–65.0)		19	10.9 ± 2.1 (7.2–17.1)	
Hybrids	F	15	77.7 ± 7.3 (63.0–91.0)	1.06	9	16.6 ± 4.0 (10.3–23.7)	1.03
	M	10	73.4 ± 6.9 (65.0–85.0)		6	16.1 ± 3.7 (10.9–21.6)	

Note: The data presented are average values with standard deviation; range is presented in brackets as minimum and maximum values. Sexual Size Dimorphism index (SSD) is presented for both SVL and BM.

N, sample size

*Females were significantly larger than males ($p < 0.01$).

microscope-mounted camera (Olympus CX 31 microscope). The resulting imagery was analyzed with Quick Photo Micro 2.3 software.

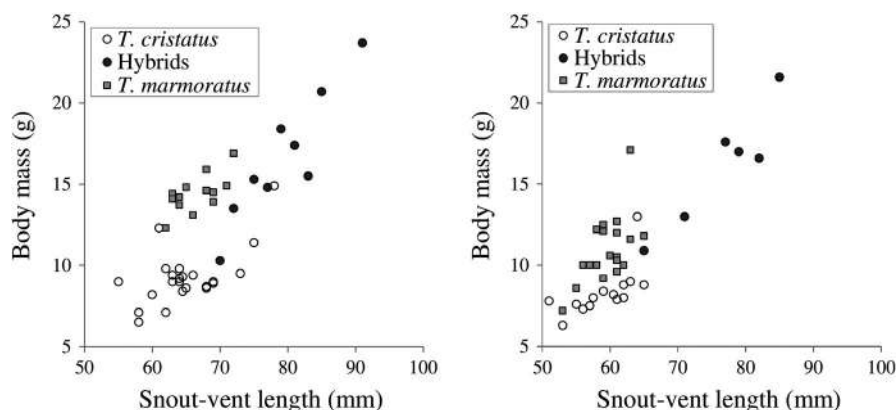
Two observers (DC and FS) independently recorded the presence/absence of the line of metamorphosis (LM) and counted the lines of arrested growth (LAGs) in three to five sections per individual. LM is a fine line within the first year's growth zone, separating larval tissue (usually stained darker) from the post-metamorphic bone, caused by the metamorphic process itself and the transition to the terrestrial environment (Rozenblut & Ogielska, 2005). The presence of a LM indicates that no full resorption occurred.

2.3 | Data analysis

Apart from our own dataset, we used for comparison the original data (i.e., age and SVL) from Francillon-Vieillot et al. (1990), for which we performed additional analyses. We first tested the data for normality and homoscedasticity and selected the statistical tests accordingly. Data transformations did not help to attain normality

in BM and age. Normally distributed data (i.e., SVL) were analyzed using a one-way ANOVA. Otherwise the data (i.e., BM and age) were tested with non-parametric substitutes (Mann-Whitney U test, Kruskal-Wallis H test). All tests were done using the statistical software IBM SPSS Statistics 20 (IBM Corp., 2011). We computed a sexual size dimorphism index (SSD) for SVL and body mass, with the results arbitrarily defined as positive when females are the larger sex and negative in the converse situation (Lovich & Gibbons, 1992).

We estimated the following parameters: (a) average lifespan, as the mean age of breeding individuals; (b) age at sexual maturity, as the minimum age of breeding individuals; (c) longevity, as the maximum age observed; and (d) potential reproductive lifespan (PRLS), as the difference between longevity and age at sexual maturity. We also computed: (e) the annual adult survival rate (S) according to Robson and Chapman's formula (see Maud, Guyétant, & Elmberg, 1999): $S = T / (R + T - 1)$, where $T = N_1 + 2N_2 + 3N_3 + 4N_4 + \dots$, $R = \sum N_i$, N_i number of individuals in age group i , and (f) adult life expectancy (ESP), the expected total longevity of individuals which have reached

**FIGURE 1** The increase in body mass according to SVL follows the pattern *T. cristatus* – *T. marmoratus* – hybrids in both males (left) and females (right)

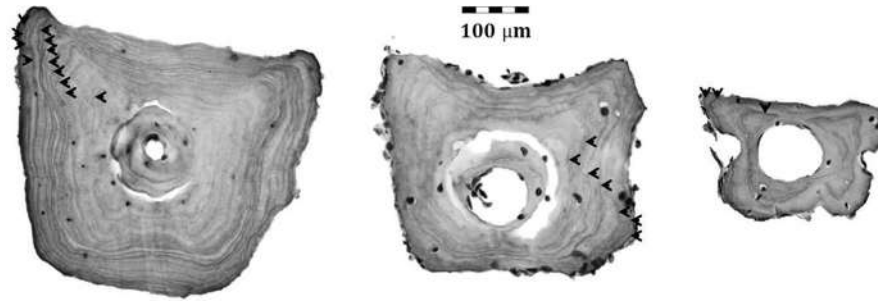


FIGURE 2 Cross sections through phalanges from a 12-year-old hybrid female (left), a 7-year-old *T. marmoratus* female (middle) and a 3-year-old *T. cristatus* male (right). Arrows indicate LAGs

maturity, using Seber's formula (Seber, 1973): $ESP = 0.5 + 1/(1 - S)$. ESP is the expected average age and differs from the “longevity” value that is simply the highest recorded age, which can be affected by sample size since the probability of encountering older specimens increases with sample size.

We computed von Bertalanffy's growth model (von Bertalanffy, 1938) following Cogălniceanu and Miaud (2003): $SVL_t = SVL_{max} - (SVL_{max} - SVL_0) \times e^{-k \times t}$, where SVL_t is the expected or average SVL at time (or age) t , SVL_{max} is the asymptotic average SVL, SVL_0 is the average SVL at time zero (i.e., metamorphosis), and k is the growth coefficient, a measure of the exponential rate of approach to the asymptotic size. We tested the growth models with a series of values of SVL at metamorphosis provided by literature: 32.7–35.8 mm (Arntzen, 2000) and 34 ± 3.87 mm (range 29.88–37.63) (Furtula, Todorović, Simić, & Ivanović, 2009) for *T. cristatus*, and 20–29.1 mm (Alvarez, Salvador, Lopez, & Martin, 1989) and 31.5 ± 6.6 mm (Jakob, Miaud, Crivelli, & Veith, 2003) for *T. marmoratus*. The best parameter estimates (i.e., with the narrowest confidence intervals and lowest standard error) were obtained

when SVL_0 value was set to 30 mm for both species and hybrids. The sample sizes of males and females for each species were small and resulted in unreliable estimates (i.e., high standard error, large confidence intervals, and negative values); therefore, we pooled the samples of males and females per group and per dataset for each group. We fitted von Bertalanffy's growth model and estimated growth parameters (henceforth VBGP) by non-linear least squares regression. Two estimated VBGP were considered significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap. We used the packages FSA (Ogle, 2016) and nlstools (Baty et al., 2015) in R Studio version 1.1.423 (R Core Team, 2017).

3 | RESULTS

3.1 | Size

The descriptive statistics of SVL and BM for the two parental newt species and the hybrids are presented in Table 1. Overall, both SVL and BM increase in the order *T. cristatus* – *T. marmoratus* – hybrids,

TABLE 2 Age-related parameters (expressed in LAGs) in *T. cristatus*, *T. marmoratus* and hybrids

Species	Sex	N	Mean age ± SD	AM	Longevity	PRLS	S	ESP
Present study								
<i>T. cristatus</i>	F	25	3.5 ± 1.8	2	8	6	0.62	3.15
	M	14	3.4 ± 1.4	2	7	5	0.61	3.04
<i>T. marmoratus</i>	F	13	5.4 ± 0.9	4	7	3	0.60	3.00
	M	19	5.2 ± 1.0	4	8	4	0.57	2.83
Hybrids	F	19	6.5 ± 2.7	3	12	9	0.78	5.17
	M	16	6.3 ± 2.0	4	11	7	0.71	3.97
Francillon-Vieillot et al., 1990								
<i>T. cristatus</i>	F	36	3.9 ± 1.6	2	8	6	0.66	3.44
	M	41	4.7 ± 3.0	2	14	12	0.73	4.27
<i>T. marmoratus</i>	F	37	6.4 ± 2.1	4	13	9	0.82	6.12
	M	32	6.1 ± 3.3	2	14	12	0.80	5.69
Hybrids	F	33	6.4 ± 3.3	2	17	15	0.81	5.95
	M	24	8.4 ± 3.9	3	15	12	0.85	7.50

Note: Top panel – present study; bottom panel data from Francillon-Vieillot et al. (1990).
Abbreviations: AM, age at sexual maturity; ESP, adult life expectancy; N, sample size; PRLS, potential reproductive lifespan; S, annual survival rate.

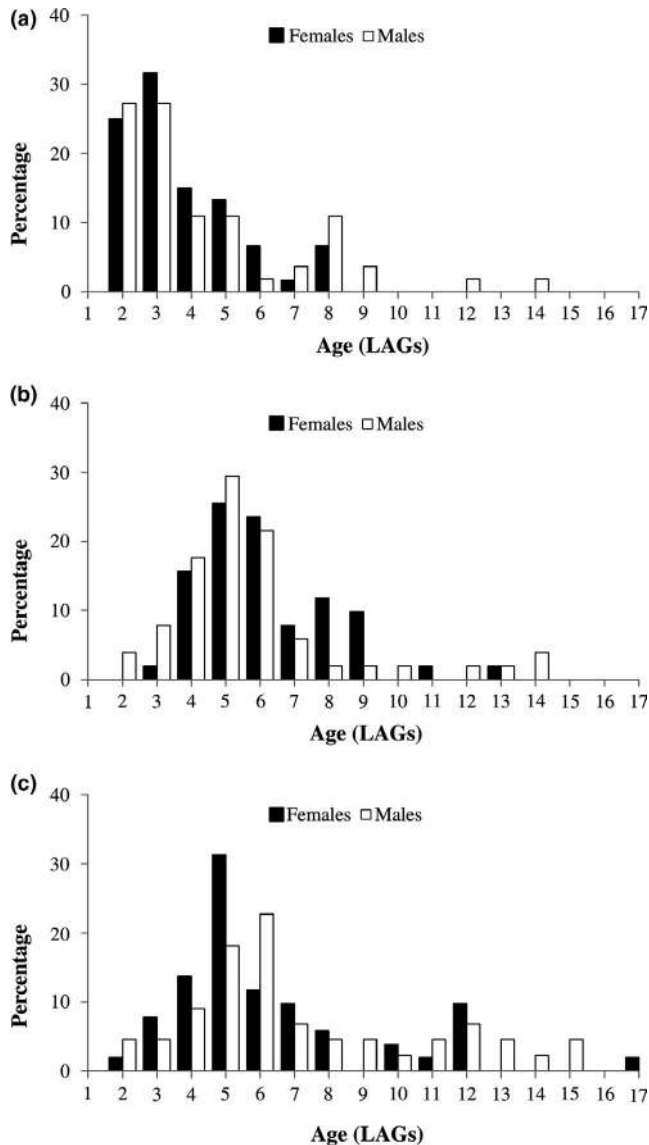


FIGURE 3 Age structure (pooled datasets) observed in *T. cristatus* (a), *T. marmoratus*, (b) and hybrids (c)

for males as well as for females (Figure 1). We observed a slight, yet significant sexual size dimorphism in SVL and BM, with females significantly larger and heavier than males in both parental species, but not in the hybrids (Table 1). Snout-vent length differed significantly among groups (ANOVA, males, $F_{2,40} = 36.589$, $p < 0.001$; females, $F_{2,49} = 19.002$, $p < 0.001$). Post hoc pairwise comparisons showed significant differences in SVL between males of hybrids and the parental species (Tamhane test: hybrids-*T. cristatus*, mean difference = 14.400, $p < 0.001$; hybrids-*T. marmoratus*, mean difference = 13.821, $p < 0.001$), but not between males of the parental species (*T. marmoratus*-*T. cristatus*, mean difference = 0.579, $p = 0.962$). This pattern was similar in females (LSD pairwise comparisons, hybrids-*T. cristatus*, mean difference = 11.916, $p < 0.001$; hybrids-*T. marmoratus*, mean difference = 11.205, $p < 0.001$; *T. marmoratus*-*T. cristatus*, mean difference = 0.711, $p = 0.962$).

Body mass also differed significantly among species, both in males (Kruskal-Wallis H, $\chi^2(2) = 21.648$, $p < 0.001$; Dunn-Bonferroni post hoc pairwise comparisons: *T. cristatus*-*T. marmoratus* = -3.209, $p = 0.004$, *T. cristatus*-hybrids = -4.398, $p < 0.001$, *T. marmoratus*-hybrids = -2.169, $p = 0.090$) and females (Kruskal-Wallis H, $\chi^2(2) = 29.880$, $p < 0.001$; Dunn-Bonferroni post hoc pairwise comparisons: *T. cristatus*-*T. marmoratus* = -4.145, $p < 0.001$, *T. cristatus*-hybrids = -4.698, $p < 0.001$, *T. marmoratus*-hybrids = -0.943, $p = 1.000$).

To ascertain that the pattern of size heterosis was not biased due to the current small sample size, we pooled our SVL data with those from Francillon-Vieillot et al. (1990). We obtained similar results: the hybrids were significantly larger than the parental species in both males ($F_{2,137} = 16.698$, $p < 0.001$; Tamhane post hoc tests: hybrids-*T. cristatus*, mean difference = 6.613, $p < 0.001$; hybrids-*T. marmoratus*, mean difference = 7.568, $p < 0.001$) and females ($F_{2,155} = 7.673$, $p = 0.001$; Tamhane post hoc tests: hybrids-*T. cristatus*, mean difference = 4.354, $p = 0.021$; hybrids-*T. marmoratus*, mean difference = 5.170, $p = 0.003$). The SVL of parental species was similar (Tamhane post hoc tests, males: *T. marmoratus*-*T. cristatus*, mean difference = -0.954, $p = 0.792$; females: *T. marmoratus*-*T. cristatus*, mean difference = -0.816, $p = 0.843$).

3.2 | Age, growth and survival

Age could be established in 106 adults and 11 juveniles where LAGs were clearly expressed (Figure 2). In 14 adults, age could not be estimated with certainty or slides were unavailable due to technical errors.

We found no significant differences between the average lifespan of males and females of the parental species (Mann-Whitney U test, *T. cristatus*, $U = 167.5$, $p = 0.821$; *T. marmoratus*, $U = 139.0$, $p = 0.528$) and their hybrids ($U = 142.5$, $p = 0.749$). Since males and females had similar ages in each group, we pooled them for the subsequent analyses. Average lifespan differed significantly among the two parental species and their hybrids (Kruskal-Wallis H, $\chi^2(2) = 41.143$, $p < 0.001$), showing a similar pattern as size (i.e., SVL and BM), increasing from *T. cristatus* - *T. marmoratus* - hybrids (Dunn-Bonferroni post hoc pairwise comparisons: *T. cristatus*-*T. marmoratus* = -4.583, $p < 0.001$; *T. cristatus*-hybrids = -6.097, $p < 0.001$; *T. marmoratus*-hybrids = -1.334, $p = 0.546$) (Table 2, Figure 3). We found the same pattern for the 1980s dataset (Kruskal-Wallis H, $\chi^2(2) = 37.519$, $p < 0.001$; Dunn-Bonferroni post hoc pairwise comparisons: *T. cristatus*-*T. marmoratus* = -4.874, $p < 0.001$; *T. cristatus*-hybrids = -5.501, $p < 0.001$; *T. marmoratus*-hybrids = -0.856, $p = 1.000$). Average lifespan was similar across the two datasets (Mann-Whitney U test, *T. cristatus*: $U = 1783.5$, $p = 0.091$; *T. marmoratus*: $U = 1,301.5$, $p = 0.141$; hybrids: $U = 1,063.0$, $p = 0.594$). Longevity, survival rate and ESP were lower in the present material compared to the data of Francillon-Vieillot et al. (1990).

Von Bertalanffy growth models fitted age and SVL data in both species and hybrids (Table 3, Figure 4). Growth models were similar in the parental species (i.e., no significant differences in SVL_{max} and k , Table 3). SVL_{max} was significantly larger in hybrids compared to both parental species. The growth coefficient was significantly lower in hybrids compared to *T. cristatus*, but not *T. marmoratus*.

Species	N	SVL _{max}	k	Dataset
<i>T. cristatus</i>	^a 49	66.67 ± 2.42 (61.68–71.45)	0.70 ± 0.14 (0.41–1.01)	Present study
	^a 87	69.07 ± 1.15 (66.78–71.37)	1.00 ± 0.14 (0.72–1.28)	Francillon-Vieillot et al., 1990
	^a 136	68.52 ± 1.15 (6.25–70.81)	0.82 ± 0.10 (0.63–1.01)	Pooled datasets
<i>T. marmoratus</i>	32	68.55 ± 4.29 (59.78–77.32)	0.36 ± 0.12 (0.12–0.59)	Present study
	69	66.59 ± 1.01 (64.57–68.61)	0.72 ± 0.17 (0.39–1.05)	Francillon-Vieillot et al., 1990
	101	65.97 ± 0.97 (64.05 – 67.90)	0.64 ± 0.12 (0.40 – 0.88)	Pooled datasets
Hybrids	25	85.94 ± 3.11 (79.50–92.38)	0.28 ± 0.04 (0.19–0.37)	Present study
	57	72.56 ± 1.79 (68.96–76.16)	0.46 ± 0.09 (0.28–0.64)	Francillon-Vieillot et al., 1990
	82	75.93 ± 1.69 (72.57–79.29)	0.40 ± 0.06 (0.28–0.52)	Pooled datasets

TABLE 3 Growth parameters in *T. cristatus*, *T. marmoratus*, and their hybrids estimated from Von Bertalanffy's equation

Note: The raw data from the study of Francillon-Vieillot et al. (1990) were also used to compute von Bertalanffy's growth parameters, either separately or pooled with our data.

N, sample size; SVL_{max}, the maximum asymptotic SVL; k, the growth coefficient

Values are given as estimate ± standard error; the 2.5%–97.5% confidence interval for each estimate is presented between brackets.

^aJuveniles were also included in the analysis.

4 | DISCUSSION

The results of our study support the hybrid superiority hypothesis, showing that hybrids of crested and marbled newts grow larger

and frequently outlive both parental species. This conclusion is in agreement with other studies, like Arnold and Hodges (1995) who reported that for some plant and animal hybrid genotypes fitness estimates are equal or even greater than those of the parental species, suggesting a significant role for natural hybridization in adaptive evolution. A similar result was reported in triploid hybrids of rock lizards (genus *Darevskia*), with hybrids growing faster and being larger compared with parental species, despite having similar longevities (Arakelyan, 2002).

In their area of range overlap, marbled (*T. marmoratus*) and crested (*T. cristatus*) newts show practically no backcrossing (Arntzen & Wallis, 1991). Attempts to obtain F₂ generation in captivity failed (Lantz, 1947). This result has been attributed to the observation that F₁ hybrids frequently fail to form mature sperm due to meiotic anomalies (Lantz & Callan, 1954). Also the frequency of severe malformations in the skeleton was higher in hybrids than in the parental species (Vallée, 1959). A comparative study of the variation patterns in limbs showed that digital variation in hybrids was significantly elevated compared with the parental species (Arntzen & Wallis, 1991; Rienesl & Wagner, 1992). At the chromosome level, the two species suffered structural rearrangements affecting one arm of the longer chromosome (number 1) that effectively suppresses synapsis and crossing-over. Thus, males and females of both parental species are consistently heteromorphic for different forms of chromosome 1, which is essential for normal development. Individuals that have two identical chromosomes 1 die at late tail bud stage of development (Macgregor, 1982). The same lethality factor(s) that

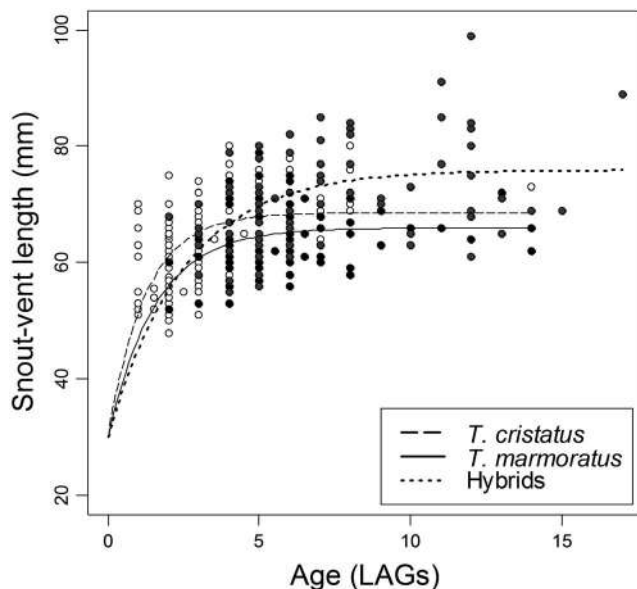


FIGURE 4 Growth described by von Bertalanffy's model (pooled datasets) in *T. cristatus* (white circles), *T. marmoratus* (black circles), and hybrids (gray circles). SVL at metamorphosis (i.e., age = 0 LAGs) was set to 30 mm

operate in homomorphic embryos for chromosome 1 also probably operate(s) in F_1 hybrids (Macgregor & Sessions, 1986). Since *T. cristatus* – *T. marmoratus* hybrids are largely sterile and introgression is near-absent, hybridization between these two species appears to be an evolutionary dead-end (Arntzen et al., 2009, 2018). This supports the theory of hybrid breakdown in the F_2 , similar to the study of Stelkens et al. (2015) on cichlid fish hybrids. At the other side of the coin, heterosis appears to be absent when there is gene flow, as in the hybrid population of *T. macedonicus* and *T. ivanbureschi* (Arntzen et al., 2018).

Studies from a wide range of taxa have shown a negative relationship between genetic compatibility and the divergence time of hybridizing genomes (Stelkens et al., 2015), since selection acts against hybrid individuals, probably due to disadvantageous combinations of co-adapted gene complexes derived from either parental species, the so-called Dobzhansky–Muller model of hybrid incompatibility (e.g., Parsons, Olson, & Braun, 1993; Wu & Ting, 2004). Theory predicts the main breakdown of fitness to happen after the F_1 hybrid generation, when heterosis subsides and recessive allelic incompatibilities are increasingly unmasked and cause hybrid sterility. Transgressive segregation, commonly found among hybrids (Stelkens & Seehausen, 2009), results from hybridization when multiple alleles with opposing phenotypic effects become fixed in parental lineages and recombination breaks down their compensating combinations (e.g., Seehausen, 2004). Thus, heterosis can be considered a deviation from the evolutionary optimal performance, requiring a safeguard mechanism to prevent extensive vigor (e.g., excessive production of biomass or faster growth), and to maintain the integrity of the organisms. It has been argued that hybrid vigor and hybrid incompatibility have both resulted from the impairment of evolved growth-limiting protective mechanisms (Bar-Zvi et al., 2017). This is consistent with the hypothesis that across the genus *Triturus* fertile hybrids allocate resources to reproduction, while infertile hybrids allocate resources to growth (Arntzen et al., 2018). Our study supports this hypothesis since the infertile hybrids reached larger body size and lived longer compared to the parental species. The growth patterns fit the life history of each species. *Triturus cristatus* approaches maximum SVL and attains sexual maturity rapidly, but has low longevity. *Triturus marmoratus* grows slower, matures later at a larger size and has similar longevity. Finally, hybrids mature late, grow slow, but reach larger size and live longer than parental species.

There are significant geographical and ecological differences in age-related parameters between studies (Cogălniceanu & Miaud, 2003; Lukanov & Tzankov, 2016), outside the hybridization area: Jakob et al. (2003) reported that age at sexual maturity was reached at 2 years in *T. marmoratus*, while Caetano, Castanet, and Francillon (1985) and Caetano and Castanet (1993) reported 4–5 years, similar to our study. Longevity was higher in both studies reaching 15 years of age. Longevity was also higher in *T. cristatus* as reported by Miaud, Joly, and Castanet (1993): 16 years for females and 17 years for males, Hagström (1977): 13 years for females and 16 years for males, or Dolmen (1983): 15 years for

both sexes. A similar longevity, of 13 years in males and 11 years in females, was reported from an admixed *Triturus macedonicus*–*T. ivanbureschi* population (Arntzen et al., 2018). From a life-history perspective, our study indicates *T. cristatus* as being more successful in the competitive arena when compared with *T. marmoratus* and hybrids, since they attain faster sexual maturity and have a longer potential lifespan than *T. marmoratus* (Table 2).

ACKNOWLEDGEMENTS

FS was supported by a Fulbright Senior Award through the Core Fulbright Visiting Scholar Program, from the Romanian–U.S. Fulbright Commission (grant no. 632). The skeletochronological procedure was approved by the Ethics Committee of the Faculty of Natural and Agricultural Sciences, Ovidius University, Constanța. A collecting permit (arrêt no. 2003-A-207) was issued by the “Service Aménagement Environnement – Chasse” of the “Direction départementale de l'Agriculture et de la Forêt de la Mayenne.”

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedure for the skeletochronological study was approved by the Ethics Committee of the Faculty of Natural and Agricultural Sciences, Ovidius University, Constanța.

DATA AVAILABILITY STATEMENT

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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How to cite this article: Cogălniceanu D, Stănescu F, Arntzen JW. Testing the hybrid superiority hypothesis in crested and marbled newts. *J Zool Syst Evol Res*. 2019;00:1–9. <https://doi.org/10.1111/jzs.12322>



Release and distress calls in European spadefoot toads, genus *Pelobates*

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ABSTRACT

The acoustic repertoire of most anurans, even common species, is still insufficiently known. Here, we present a thorough quantitative description of acoustic parameters in release and distress calls in the three European spadefoot toad species, genus *Pelobates*. We also explored the intra- and interspecific variation and effects of body size on acoustic traits. We analysed a total of 749 release calls of 34 individuals of *Pelobates fuscus* and *P. syriacus*, and 312 distress calls of 36 individuals of *P. fuscus*, *P. syriacus* and *P. cultripes*. Release calls consisted of a note with one to three initial pulses of lower energy and a group of consecutive pulses characterized by higher energy in both species analysed. The structure of the distress calls was similar in all three species: loud, explosive, cat meow-like, single multi-pulsed notes rich in harmonics. Some of the variation among species in acoustic parameters in the release and distress calls is related to body size. Acoustic properties for both release and distress calls showed high levels of variability in all species. This pattern of variation is different from what is regularly found in the more conservative advertisement calls, and we advocate that such high variability is possible because release and distress call are not signals with divergent evolutionary history among related species.

ARTICLE HISTORY

Received 2 October 2017
Accepted 4 January 2018

KEYWORDS

Pelobates cultripes; *Pelobates fuscus*; *Pelobates syriacus*; vocalization; acoustic parameters

Introduction

Most of the acoustic signals observed in anuran amphibians occur in the context of mating: advertisement, reciprocation and release calls (Toledo et al. 2015). Release calls are normally short in duration, highly variable in structure and seem to be specific to anurans, emitted when sexually unresponsive individuals are forced into amplexus (Gerhardt 1994). Release calls are an important component of the mate-recognition system, by providing information about the identity of the emitter (Duellman and Trueb 1994; Di Tada et al. 2001; Toledo et al. 2015); hence, they allow energy conservation in cases of erroneous, undesired amplexus

during breeding events (e.g. male–male, male–unreceptive female, male–heterospecific partner). Similar to the advertisement calls, release calls are phylogenetically informative, which emphasizes the importance of its description (Brown and Littlejohn 1972; Sullivan and Wagner 1988; Sullivan 1992; Di Tada et al. 2001).

Distress calls, on the other hand, are acoustic signals used in defensive behaviour, and have been described from a wide range of vertebrates, such as fish (Ladich 1997), amphibians (Duellman and Trueb 1994), reptiles (Staton 1978), birds (Conover 1994) and mammals (Russ et al. 2004). In anuran amphibians, such defensive display is considered an ancestral behaviour, described in more than 100 species (L. Forti, unpublished data). Distress calls are usually loud, sudden sounds, emitted when frogs are grasped by a predator (Toledo and Haddad 2009). This defensive signal was observed in males, females, as well in juveniles and tadpoles (Toledo and Haddad 2009; Natale et al. 2011). In spite of being a conspicuous behaviour, many aspects of its evolution and social functioning are still understudied, even in the most well-studied anuran species. Apparently, the distress calls in anurans did not evolve as an intraspecific warning signal, and its emission is considered to have the purpose of startling an attacking predator of self or spawn (i.e. parental distress call) and perhaps to attract secondary predators (Hödl and Gollmann 1986; Sestito et al. 2016).

The spadefoot toads genus *Pelobates* consists of four cryptic, fossorial and nocturnal anurans: *P. varaldii*, *P. cultripes*, *P. fuscus* and *P. syriacus*, distributed across Europe, Western Asia and North Africa (Salvador et al. 2004; Agasyan et al. 2009a, 2009b; Beja et al. 2009). Two of these species, *P. syriacus* and *P. fuscus*, overlap their ranges along the lower course of Danube River and the western coast of the Black Sea. Generally, life history information across the genus *Pelobates* is scarce, and few studies have looked into their acoustic repertoire: Schneider (1966) was the first to briefly describe the advertisement calls in *P. f. fuscus*, followed by Müller (1984) who provided a more exhaustive description of the acoustic repertoire of the same species; only brief and mostly qualitative descriptions of the advertisement call are provided for *P. f. fuscus* (Nöllert 2003) and *P. syriacus* (Nöllert 2003; Sofianidou 2012; Yegiasaryan 2014). More recently, ten Hagen et al. (2016) described the acoustic repertoire of *P. f. fuscus* juveniles, which might be linked to a foraging context specific to this life stage; Andreone and Piazza (1990) and Seglie et al. (2013) provided thorough descriptions of the vocal repertoire of *P. f. insubricus*, the endemic *P. fuscus* subspecies from Northern Italy; Lizana et al. (1994) were the first to describe the advertisement and briefly, the distress calls of *P. cultripes*. Meanwhile, information regarding vocalizations remains incipient in *P. syriacus* (Nöllert 2003; Sofianidou 2012; Yegiasaryan 2014) and unknown in *P. varaldii*. Our study fills a knowledge gap regarding the vocal repertoire of spadefoot toads, by providing a thorough quantitative description of the acoustic parameters in release and distress calls in *P. fuscus*, *P. syriacus* and *P. cultripes*. In addition, we analysed the relationship between the acoustic traits in release and distress calls, and explored their intra- and interspecific variations.

Materials and methods

Data collection

We collected 18 *P. fuscus* (4 females, 12 males and 2 subadults) and 16 *P. syriacus* (6 females, 7 males and 3 subadults) from their area of sympatry, the coastal wetlands of the Black

Sea in south-east Romania (44°26'04.2"N 28°44'06.6"E, -1 m a.s.l., 6–7 November 2012). We measured snout-vent length (SVL) with digital callipers (precision 0.1 mm), and body mass (BM) with a portable electronic balance (Triton T2, MyWeigh, precision 0.01 g). In both species, males were identified by the presence of humeral pads, and subadults based on SVL and BM thresholds, following Cogălniceanu et al. (2014).

Acoustic samples were obtained in the laboratory at 21 ± 1 °C. Release calls were obtained by simulating amplexus, following Di Tada et al. (2001): we set each individual facing the tip of the microphone and gently compressed their body sides, using the thumb and forefinger. In order to elicit distress calls, we used a pair of long, metallic tweezers enveloped in sterile mesh to grab one or both the hind legs of the frog, with a swift and precise move; we repeated this action three times for each individual. The mesh acted as a soft interface with the tweezers and the procedure produced no visible harm and caused no lesions to the animals which could walk and jump immediately after being handled. Individuals were kept in the laboratory for two consecutive nights, in 10-L plastic boxes covered with mesh, half filled with moist, sandy soil. All individuals were released at the capture site after obtaining the recordings. Sounds were recorded at 44.1 kHz sampling frequency and 16-bit resolution, using a PMD660 Marantz portable solid state recorder and a ME66 Sennheiser unidirectional microphone in the case of *P. fuscus* and *P. syriacus*.

We used for comparison *P. cultripes* samples of distress calls from a population in Los Montalvos, Salamanca, Spain deposited in Fonoteca Zoológica – www.fonozoo.com, at Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain (FonoZoo ID FZ-0884-0885 and FZ-P-1-22, FZ-P-1-23; Márquez and Matheu 2004) and a recording from the sound guide of France, Switzerland, Belgium and Luxembourg (FZ-P-646-6; Déroussen 2002). In addition, we used a sound sample of *P. fuscus* distress call from the sound guide of France, Belgium and Luxembourg (FonoZoo ID FZ-P-380-6; Duguet and Melki 2003).

Acoustic analyses

We carried out the acoustic analyses using the software Raven Pro 1.4 (Bioacoustics Research Program 2011; Cornell Lab of Ornithology). We used a bandpass filter to decrease background noise; in addition, calls were individually normalized (peak -1.0 dB) with Audacity® v. 2.1.1 software (Audacity Team 2015). Temporal variables were measured in the oscillograms. For spectral measurements, we used FFT (Fast Fourier Transformation) 1024 points, and 50% of window overlap. Using the functions 'Bandwidth 90% (Hz)', 'Frequency 5% (Hz)', 'Frequency 95% (Hz)', 'Peak Frequency (Hz)', 'Delta Time (s)' and 'Max Amplitude (U)' in the 'choose measurements' menu in Raven, we measured the following acoustic properties: range frequency (RF); minimum frequency (MinF); maximum frequency (MaxF); dominant frequency (DF); fundamental frequency (FF); call duration (CD). We computed rise time proportion (RT) as the ratio between the period from the onset of the sound and the moment of maximum amplitude within the analysed call, and the call duration (Márquez et al. 2005). We computed the frequency modulation within a call (FM) as the difference between the dominant frequencies at the beginning and at the end of the call (Márquez et al. 1996). For a better understanding of the frequency modulation patterns, we generated figures with the peak frequency contour within release and distress calls for each species

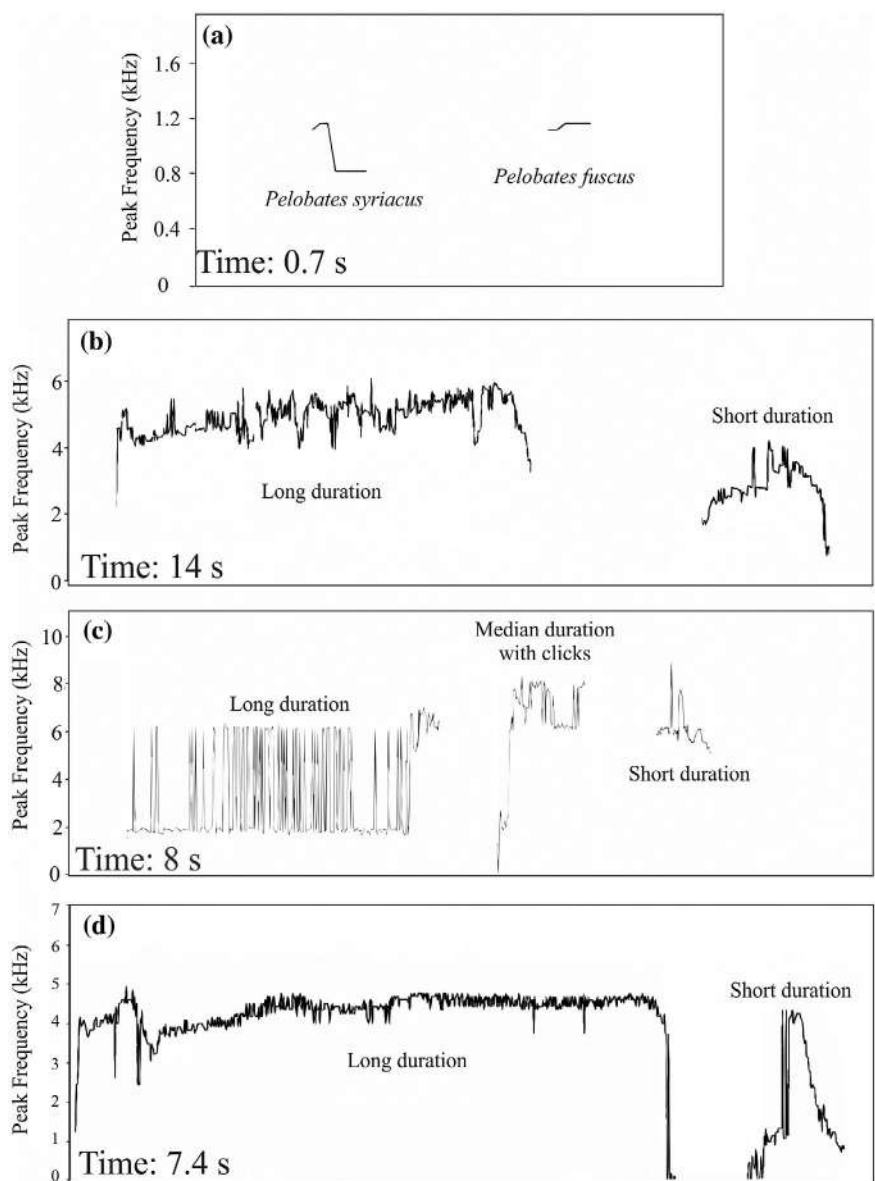


Figure 1. Patterns of frequency modulation by peak frequency contour in (a) release calls of *P. syriacus* and *P. fuscus*, and distress calls of (b) *P. syriacus*, (c) *P. fuscus*, and (d) *P. cultripes*.

(Figure 1). Following Gerhardt (1991), we computed a coefficient of variation ($CV\% = SD / \text{mean} \times 100$) to differentiate between static ($CV\% < 5$) and dynamic ($CV\% > 12$) acoustic parameters.

Following the recommendations of Toledo et al. (2015), the recordings were deposited in Fonoteca Zoológica – www.fonozoo.com, at Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain (FonoZoo ID *P. fuscus*: 8198–8203, *P. syriacus*: 8204–8209). The terminology used in this paper follows Koehler et al. (2017).

Statistical analyses

We performed all statistical analyses using PASW Statistics for Windows, version 18.0 (Chicago: SPSS Inc. 2009). The significance level was set at $\alpha = 0.05$.

To better explain the relationship between the acoustic parameters describing release and distress calls, and to avoid multicollinearity in further analyses, we performed Principal Component Analyses (PCAs) based on a rotated component matrix (rotation method: Varimax with Kaiser Normalization) and retained those principal components (PCs) with eigenvalues > 1.0 . The extracted PCs were further used in multivariate analyses, as described in the following steps.

Data regarding gender and size were only available for *P. fuscus* and *P. syriacus*. Therefore, we used multivariate GLMs to test the effect of species and gender on the PCs of release and distress calls in *P. fuscus* and *P. syriacus*. SVL and BM were highly correlated (Pearson $r = 0.984$, $p = 0.000$), and since body mass is prone to higher variability, we chose SVL as a robust proxy of body size. Thus, we introduced SVL as a covariate into the GLMs in order to control for the effect of body size on the acoustic parameters.

We performed an additional multivariate GLM to test only the effect of species on the acoustic parameters of distress calls in all three species: *P. fuscus*, *P. syriacus* and *P. cultripipes*. Since we had no data available regarding size or gender for *P. cultripipes*, we could not account for the effect of size and gender in this latter analysis.

Significant results of the multivariate GLMs were followed-up by *post hoc* tests of Between-Subjects Effects and Bonferroni pairwise comparisons.

Results

Release calls of *P. fuscus* and *P. syriacus*

We analysed a total of 361 release calls for 18 *P. fuscus* (12 males, 4 females, 2 subadults) and 388 calls for 16 *P. syriacus* (7 males, 6 females, 3 subadults). The general structure of the release calls was similar in both species: a pulsed note composed of one to three initial pulses of lower energy and a group of consecutive pulses characterized by higher energy (Figures 2 and 3). The release calls were emitted with the mouth closed, and individuals used their hind members to push themselves away from the fake amplexus. The quantitative description of the release calls is presented in Table 1.

In *P. fuscus*, minimum frequency and number of visible pulses were considered static parameters (CV $< 5\%$), call duration and dominant frequency were intermediary (CV between 5 and 12%), while all other acoustic parameters were dynamic (CV $> 12\%$). In *P. syriacus*, only the number of visible pulses was static, while minimum frequency and call duration were intermediary and all other acoustic properties were dynamic (Table 1). Only minimum frequency in *P. fuscus* and call duration in *P. syriacus* presented a low coefficient of variation between individuals (CV $< 12\%$).

The correlation matrix in the PCA showed that the tested acoustic parameters were inter-related (Table 2). The PCA revealed three PCs with eigenvalues > 1.0 , explaining 76.03% of the total variance: PC1, eigenvalue = 3.163, 39.54% of variance explained; PC2, eigenvalue = 1.727, 21.59% of variance explained; PC3, eigenvalue = 1.192, 14.90% of variance explained. PC1 was highly loaded with temporal parameters, while PC2 and PC3 were loaded with spectral parameters (Table 3).

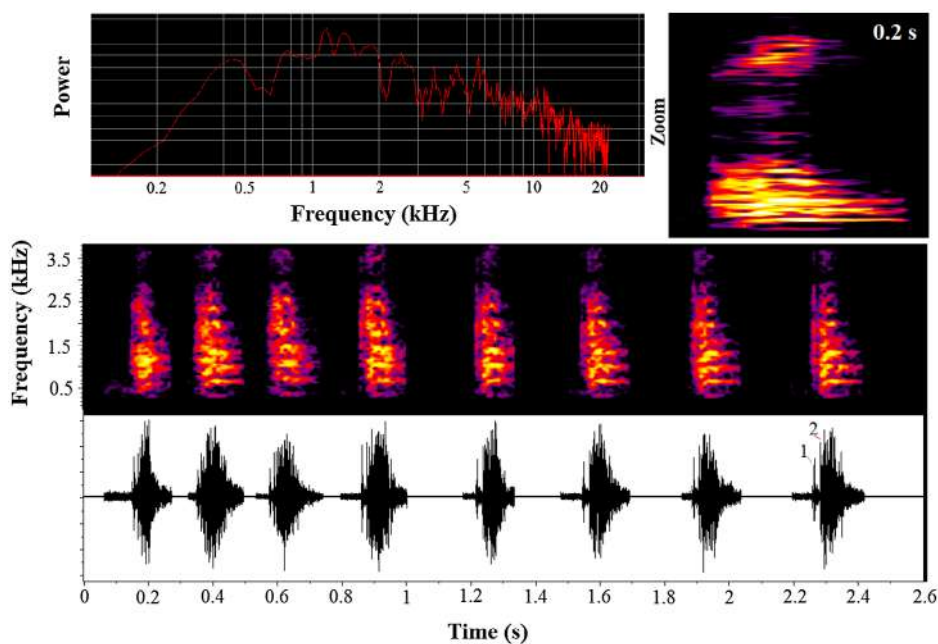


Figure 2. Power spectrum from a slice on zoomed release call, audiospectrogram and oscillogram of a sequence of release calls in *P. syriacus*.

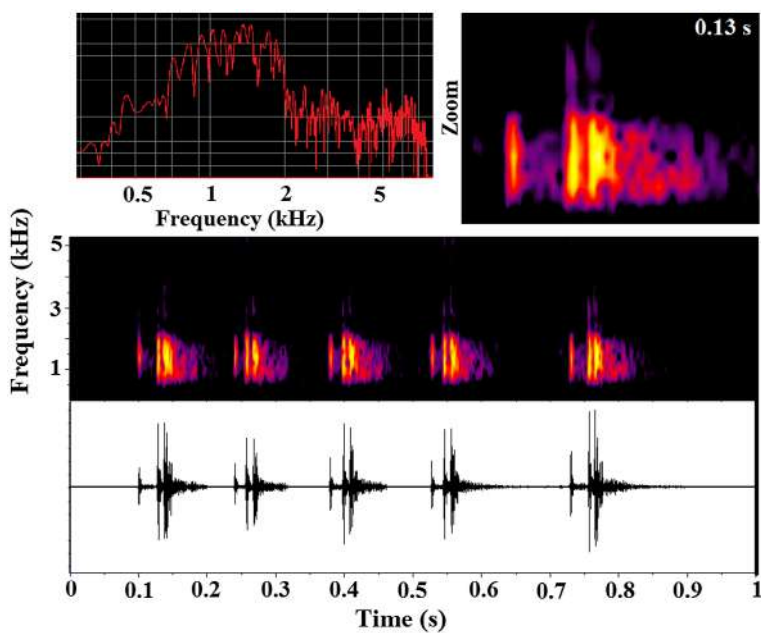


Figure 3. Power spectrum from a slice on zoomed release call, audiospectrogram and oscillogram of a sequence of release calls in *P. fuscus*.

Table 1. Summary of the quantitative description of the release calls in *P. fuscus* ($n = 18$ individuals, 361 calls analysed) and *P. syriacus* ($n = 16$ individuals, 388 calls analysed).

Species		RF (Hz)	MinF (Hz)	MaxF (Hz)	DF (Hz)	FM (Hz)	RT (s)	CD (s)	P
<i>P. fuscus</i>	Mean	1840.0	835.7	2675.7	1214.9	210.7	0.034	0.061	3.2
	±SD	1135.5	104.4	1110.9	264.5	197.8	0.010	0.016	0.5
	CVbetween%	44.8	11.4*	28.9	17.8	93.9	22.1	23.3	16.0
	CVwithin%	21.5	4.1**	14.6	8.8*	118.8	20.0	11.4*	2.0**
<i>P. syriacus</i>	Mean	3016.0	702.5	3718.5	1200.2	296.0	0.050	0.108	2.4
	±SD	1854.9	119.7	1839.9	355.3	201.4	0.015	0.014	0.5
	CVbetween%	55.9	15.0	44.8	20.0	68.1	18.0	9.2*	20.1
	CVwithin%	21.9	7.3*	16.8	20.5	91.4	24.3	8.5*	0.7**

Notes: Mean ± SD and CV% – between-individuals and within-individual coefficients of variation, are presented for each studied parameter: RF = range frequency, MinF = minimum frequency, MaxF = maximum frequency, DF = dominant frequency, FM = frequency modulation, RT = rise time proportion, CD = call duration, P = number of visible pulses. Intermediary parameters (CV among 5 and 12%) are marked with *, and Static parameters (CV < 5%) with **.

Table 2. PCA correlation matrix of the acoustic parameters of release calls.

	RF (Hz)	MinF (Hz)	MaxF (Hz)	DF (Hz)	FM (Hz)	RT (s)	CD (s)	P
RF (Hz)								
MinF (Hz)	-0.442							
	0.004							
MaxF (Hz)	0.997	-0.370						
	0.000	0.016						
DF (Hz)	0.128	0.236	0.153					
	0.235	0.090	0.194					
FM (Hz)	0.283	-0.238	0.273	0.329				
	0.052	0.087	0.059	0.029				
RT (s)	0.190	-0.353	0.166	-0.102	0.139			
	0.141	0.020	0.174	0.284	0.216			
CD (s)	0.369	-0.452	0.342	0.073	0.232	0.792		
	0.016	0.004	0.024	0.341	0.094	0.000		
P	-0.060	0.546	-0.015	0.047	-0.129	-0.305	-0.570	
	0.368	0.000	0.467	0.396	0.233	0.040	0.000	

Notes: Rf = range frequency, MinF = minimum frequency, MaxF = maximum frequency, DF = dominant frequency, FM = frequency modulation, RT = rise time proportion, CD = call duration, P = number of visible pulses. For each parameter, the values of the correlation coefficient and Sig. (1-tailed) are provided. Significant correlations are marked in bold.

Table 3. Rotated component matrix resulted from the PCA performed on the acoustic parameters of release calls.

Parameters	Components		
	1	2	3
CD (s)	0.867	0.217	0.191
P	-0.789	0.069	0.003
RT (s)	0.785	0.080	0.033
MinF (Hz)	-0.644	-0.448	0.214
RF (Hz)	0.128	0.976	0.131
MaxF (Hz)	0.077	0.971	0.154
DF (Hz)	-0.138	0.008	0.891
FM (Hz)	0.224	0.228	0.679

Notes: Rotation method: Varimax with Kaiser Normalization. Rotation converged in 5 iterations. Rf = range frequency, MinF = minimum frequency, MaxF = maximum frequency, DF = dominant frequency, FM = frequency modulation, RT = rise time proportion, CD = call duration, P = number of visible pulses.

The GLM using the three PCs as dependent variables showed a significant effect of the main factor ‘species’ ($F_{3,25} = 4.920$, $p = 0.008$, Wilks’ $\lambda = 0.629$, partial $\eta^2 = 0.371$), but not ‘gender’ ($F_{6,50} = 2.162$, $p = 0.062$, Wilks’ $\lambda = 0.630$, partial $\eta^2 = 0.206$) or ‘species x gender’ interaction ($F_{6,50} = 0.383$, $p = 0.886$, Wilks’ $\lambda = 0.914$, $\eta^2 = 0.044$), after controlling for the effect of SVL. The follow-up *post hoc* tests of Between-Subjects Effects showed that there were significant differences in PC2 between the two species ($F_{1,33} = 8.125$, $p = 0.008$, $\eta^2 = 0.231$).

Distress calls

We analysed a total of 17 distress calls from 5 *P. cultripipes* individuals (sex unknown), 114 calls from 15 *P. fuscus* (10 males, 3 females, 1 subadult, 1 sex unknown), and 181 calls from 16 *P. syriacus* (7 males, 6 females, 3 subadults). The structure of the distress calls was similar in all three species: loud, explosive, cat meow-like, single multi-pulsed notes, rich in harmonics (Figures 4–6). The distress calls were emitted with the mouth open, with the individual adopting a stiff posture, while inflated with air, spreading wide or standing on all four members; the emission of sounds was accompanied by consecutive jumps in random directions. In some cases, short and loud click-like multi-pulsed calls were also emitted (Figure 5c). The descriptive statistics of the distress calls is presented in Table 4. All acoustic parameters for the three species presented dynamic variations (CV > 12%), except maximum frequency in *P. syriacus*, which was intermediary (Table 4).

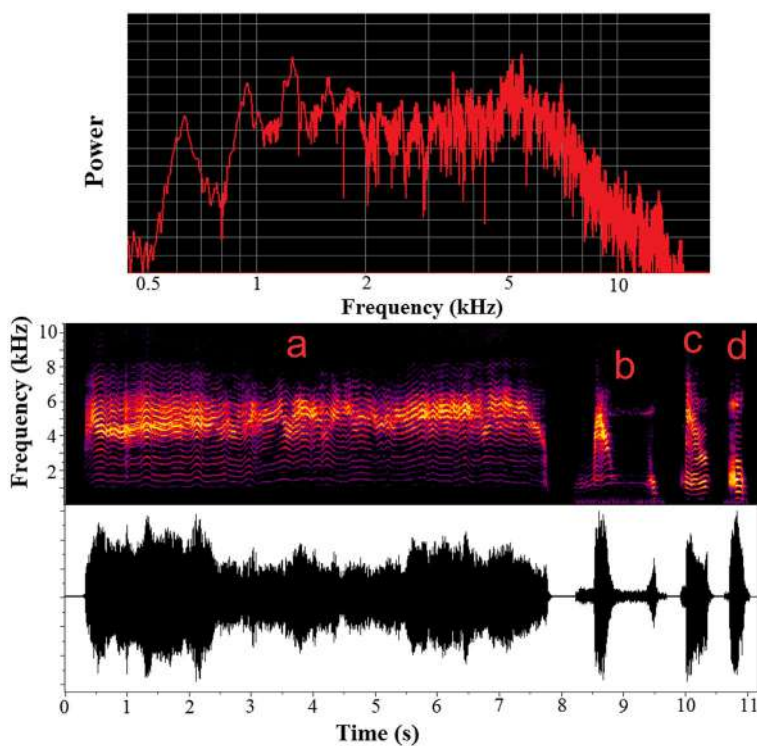


Figure 4. Power spectrum from a slice on longest distress call, audiospectrogram and oscillogram of four variations of distress calls in *P. syriacus* (a – long duration, b – two notes call, c – short duration 1, and d – short duration 2).

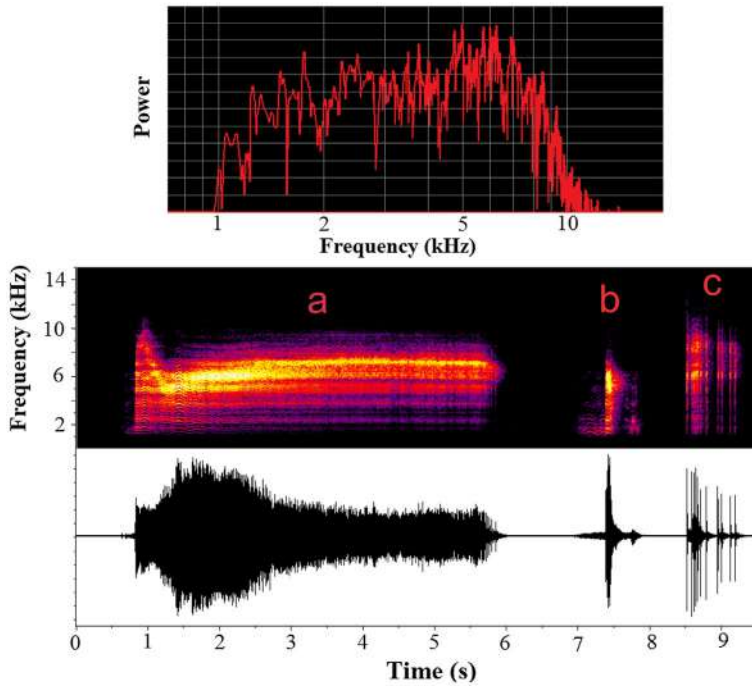


Figure 5. Power spectrum from a slice on longest distress call, audiospectrogram and oscillogram of three variations of distress calls in *P. fuscus* (a – long duration, b – short duration, and c – click-like multi-pulsed calls).

The correlation matrix in the PCA showed that all tested variables were interrelated (Table 5). The PCA revealed two PCs with eigenvalues > 1.0 , explaining 73.77% of the total variance: PC1, eigenvalue = 4.073, 50.91% of variance explained; PC2, eigenvalue = 1.829, 22.86% of variance explained). PC1 was highly loaded with spectral parameters, while PC2 was loaded mainly with temporal parameters (Table 6).

The GLM using the two PCs as dependent variables, showed no significant effects of the main factors 'species' ($F_{2,18} = 2.902$, $p = 0.081$, Wilks' $\lambda = 0.756$, $\eta^2 = 0.244$) and 'gender' ($F_{4,36} = 0.532$, $p = 0.713$, Wilks' $\lambda = 0.891$, partial $\eta^2 = 0.056$), or 'species x gender' interaction ($F_{4,36} = 0.661$, $p = 0.623$, Wilks' $\lambda = 0.868$, partial $\eta^2 = 0.068$), after controlling for the effect of SVL, in *P. fuscus* and *P. syriacus*.

The GLM performed for all three species, using the two PCs as dependent variables, showed significant differences between species ($F_{4,56} = 5.237$, $p = 0.001$, Wilks' $\lambda = 0.530$, partial $\eta^2 = 0.272$). The follow-up *post hoc* tests of Between-Subjects Effects showed that there were significant differences in both PC1 ($F_{2,31} = 6.344$, $p = 0.005$, partial $\eta^2 = 0.304$) and PC2 ($F_{2,31} = 3.451$, $p = 0.045$, partial $\eta^2 = 0.192$), between the three species. A Bonferroni *post hoc* pairwise comparison showed that there were significant differences in PC1 between *fuscus-cultripes* (mean difference = 1.316, $p = 0.023$) and *fuscus-syriacus* (mean difference = 1.032, $p = 0.013$), and in PC2 between *fuscus-syriacus* (mean difference = -0.923 , $p = 0.047$). However, it must be noted that this model did not account for body size or gender, since this information was not available for *P. cultripes*.

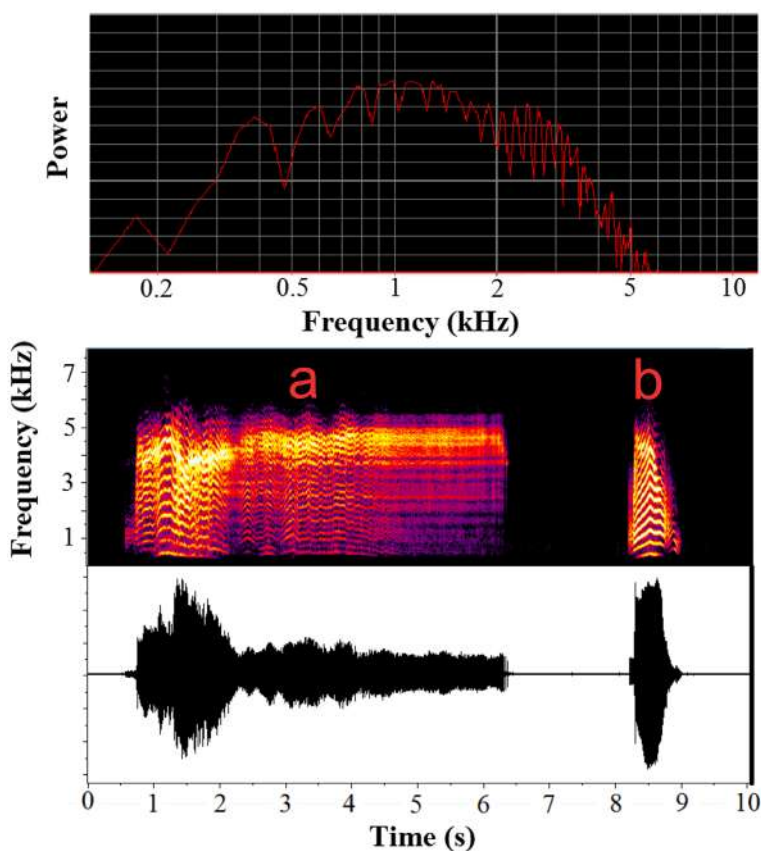


Figure 6. Power spectrum from a slice on longest distress call, audiospectrogram and oscillogram of the two distress calls in *P. cultripipes*.

Discussion

This study provides a first thorough quantitative description of acoustic parameters in release and distress calls of the three species of European spadefoot toads. Although the general structure of release calls is similar, we found significant differences in the spectral acoustic parameters of these calls between *P. fuscus* and *P. syriacus*. Such differences among related species is expected because the release call is a close-range signal with reproductive meaning (Toledo et al. 2015), which alerts unavailability to conspecific and heterospecific individuals (Wells 2007). For this reason, in some occasions the release call is used as a diagnostic feature for species identification (Brown and Littlejohn 1972; Grenat and Martino 2013) and different genetic populations (Castellano et al. 2002). However, release calls of anurans are acoustically simple, of low intensity and generally composed by a single, multi-pulsed note (Brown and Littlejohn 1972; Grenat and Martino 2013; Dias et al. 2014; Nali et al. 2015; Mângia et al. 2017).

Release and advertisement calls have a similar structure in *P. fuscus* and *P. syriacus*. However, affected by different social contexts, it is possible that these two different signals followed distinct evolutionary paths: advertisement calls tend to divergent evolution, while release calls face convergent character displacement (Leary 2001). Schneider (1966) and

Table 4. Summary of the quantitative description of the distress calls in *P. cultripes* ($n = 5$ individuals, 17 calls analysed), *P. syriacus* ($n = 16$ individuals, 181 calls analysed) and *P. fuscus* ($n = 15$ individuals, 114 calls analysed).

Species		RF (Hz)	MinF (Hz)	MaxF (Hz)	DF (Hz)	FF (Hz)	FM (Hz)	RT (s)	CD (s)
<i>P. fuscus</i>	Mean	4018.6	3766.5	7785.1	5971.9	1232.3	770.8	0.308	1.816
	±SD	985.0	1168.8	1426.1	1485.7	307.6	518.8	0.282	1.908
	CVbetween%	24.5	31.0	18.3	24.9	25.0	101.7	91.5	105.1
	CVwithin%	26.5	24.5	12.6	13.4	13.7	94.7	72.1	59.8
<i>P. syriacus</i>	Mean	3589.1	2273.9	5863.0	4101.3	762.6	992.5	0.454	1.513
	±SD	560.8	932.8	689.2	1224.4	296.4	391.2	0.275	0.909
	CVbetween%	15.6	41.0	11.8*	29.9	38.9	90.5	60.6	60.1
	CVwithin%	28.8	36.0	11.8*	27.2	23.2	85.6	67.8	42.7
<i>P. cultripes</i>	Mean	2838.7	2061.5	4900.1	3973.2	864.0	340.0	0.351	1.566
	±SD	1331.6	1552.9	2802.0	2439.7	677.3	263.0	0.095	0.627
	CVbetween%	46.9	75.3	57.2	61.4	78.4	71.9	27.2	40.0
	CVwithin%	23.4	33.5	14.8	25.1	23.7	60.8	60.5	59.8

Notes: Mean ± SD and CV% – between-individuals and within-individual coefficients of variation, are presented for each studied parameter: Rf = range frequency, MinF = minimum frequency, MaxF = maximum frequency, DF = dominant frequency, FF = fundamental frequency, FM = frequency modulation, RT = rise time, CD = call duration. Intermediary parameters (CV among 5 and 12%) are marked with *, and Static parameters (CV < 5%) with **.

Table 5. PCA correlation matrix of the acoustic parameters of distress calls.

	RF (Hz)	DF (Hz)	FF (Hz)	FM (Hz)	RT (s)	CD (s)	MinF (Hz)	MaxF (Hz)
RF (Hz)								
DF (Hz)	0.592							
	0.000							
FF (Hz)	0.587	0.789						
	0.000	0.000						
FM (Hz)	0.377	0.189	0.017					
	0.017	0.151	0.464					
RT (s)	0.026	0.006	-0.191	0.367				
	0.445	0.486	0.148	0.019				
CD (s)	-0.014	0.205	0.122	0.270	0.569			
	0.470	0.130	0.253	0.067	0.000			
MinF (Hz)	0.358	0.916	0.754	0.043	-0.129	0.161		
	0.022	0.000	0.000	0.408	0.242	0.190		
MaxF (Hz)	0.763	0.939	0.824	0.224	-0.076	0.104	0.877	
	0.000	0.000	0.000	0.109	0.340	0.285	0.000	

Notes: Rf = range frequency, MinF = minimum frequency, MaxF = maximum frequency, DF = dominant frequency, FF = fundamental frequency, FM = frequency modulation, RT = rise time, CD = call duration. For each parameter, the values of the correlation coefficient and Sig. (1-tailed) are provided. Significant correlations are marked in bold.

Müller (1984) described the advertisement call of *P. f. fuscus* as a group of usually three, rarely two or four consecutive notes, each note consisting of two or three groups of pulses. Similar to the release calls, the first pulse group of each note has the lowest amplitude within the note. The mean duration of these notes varied between 0.0538 and 0.0558 s (Schneider 1966), which is similar to the mean duration of 0.061 s in the notes composing the release calls. Müller (1984) showed that the temporal parameters (i.e. call duration, note duration, interval between call duration) in advertisement and release calls are correlated with size in *P. fuscus*, which is consistent with our own results. We did not find significant gender-related differences in the release calls of the two species, but given the significant sexual size dimorphism in *P. f. fuscus* we suspect that release calls may convey information regarding gender in this species, similar to the results reported by Andreone and Piazza

Table 6. Rotated component matrix resulted from the PCA performed on the acoustic parameters of distress calls.

Parameters	Components	
	1	2
MaxF	0.986	0.070
DF	0.949	0.130
FF	0.894	−0.076
MinF	0.892	−0.025
Rf	0.714	0.169
RT	−0.152	0.867
CD	0.094	0.774
FM	0.166	0.675

Notes: Rotation method: Varimax with Kaiser Normalization. Rotation converged in 3 iterations. Rf = range frequency, MinF = minimum frequency, MaxF = maximum frequency, DF = dominant frequency, FF = fundamental frequency, FM = frequency modulation, RT = rise time, CD = call duration.

(1990) in *P. f. insubricus*. The note structure of the release calls in *P. f. fuscus* is also similar with the note structure of the advertisement and release calls in *P. f. insubricus* (Andreone and Piazza 1990; Seglie et al. 2013).

A complete temporal and spectral analysis of the advertisement call of *P. syriacus* is not yet available, but brief descriptions were published by several authors (e.g. Nöllert 2003; Sofianidou 2012; Yegiasaryan 2014). As in *P. fuscus*, the structure of the notes composing the release call is similar to the advertisement call, the first pulse group having the lowest amplitude in the note. Sofianidou (2012) found that the mean note duration of release calls was 0.100 s, which is similar to our results of 0.108 s, while the advertisement call had a longer duration, of 0.132 s.

The distress calls of all three spadefoot toads had a similar structure, and were characterized by a high variability in all acoustic parameters analysed. Such results were expected, since the basic acoustic structure with loud and single multi-pulsed harmonic notes is similar for many anurans species (Toledo and Haddad 2009). We found that differences in acoustic parameters of distress calls among *P. fuscus* and *P. syriacus* are related to variations in body size. When body size was not accounted for, the differences between the acoustic parameters of distress calls were significant between the three species of *Pelobates* compared. However, we conclude that such differences should be related to variation in body size among species (Toledo and Haddad 2009; Santana et al. 2013), which apparently affected acoustic parameters in other call types as well.

The high variability in acoustic parameters of distress calls is likely related to the ecological role of such behaviour, once distress calls are interspecific signals applied as a defensive mechanism against common predators (Toledo et al. 2007, 2015). Release and distress calls probably do not face divergent evolution as it occurs in advertisements call, which usually present static variation in spectral parameters (Márquez and Eekhout 2006; Forti et al. 2010, 2015, 2016; Forti, Lingnau et al. 2017). A similar variability pattern in distress calls was found for Leptodactylidae species (Forti, Sousa et al. 2017). In this context, and considering the high variability of the acoustic parameters, distress calls are yet a questionable source for species recognition.

Acknowledgements

We are grateful to Paul and Diana Székely for their logistic support and to an anonymous reviewer for improving our previous version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work received support from the SYNTHESYS Project <http://www.synthesys.info/> which is financed by European Community Research Infrastructure Action under the FP7 ‘Capacities’ Programme, at the Museo Nacional de Ciencias Naturales (CSIC) [grant numbers SYNTHESYS ES-TAF-2814, SYNTHESYS ES-TAF-6704]; the Romanian National Authority for Scientific Research, CNCS – UEFISCDI [grant number PN-II-ID-PCE-2011-3-0173] and CCCDI-UEFISCDI – project No. 105 BM/2017; Sao Paulo Research Foundation (FAPESP) by a doctoral grant [grant number #2009/13987-2] and a fellowship [grant number #2013/21519-4]; The National Council for Scientific and Technological Development (CNPq) provided a fellowship [grant number #150041/2017-9]. Partial funding for acoustical analyses was provided by Ministerio de Ciencia e Innovación, Spain, projects TATANKA CGL2011-25062, CGL2010-09700; Ministerio de Economía y Competitividad, Spain project CGL2011-16159-E.

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Contrasting life-histories in two syntopic amphibians of the *Leptodactylus fuscus* group (Heyer 1978)

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Manuscript received on May 23, 2018; accepted for publication on August 25, 2018

How to cite: MARANGONI F, COURTIS A, PIÑEIRO JM, INGARAMO MDR, CAJADE R AND STĂNESCU F. 2019. Contrasting life-histories in two syntopic amphibians of the *Leptodactylus fuscus* group (Heyer 1978). *An Acad Bras Cienc* 91: e20180507. DOI: 10.1590/0001-3675201920180507.

Abstract: We used skeletochronology to compare age, size, reproductive parameters and growth patterns of two related, anuran amphibians from Northern Argentina: *Leptodactylus bufonius* (n=69) and *L. latinasus* (n=56), in order to better understand their coexistence in syntopy. Previous studies showed that the two species overlap in their dietary requirements and utilize the same habitats for feeding and breeding. We found that their life-history patterns are significantly different, *L. bufonius* being larger, and having a higher reproductive output and lifespan, compared to the smaller and shorter-living *L. latinasus*. Since none of the species exhibited sexual size dimorphism, and both acquired sexual maturity after the first year of life, we suggest that the differences in the observed life-history parameters must appear during early stages of development, during larval and/or juvenile stages.

Key words: age, Anura, body size, growth, *Leptodactylus bufonius*, *Leptodactylus latinasus*.

INTRODUCTION

Body size, growth and lifespan are central life history traits related to fitness, and ultimately to reproductive success and survival (Calder 1984, Schmidt-Nielsen 1984, Brown et al. 1993, Stearns 2000, Metcalfe and Monaghan 2003), which makes the study of intra- and interspecific variation in these traits an essential goal for understanding life-

history evolution. In amphibians, several factors may contribute to the variation in adult body size, either within species or among them. Traditionally, it has been considered that variation in body size can simply reflect an underlying pattern of variation in the age structure of populations (Díaz-Paniagua and Mateo 1999, Miaud et al. 1999, Laugen et al. 2005). Variations in juvenile growth rates to sexual maturity and age at maturity may promote divergences in adult body size between species and populations or sexual size dimorphism within a population (Hemelaar 1985, Monnet and

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Cherry 2002). Thus, an important factor in the analysis of variation of body size in amphibians is the indeterminate growth pattern that they exhibit, which becomes asymptotic when sexual maturity is reached (Halliday and Verrel 1988). Fast growth and early sexual maturity is one of the life strategies that determines increased efficacy at the expense of reaching a smaller adult size (Roff 1993, Stearns 1992). In contrast, the benefits of delaying reproduction are generally related to the benefits associated with large body size, which is positively related to fecundity and breeding success (Howard 1980, Berven 1981), jumping performance (Tejedo et al. 2000) and competition (Tejedo 1988). Large body size is also associated with higher survivorship and clutch size (Berven 1982a, Gibbons and McCarthy 1986, Begon et al. 1990, Stearns 1992). In addition, the environmental conditions experienced by the mother (non-genetic factors), are an important determinant of offspring adult body size, due to the maternally induced variation in egg size (Kaplan 1998). For example, a smaller egg size may determine a smaller size at metamorphosis (Bernardo 1996) and, when compensatory growth does not take place (Metcalf and Monaghan 2003, Hector and Nakagawa 2012), it can determine small adult body size (Bernardo 1996, Räsänen et al. 2003, 2005, Laugen et al. 2005, Marangoni 2006).

Skeletochronology is a useful technique to estimate individual age in amphibians, and discriminate variations in growth rates and age-related parameters such as age and size at sexual maturity, longevity, and potential reproductive lifespan (Sinsch 2015). These life-history parameters also allow explaining the actual pattern of sexual size dimorphism in amphibians (Marangoni et al. 2012, Cajade et al. 2013, Quiroga et al. 2015). Skeletochronology is based on the presence of cyclic and annular bone growth, which can be visualized in cross-sections of bones (Castanet 1982, Castanet and Smirina 1990). This

method is commonly and successfully used for evaluating the age of many species of amphibians and reptiles, providing an age estimate through non-lethal means (Castanet and Smirina 1990, Marangoni et al. 2009, 2012, Sinsch 2015).

The comparative study of life-history traits in related amphibian species which undergo similar environmental conditions is a good way to understand interspecific interactions and explain how differences in life-history strategies allow the coexistence of these species (MacArthur and Levin 1967, MacArthur 1970, Pianka 1975, Toft 1980, 1981). We used skeletochronology to compare the life-history patterns of two closely-related species of the *Leptodactylus fuscus* group (Heyer 1978), which occupy the same habitats in the wet Chaco region of northern Argentina. Specifically, we estimated and compared body size, age, growth and reproductive parameters for the two species, aiming to explain the life-history strategies that allow their coexistence.

MATERIALS AND METHODS

STUDY SPECIES

The monophyletic genus *Leptodactylus* (Fitzinger 1826) has a predominantly Neotropical distribution and is composed of 75 currently recognized species included in four groups: *Leptodactylus fuscus* (30 species), *L. melanonotus* (17 species), *L. latrans* (8 species), *L. pentadactylus* (17 species) and three species unassigned to any species group (De Sá et al. 2014). The study species, *Leptodactylus latinasus* (Jiménez de la Espada 1875) and *L. bufonius* (Boulenger 1894), belong to the monophyletic *L. fuscus* clade (De Sá et al. 2014). *Leptodactylus latinasus* is distributed in Argentina (Vaira et al. 2012), Bolivia, Paraguay, south and east throughout Uruguay and southern Brazil (Lavilla et al. 2004). *Leptodactylus bufonius* is distributed in Argentina (Cabrera and Willink 1980, Carnevali 1994, Vaira et al. 2012), southern Bolivia, Paraguay, and central

Brazil (Heyer 1978). The two species are common, and sharing habitats scattered throughout the Chaco and Espinal ecoregions (Cabrera and Willink 1980, Carnevali 1994, Vaira et al. 2012). Males of both species construct mud nests at the edge of ponds and other low-lying depressions, and call near semi-permanent or ephemeral water bodies, from inside or close to the chambers (Heyer 1978, Cei 1980, Faggioni et al. 2017). The two species share the same reproductive mode (Duellman and Trueb 1986) and their trophic niche overlaps to a great extent (Duré and Kehr 2004).

STUDY SITE

Fieldwork was carried out in the area called “El Perichón” (27°25'53.1" S, 58°44'44.8" W), 10 km northeast from Corrientes city, Argentina, where *L. latinasus* and *L. bufonius* live in syntopy. This area is characterized by the presence of numerous temporary and semi-permanent ponds. Mean annual temperature is 21.5°C and the mean annual precipitation is 1500 mm, without a pronounced dry season, although periods of rain shortages occur every 4-6 years (Carnevali 1994). The original vegetation was *Schinopsis balansae* “quebracho” forest, which is currently extremely degraded and largely replaced by sclerophyllous forest, with prevalence of *Acacia caven*, *Celtis* spp., *Prosopis affinis*, *Prosopis nigra*, and numerous colonies of *Aechmea distichantha* and *Bromelia* spp. (Carnevali 1994). Eight out of thirteen species of *Leptodactylus* genus reported for Argentina are present in the study area: five belong to *L. fuscus* group (*L. bufonius*, *L. elenae*, *L. gracilis*, *L. latinasus*, and *L. mystacinus*), two to the *L. latrans* group (*L. latrans* and *L. chaquensis*) and one to the *L. melanonotus* group (*L. podicipinus*).

SAMPLING

We sampled 56 *L. latinasus* (34 males, 17 females and five juveniles), and 69 *L. bufonius* (56 males, 9

females and four juveniles), from autumn 2007 to late spring 2008. The frogs were captured between 20:00 and 23:00 h. The sampling followed the ethical standards imposed by the Dirección de Recursos Naturales of the Corrientes province, Argentina. Most males (26) were captured by hand when they were calling on the ground away from ponds, hidden in crevices (*L. latinasus*) or near the cone-shaped nests (*L. bufonius*). Remaining males and females were collected during migration or at the edge of the breeding ponds. In these cases, sex and sexual maturity was determined by the presence of dark vocal sac (males), or ova that could be visualized through the skin (females). Frogs were separated by sex, placed in independent plastic containers (12 cm diameter x 6.5 cm height), and brought to the laboratory.

We measured snout-vent length (SVL) and right hind-limb length (HL) by placing each frog on laminated graph paper (accuracy ± 1 mm). We measured the head width (HW), arm length (AL) and tibia length (TL) to the nearest 0.1 mm with digital calipers. We measured body mass (BM) to the nearest 0.01 g, using an Acculab electronic balance (Acculab Scales, Titusville, NJ). In addition, we measured 67 specimens of related *Leptodactylus* species from the Collection of Laboratorio de Genética Evolutiva (Instituto de Biología Subtropical (CONICET-UNaM), Posadas, Misiones, Argentina), which we considered useful for further comparisons: 13 *Leptodactylus furnarius*, 18 *L. laticeps* and 36 *L. plaumanni* (Appendix A, B). All measurements were taken according to Duellman (1970).

Most individuals (109 out of 125; 87%) were released back into their original ponds within 24-48 h after their capture. Ten *L. latinasus* and six *L. bufonius* females were preserved for the analysis of reproductive traits, and further genetics and morphological studies, and deposited in the Collection of Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-

UNaM), Posadas, Misiones, Argentina (see Appendix A for specimen codes).

SEXUAL SIZE DIMORPHISM

We checked for significant differences in size parameters (i.e. SVL, BM, HW, AM and TL) between sexes, using multi- and univariate analyses of variance (with type III Sum of Squares). We used Pearson correlation coefficient adjusted for small sample sizes (radj) to analyze the associations between these parameters.

We assessed the sexual size dimorphism (SSD) for each body measurement using the sexual dimorphism index (SDI), following Lovich and Gibbons (1992): $SDI = \text{mean size}_{\text{larger sex}} / \text{mean size}_{\text{smaller sex}}$, with the result arbitrarily defined as positive when females are larger than males, and negative when males are larger.

SKELETOCHRONOLOGY

We clipped the third toe of the right leg of 28 *L. latinasus* (12 males, 12 females, four juveniles) and 35 *L. bufonius* (24 males, 7 females, four juveniles) (Table II), and stored them in 70% ethanol at room temperature for age estimation through skeletochronology. We followed the standard protocols used in skeletochronology (e.g. Smirina 1972). We selected the third phalanx of the toe, which was washed in water for 30 min, and decalcified in 5% nitric acid for 30-45 min. Afterwards, the samples were washed in running tap water for 5 min and kept overnight in distilled water. Then, the phalanges were frozen (Tissue-Tek O.C.T. Compound, Sakura Finetek) and cross-sectioned at 16 µm using a cryo-microtome. Sections were stained for 3-6 h at room temperature with Ehrlich's hematoxylin (Tejedo et al. 1997). 15 to 20 of these sections were permanently mounted in aqueous synthetic resin (Aquatex®, Merck KGaA, Germany) on glass microscope slides. Cross sections were

examined light microscopically at magnifications of 20x using a Nikon Optiphot microscope. A Canon PowerShot A570 was used to take digital images from those diaphysis sections in which the size of the medullar cavity was at its minimum and that of periosteal bone at its maximum. Cross sections were viewed and measured using the computer package Image-Pro Plus Version 1.1 (Media Cybernetics 1993-1994). In a first step of the analysis, we recorded the presence/absence of the line of metamorphosis (LM) and of lines of arrested growth (LAGs). The number of LAGs visible in each cross section was assessed by FM and independently by AC. In those frogs with no remnant of the line of metamorphosis we estimated the degree of resorption by osteometrical analysis, following the method of Sagor et al. (1998). In a second step, we distinguished annual growth marks (LAGs *sensu stricto*) from non-annual ones (irregular interruptions of the aestivation periods), following Sinsch et al. (2007). The age of maturity was defined as the lowest age recorded in a reproductive frog of a given population.

AGE-RELATED PARAMETERS

We computed the following age-related parameters (*sensu* Leskovar et al. 2006): (1) age at maturity: the minimum number of LAGs counted in breeding individuals; (2) longevity: the maximum number of LAGs counted in breeding individuals; (3) potential reproductive lifespan: the difference between longevity and age at maturity; (4) mean lifespan; (5) size at maturity: the average snout-vent length of all individuals with the minimum number of LAGs. We used a two-sample Kolmogorov-Smirnov and Mann-Whitney test to check for differences in the shape of age distribution and median age between males and females. We used linear regressions to analyze the associations between age and body size parameters.

GROWTH PATTERNS

We used the packages FSA (Ogle 2018) and nlstools (Baty et al. 2015) in R Studio version 1.1.423 (© 2009-2018 RStudio, Inc.) to compute von Bertalanffy's growth model (Bertalanffy 1938) following Beverton and Holt (1957): $SVLt = SVL_{max} \times (1 - e^{-k \times (t - t_0)})$, where $SVLt$ 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. We fitted von Bertalanffy growth model and estimated growth parameters (VBGP) by nonlinear least squares regression. Two estimated VBGP were considered significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap. We used the value of 10.9 mm as the mean size at metamorphosis (0 LAGs) found in *L. bufonius* by Vera and Ponssa (2014), to adjust the growth model, since no freshly metamorphosed individuals could be captured from the studied area. Based on the known life-history patterns of the species (i.e. breeding period and larval development) and the moment of sampling, we assigned the age of 0.25 LAGs to *L. bufonius* and *L. latinasus* juveniles, assuming that only 3 months elapsed since their metamorphosis.

REPRODUCTIVE TRAITS

Reproductive traits were measured in 16 females: ten *L. latinasus* and six *L. bufonius*. We determined the ovarian mass (OM) as the difference between the body mass before and after ovary removal. The ovarian complement (OC) represents the total number of mature ova from each gravid female and is considered a measure of their fertility or reproductive potential (Crump 1974, Basso 1990). We removed and weighed approximately 10% of each ovary and counted the mature ova under a Nikon C-DS magnifying glass. Mature ova had well-defined black and yellow poles and pronounced

larger size, consistent with the post-vitellogenesis class (Crump 1974). We photographed a random sample of about 200 ova from each ovary with a digital Nikon Coolpix S10 camera, mounted on a Nikon C-DS magnifying glass. We measured the longest and shortest perpendicular axes of 100 ova per sample to the nearest 0.01 mm using Image-Pro Plus 1.1 (Media Cybernetics 1993-94). We determined mature ovum size (OS) by square rooting the product of the two axis measurements. We estimated the ovarian size factor (OSF) which correlates the number and size of mature ova to body length, following Duellman and Crump (1974): $OSF = (OC \times OS)/SVL$. Finally, we estimated the reproductive effort (RE) following Prado et al. (2000): $RE = (OM/BM) \times 100$, where the body mass is the final weight of the female after oviposition. We used Pearson product-moment correlation coefficient adjusted for small sample sizes (r_{adj}) to analyze the associations between size and reproductive parameters.

STATISTICAL ANALYSIS

Body size variables were log-transformed in order to achieve normality. We tested all data for normality and homoscedasticity using Shapiro-Wilk and Levene tests and chose the statistic tests accordingly. We used multi- and univariate analyses of variance to test for differences in body size between the sexes. We used linear regressions to test the association between body size, age and clutch characteristics. All statistical analyses were performed using STATISTICA 6.0 package (StatSoft Inc. 2001).

RESULTS

BODY SIZE AND SEXUAL SIZE DIMORPHISM

Both species (Table I) showed no significant effect on all measured morphometric variables (*L. latinasus*: Wilk's $\lambda=0.771$, $F_{6,39}=1.920$, $P=0.101$; *L. bufonius*: Wilk's $\lambda=0.846$, $F_{6,58}=1.750$, $P=0.125$).

TABLE I
Mean $\pm$ SD values of body mass (BM), snout-vent length (SVL), right hind leg length (HLR), head width (HW), tibia length (TL) and arm length (AL) of male and female *Leptodactylus latinasus* and *L. bufonius* from northeastern Argentina. SDI = sexual dimorphism index. Sample size is provided in parentheses.

Sex/trait	<i>L. latinasus</i>				<i>L. bufonius</i>			
	Male	Female	SDI	Juvenile	Male	Female	SDI	juvenile
BM	2.46 $\pm$ 0.57 (34)	2.65 $\pm$ 0.65 (17)	1.08	0.8 $\pm$ 0.08 (5)	11.21 $\pm$ 1.75 (56)	12.83 $\pm$ 1.28 (9)	1.14	1.63 $\pm$ 0.48 (4)
SVL	32.38 $\pm$ 2.94 (34)	33.02 $\pm$ 3.15 (17)	1.02	22.04 $\pm$ 2.83 (5)	55.3 $\pm$ 1.8 (56)	56.33 $\pm$ 2 (9)	1.02	29.25 $\pm$ 2.5 (4)
HLR	45.1 $\pm$ 2.45 (30)	46.43 $\pm$ 2.78 (16)	1.03	32.5 $\pm$ 1.91 (4)	63.14 $\pm$ 2.43 (56)	64.66 $\pm$ 3.53 (9)	1.02	35.75 $\pm$ 3.4 (4)
HW	10.35 $\pm$ 0.43 (30)	10.33 $\pm$ 0.62 (16)	-1.00	7.63 $\pm$ 0.15 (4)	16.76 $\pm$ 1.34 (56)	17.22 $\pm$ 0.74 (9)	1.09	10.05 $\pm$ 0.94 (4)
TL	14.27 $\pm$ 0.73 (30)	14.69 $\pm$ 0.91 (16)	1.03	10.13 $\pm$ 0.52 (4)	20.99 $\pm$ 0.66 (56)	21.39 $\pm$ 0.25 (9)	1.02	11.13 $\pm$ 1.17 (4)
AL	6.39 $\pm$ 0.45 (30)	6.5 $\pm$ 0.5 (16)	1.02	4.3 $\pm$ 0.34 (4)	11.65 $\pm$ 0.51 (56)	11.58 $\pm$ 0.57 (9)	-1.01	6.33 $\pm$ 0.52 (4)

The values of sexual dimorphism index (SDI) were negative for head width (-1.00) in *L. latinasus* and arm length (-1.01) in *L. bufonius*, showing that the males were larger than females in these variables, whereas the females were larger than males in the remaining variables studied (Table I). We found a positive and significant correlation between body mass and SVL in both species. This correlation showed differences in the slope between the two species, with body mass increasing faster with SVL in *L. bufonius* compared to *L. latinasus* (Fig. 1).

AGE-RELATED PARAMETERS

All sections showed recognizable bone structures that allowed age determination. We found well-defined LAGs in the periosteal bone of these sections, and they were relatively easy to count in order to assess individual age (Fig. 2). The descriptive statistics of the studied life history-traits are summarized in Table II, and the age structure is presented in Fig. 3. The minimum number of LAGs found in reproductive individuals was one in both species. One-year old *L. latinasus* males were on average smaller than one-year old females, whereas in *L. bufonius* males were bigger than females within the one-year old age class. On average, males were older than females in both species; however, the differences in the median lifespan between sexes were significant only in *L. latinasus* (Mann-Whitney *U* test, $Z = 2.849$, $P = 0.004$). Longevity in *L. latinasus* was three years in males and two years in females, while females were older in *L. bufonius* (five years in females and four years in males). Thus, the difference in the potential reproductive lifespan (PRLS) between sexes was one year in both species. We found no LAGs in the cross sections of juveniles.

GROWTH PATTERNS

Since MANOVA on all morphometric variables measured showed no significant effects of sex

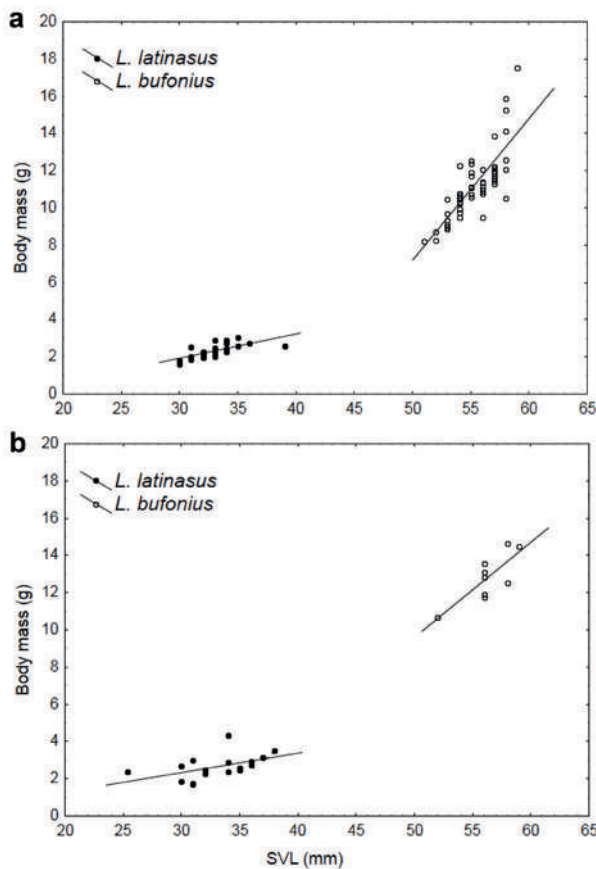


Figure 1 - Snout-vent length (SVL) and body mass (BM) relationships in *Leptodactylus latinasus* and *L. bufonius* males (a) and females (b).

in both species, we computed Von Bertalanffy's growth model using pooled data of males and females (Fig. 4). The asymptotic average snout-vent length was significantly higher in *L. bufonius* ($SVL_{max} \pm SE = 56.22 \pm 0.43$, CI 95% = 55.35 – 57.09, $K \pm SE = 3.62 \pm 0.44$, CI 95% = 2.71 – 4.52) than in *L. latinasus* ($SVL_{max} \pm SE = 32.68 \pm 0.816$, CI 95% = 31.00 – 34.36, $K \pm SE = 2.60 \pm 0.55$, CI 95% = 1.46 – 3.75). Although we found no significant differences in the growth coefficient, the growth rates during the first year of life, from metamorphosis to sexual maturity, appear to be distinct in the two species: *L. latinasus* had a mean SVL of only 32.6 mm ($n=12$ one-year old individuals: four males, eight females), compared to *L. bufonius* which attained a mean SVL of 56.8 mm

in the same age class ($n=8$ one-year old individuals: four males, four females). In addition, there were significant differences both in the size and age distribution of the two species, *L. bufonius* being larger (Mann-Whitney U-test, $U=50$, $P<0.001$) and having a higher average lifespan compared to *L. latinasus* (M-W, $U= 257$, $P= 0.006$).

REPRODUCTIVE TRAITS VS FEMALES' SIZE

The descriptive statistics of the reproductive variables in both species are presented in Table III. Following the differences in body size between species, all reproductive traits were higher in *L. bufonius* than *L. latinasus*. However, the relationships among reproductive variables (i.e. ovarian complement, ovarian mass and ovum size), female size (i.e. snout-vent length, body mass) and age in *L. latinasus* and *L. bufonius* were statistically non-significant (Table IV). In *L. latinasus*, the mean OC increased with SVL, BM and age. Similarly, OM increased with BM and age, but the relationship with SVL was negative. OS increased with age, but decreased with SVL and BM. On the other hand, in *L. bufonius*, OC increased with age, but showed a negative relationship with SVL and BM. Moreover, OM showed a positive relationship with SVL and age, but had a negative relationship with BM. Finally, OS increased significantly with SVL, BM and age.

DISCUSSION

INTRASPECIFIC DIFFERENCES IN BODY SIZE AND AGE-RELATED PARAMETERS

Several non-mutually exclusive factors may contribute to SSD in amphibians, such as environmental conditions, phylogeny, genetic drift, or natural and sexual selection (Berven 1982a, b, Marangoni and Tejedo 2008, Cogălniceanu et al. 2014). In most anurans, females are larger than males and in overall this is explained by the positive correlation between female body size and

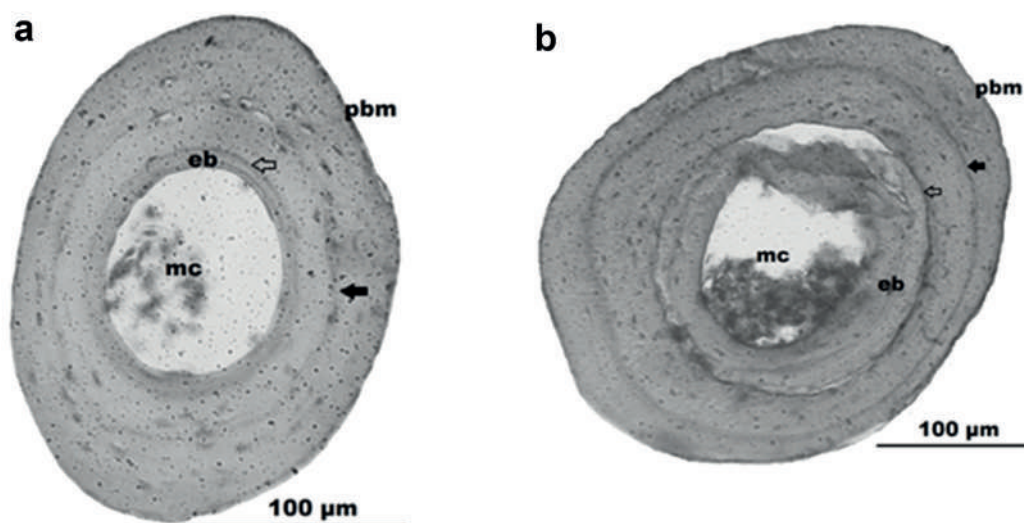


Figure 2 - Cross sections through a phalanx of *Leptodactylus latinasus* (a) and *L. bufonius* (b). An arrowhead indicates the lines of arrested growth (LAGs), medullary cavity (mc), endosteal bone (eb), periosteal bone margin (pbm).

reproductive output (Shine 1979, Duellman and Trueb 1986). However, in some cases males are larger than females or there is no SSD (Shine 1979, Silva et al. 2005, Zina and Haddad 2005). We did not find a significant SSD in the studied *L. latinasus* and *L. bufonius* populations, in any of the analyzed morphological variables. However, a female-biased SSD was reported in other populations of *L. bufonius* (Heyer 1978, Reading and Jofré 2003, Schaefer 2007, Faggioni et al. 2017, but see Duré and Kehr 2004) and *L. latinasus* (Heyer 1978, Duré and Kehr 2004, Schaefer 2007, Ponssa and Barrionuevo 2012, Attademo et al. 2014), and likewise, in the other 11 species of the genus distributed in Argentina (Appendix B). Regarding species of the *Leptodactylus fuscus* group, where males construct subterranean chambers, Heyer (1978) hypothesized a relationship between burrowing habits and sexual dimorphism, males having larger heads compared to females (Faggioni et al. 2017). We found no SSD in the head width of either species studied, but our results are similar to those obtained by Ponssa and Barrionuevo (2012).

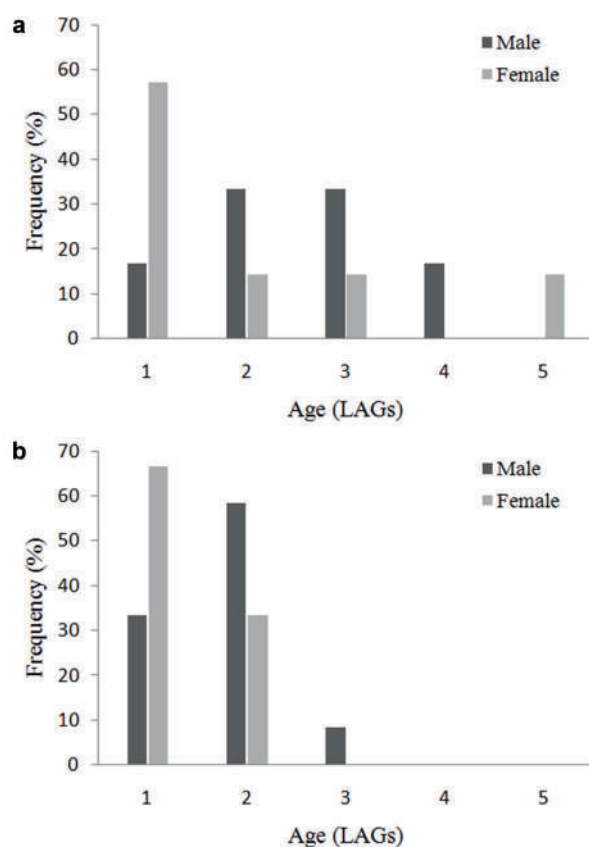


Figure 3 - Population age structure in males and females of *L. bufonius* (a) and *L. latinasus* (b).

TABLE II

Age-related traits of *Leptodactylus latinasus* and *L. bufonius*. AM = age at maturity (i.e. the minimum age in the sample, in LAGs); Longevity = maximum age in the sample (LAGs); PRLS = potential reproductive lifespan (years); Mean and maximum size at AM = mean and maximum snout-vent length of first-year breeders (mm). Sample size is provided in parentheses.

Species/Sex	Mean age ± SE	Modal age	Median age	AM	Longevity	PRLS	Mean size at AM ± SD	Maximum size at AM
<i>L. latinasus</i>								
Males (12)	1.75 ± 0.62	2(7)	2	1	3	2	31.27 ± 5.15 (4)	39 (1)
Females (12)	1.33 ± 0.49	1(8)	1	1	2	1	32.43 ± 3.51 (8)	38 (2)
<i>L. bufonius</i>								
Males (24)	2.5 ± 0.98	2-3(8)	2.5	1	4	3	57.25 ± 1.7 (4)	59 (1)
Females (7)	2.0 ± 1.52	1(4)	1	1	5	4	56.5 ± 1.0 (4)	59(5)

Several species of *Leptodactylus* exhibit male combat, a main source that have been widely proposed to explain the existence of sexual size dimorphism in anurans (Shine 1979, Blanckenhorn 2000, Monnet and Cherry 2002), but although we observed male-male interaction in *L. bufonius*, with the consequent emission of territorial calls (F. Marangoni, personal observation), we never observed male combat in either of the two species studied (F. Marangoni, personal observation). Thus, we suggest that the absence of male combat could be another possible explanation for the absence of sexual size dimorphism in these species.

Variation in age structure promoting considerable variation in adult body size has been widely demonstrated in amphibians (Díaz-Paniagua and Mateo 1999, Miaud et al. 1999, Laugen et al. 2005, Marangoni et al. 2006, 2012, Cajade et al. 2013, Quiroga et al. 2015, Sinsch et al. 2015). In addition, contrasting life-strategies related to growth rates, age and body size at sexual maturity of males versus females can also shape sexual size dimorphism in amphibians (Hemelaar 1988, Halliday and Tejedo 1995). We found that sexual maturity was reached after the first year of life in males and females of both species studied, which could also explain the absence of a significant sexual size dimorphism. Basso and Kehr (1991)

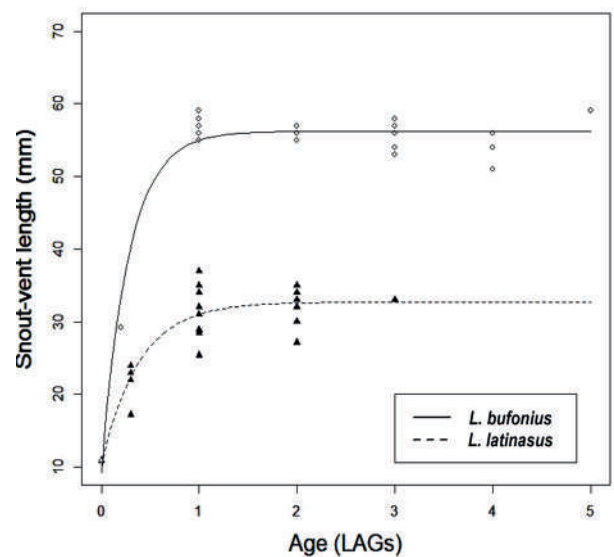


Figure 4 - Growth patterns in *Leptodactylus latinasus* (black triangles) and *L. bufonius* (open circles).

also found that *L. latinasus* attains sexual maturity after the first year of life. Similar age at maturity and longevity (one and five years, respectively) and no SSD was also found in a related species - *L. latrans*, by López et al. (2017) and the authors proposed that the lack of SSD is probably due to the lack of differences in the age structure of males and females, females having only a slightly delayed sexual maturity. In contrast, other studies found that males and females attained sexual maturity after the second year of life in *L. bufonius* (Reading and

TABLE III

Reproductive traits of *Leptodactylus latinasus* and *L. bufonius* females. SVL = snout-vent length; OM = ovarian mass; OC = ovarian complement; OS = ovum size; RE = reproductive effort; OSF = ovarian size factor. Values are presented as Mean $\pm$ SD. Sample size is provided in parentheses.

Species	SVL (mm)	OM (g)	OC	OS (mm)	RE (%)	OSF
<i>L. latinasus</i>	33.03 $\pm$ 3.16 (17)	0.20 $\pm$ 0.10 (10)	224.30 $\pm$ 51.43 (10)	1.19 $\pm$ 0.14 (10)	6.44 $\pm$ 2.97 (10)	7.95 $\pm$ 2.26(10)
<i>L. bufonius</i>	56.33 $\pm$ 2.00 (9)	1.73 $\pm$ 1.54 (6)	400.17 $\pm$ 117.89 (6)	1.29 $\pm$ 0.17 (6)	17.11 $\pm$ 16.54 (6)	9.33 $\pm$ 2.91(6)

TABLE IV

Relationship between reproductive variables (i.e. ovarian complement, ovarian mass, ovum size), body size (snout-vent length, body mass) and age in *Leptodactylus latinasus* and *L. bufonius* females. All variables were log-transformed. All relationships were statistically not significant.

Reproductive traits (y) vs. body size	<i>Leptodactylus latinasus</i>			<i>Leptodactylus bufonius</i>		
	n	Equation	r ²	n	Equation	r ²
Ovarian Comp.						
SVL	10	y = -0.8606 + 1.767 x	0.2703	6	y = 14.4744 - 2.1199 x	0.0635
BM	10	y = -0.313 + 0.2303 x	0.0968	6	y = 7.7499 - 0.5038 x	0.2628
Age	7	y = 5.3276 + 0.0573 x	0.0059	5	y = 5.9463 + 0.0833 x	0.0147
Ovarian mass						
SVL	10	y = 0.3483 - 0.0793 x	0.0323	6	y = -16.9517 + 4.3615 x	0.0932
BM	10	y = 0.7944 + 1.9593 x	0.1179	6	y = 5.0727 - 1.2619 x	0.5721
Age	7	y = 0.271 + 0.3624 x	0.0013	5	y = 0.5017 + 0.7775 x	0.5597
Ovum size						
SVL	10	y = 2.7067 - 0.7174 x	0.1791	6	y = -10.9025 + 2.7752 x	0.4827
BM	10	y = 0.2494 - 0.0855 x	0.0161	6	y = 0.0279 + 0.0625 x	0.0179
Age	7	y = 0.1725 + 0.0579 x	0.0253	5	y = 0.29 + 0.054 x	0.1160

Jofré 2003) and *L. latinasus* (Attademo et al. 2014). Attademo et al. (2014) found that age at maturity and longevity were 3 and 7 years respectively, in *L. mystacinus*.

INTERSPECIFIC DIFFERENCES IN LIFE-HISTORY TRAITS

The observed differences in adult body size paralleled the differences in age-related parameters (longevity and PRLS), all reproductive traits, and growth pattern in both species. Observed SVL of first-breeders suggest that distinct growth patterns occur before sexual maturity in the two species, *L. bufonius* achieving a larger body size compared to *L. latinasus*, during the same amount of time. This pattern is also evident from the SVL-BM

relationship, body mass increasing faster with SVL in *L. bufonius* compared to *L. latinasus* (Fig. 1). Overall, *L. bufonius* is larger than *L. latinasus*, and females have a higher reproductive investment. In addition, the potential reproductive lifespan (PRLS) is also higher in *L. bufonius*, which increases the potential reproductive success of the species (Halliday and Verrel 1988, Halliday and Tejedo 1995, Blanckenhorn 2000). Overall, our study indicates that *L. bufonius* exhibits a more successful life-history strategy and therefore has better chances to displace *L. latinasus* in competition for resources. However, Duré and Kehr (2004) showed that *L. latinasus* and *L. bufonius* exhibit niche complementarity, which means that under satisfactory levels of food and

space availability, competition should not be an issue, and thus explaining the coexistence of the two species in syntopy. Furthermore, competition is avoided through spatial segregation between *L. latinasus* and *L. bufonius*: for example, although males of both species construct mud nests at the edge of ponds and other low-lying depressions (Heyer 1978, Cei 1980), there are subtle differences in their microhabitat preferences and reproductive behavior (see Crump 1995), *L. latinasus* being usually associated to crevices in the ground, while *L. bufonius* constructs cone-shaped nests at the edge of the ponds (Shoemaker and McClanahan 1973, Crump 1995, F. Marangoni, personal observation during present study). Since both species use the same ponds for breeding, at the same time, interspecific interaction is most likely to occur during larval stages. However, little is known regarding the length of larval development, dietary requirements, foraging behavior of the tadpoles, or size at metamorphosis. Hence, studies regarding growth, diet and foraging behavior during early-stages of life in both species are required to fully understand the mechanisms that shape their life-histories and allow their coexistence.

ACKNOWLEDGMENTS

We are grateful to V. I. Gomez for invaluable help during fieldwork. We acknowledge L. Rossi and A. Ibañez for their assistance in the sample processing. We acknowledge M. Sanchez-Negrette and M. Montenegro for providing the corresponding permits to use the cryostat microtome at the Cátedra de Patología General y Sistemática and Cátedra de Histología y Embriología, Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste. We thank D. Baldo for access to the database of amphibian collections of the Laboratorio de Genética Evolutiva del Instituto de Biología Subtropical (CONICET-UNaM). We thank J. J. Neiff for providing the Microscopy

Laboratory at the Centro de Ecología Aplicada del Litoral (CECOAL, CONICET // UNNE). We thank also H. Duarte, a native speaker, for correcting the English draft of this manuscript. D. Cogălniceanu reviewed earlier versions of the manuscript and provided useful comments that helped improve it. The authors have complied with all applicable Institutional Animal Careguide lines. The collecting permit was granted by Dirección de Recursos Naturales of the Corrientes province, Argentina. This project was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, through fellowships to F. Marangoni. We are very grateful for the continuous support of the CONICET and the Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste (FACENA, UNNE).

AUTHOR CONTRIBUTIONS

FM, JMP, MRI, RC performed the fieldwork. FM and AC performed the laboratory analyses. AC performed the literature review. FM and FS performed the statistical analyses and wrote the manuscript. JMP, MRI and RC prepared the tables and figures; all authors critically revised the manuscript and approved the final version.

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APPENDIX A

Species, location and registration numbers of all individuals deposited and measured at the Collection of the Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-UNaM), Posadas, Misiones, Argentina (LGE):

L. bufonius: Charata, Chaco: 05226, 05235, 05236, 05247, 05248, 05249, 05250, 05251, 05252, 05253, 05254. Perichón, Corrientes: 20058, 20059, 20060, 20061, 20062, 20063, 20064, 20065, 20066, 20067, 20068, 20069, 20070, 20071, 12163, 12947, 12948, 12949, 12950, 12951, 13330, 13419, 13437, 13438, 13439, 13440. Fuerte Esperanza, Chaco: 13006, 05863, 05864, 05865, 05866, 05867, 05868, 05869, 05870, 05871, 05872, 05873, 05874, 05875, 05876, 05877, 05878, 05879, 05880, 05881, 05882, 05883, 05886, 05887, 05888, 05889, 05890, 05891, 05892, 05893, 05894, 05895, 05896, 05897, 05898,

05899, 13022, 13078, 13079, 13317, 13370, 13371, 13373, 13405, 50898, 5899.

L. latinasus: Perichón, Corrientes: 20072, 20073, 20074, 20075, 20076, 20077, 20078, 20079, 20080, 20081, 20082, 20083, 20084, 20085, 20086, 20087.

L. laticeps: Chaco, Formosa: 12083, 12084, 12100, 12101, 12150, 12164, 15282, 15283, 15284, 15285, 15286, 15287, 15289, 15290, 15291, 15292, 15293, 15294.

L. furnarius: Corrientes and Misiones: 03438, 03439, 03493, 03666, 03867, 04119, 04163, 04694, 07889, 12854, 12855, 12856, 12857.

L. plaumanni: Misiones: 02067, 03373, 03374, 03375, 03386, 03427, 03430, 03431, 03537, 03543, 03545, 03546, 03556, 03557, 03929, 03930, 04243, 04244, 04823, 05086, 05104, 07034, 07077, 09662, 09663, 09664, 09665, 09666, 09667, 09668, 09669, 09672, 09673, 09674, 09675, 19927.

APPENDIX B

Average SVL of males and females of the 13 species of the genus *Leptodactylus* and the SDI computed for each population. # = species belong to the *L. fuscus* group. LGE = Collection of the Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-UNaM), Posadas, Misiones, Argentina. Mean $\pm$ SD. n=sample size. Significant differences in SVL between males and females ($P < 0.05$) are marked with *; Δ = not analyzed.

Species	Country	Coordinates	Males SVL	Females SVL	SDI	Source
<i>L. bufonius</i> #	Argentina	29°48'S, 64°43'W	56.40 $\pm$ 2.58 (62)	60.00 $\pm$ 2.87 (53)	1.06*	Reading and Jofré 2003
<i>L. bufonius</i> #	Several	Several	51.60 $\pm$ 2.0	53.60 $\pm$ 2.3	1.04*	Heyer 1978
<i>L. bufonius</i> #	Argentina	27°17'34.8" S, 61°09'01.4" W	46.68 $\pm$ 1.02 (9)	45.69 $\pm$ 1.76 (3)	-1.02	LGE
<i>L. bufonius</i> #	Argentina	25°04'39.24" S, 61°37'52.33" W	53.26 $\pm$ 3.05 (36)	59.87 $\pm$ 3.64 (10)	1.12*	LGE
<i>L. bufonius</i> #	Argentina	27°30' S, 58°45' W	44.20 $\pm$ 4.2 (12)	43.80 $\pm$ 6.9 (8)	-1.01	Duré and Kehr 2004
<i>L. bufonius</i> #	Argentina	27°26' S, 58°44' W	46.10 $\pm$ 1.94 (11)	47.90 $\pm$ 2.32 (11)	1.04	Schaefer 2007
<i>L. bufonius</i> #	Argentina	27°25'53.2" S, 58°44'44.8" W	55.30 $\pm$ 1.8 (56)	56.33 $\pm$ 2 (9)	1.02	Present study
<i>L. bufonius</i> #	Brazil	21°42'39" S, 57°43'16" W	46.03 $\pm$ 2.91 (25)	47.66 $\pm$ 3.11 (31)	1.04*	Faggioni et al. 2017
<i>L. chaquensis</i>	Brazil	57°00' W, 19°34' S	71.34 $\pm$ 5.11 (34)	71.31 $\pm$ 4.51 (50)	-1.00	Prado et al. 2000
<i>L. chaquensis</i>	Argentina	27°30' S, 58°45' W	62.90 $\pm$ 5.43 (21)	65.30 $\pm$ 7.82 (14)	1.04	Schaefer et al. 2006
<i>L. chaquensis</i>	Argentina	27° 26' S, 58°44' W	63.00 $\pm$ 5.45 (27)	61.38 $\pm$ 7.67 (35)	-1.03	Schaefer 2007
<i>L. elenae</i> #	Several	Several	42.70 $\pm$ 2.5	42.80 $\pm$ 3.1	1.00*	Heyer 1978
<i>L. furnarius</i>	Brazil	18°55' S, 48°17' W	38.00 $\pm$ 1.13 (19)	42.40 $\pm$ 1.58 (52)	1.12*	Giaretta and Kokubum 2003
<i>L. furnarius</i>	Argentina	Several	36.62 $\pm$ 1.70 (11)	41.79 $\pm$ (2)	1.14 Δ	LGE

APPENDIX B (continuation)

Species	Country	Coordinates	Males SVL	Females SVL	SDI	Source
<i>L. fuscus</i>	Brazil	-	43.60 ± 2.4 (135)	45.60 ± 2.2 (13)	1.05*	Lucas et al. 2008
<i>L. fuscus</i>	Brazil	-	43.00 (39.9-46.8) (28)	43.70 (41.9-46.3) (28)	1.02	Maragno and Cechin 2009
<i>L. fuscus</i>	Brazil	2°48' N, 60°12' W	36.20 ± 1.3 (25)	39.50 ± 1.3 (25)	1.09*	Martins 1988
<i>L. fuscus</i>	Brazil	16°13'50" S, 48°04'49" W	46.90 ± 2.7 (13)	49.30 ± 2.6 (6)	1.05 [▲]	De-Carvalho et al. 2008
<i>L. gracilis</i> #	Several	Several	43.00 ± 4.8	43.00 ± 3.7	1.00	Heyer 1978
<i>L. labyrinthicus</i>	Brazil	18°55' S, 48°17' W	136.50 ± 17.2 (16)	127.30 ± 12.7 (12)	-1.07	Silva et al. 2005
<i>L. labyrinthicus</i>	Brazil	22°15' S, 47°49' W	170.00 ± 18.9 (5)	157.00 ± 10.4 (5)	-1.08	Zina and Haddad 2005
<i>L. labyrinthicus</i>	Brazil	22°16' S, 47°42' W	152.30 ± 10.6 (10)	155.00 ± 12.3 (8)	1.02	Zina and Haddad 2005
<i>L. laticeps</i>	Argentina	Several	94.61 ± 3.00 (8)	99.32 ± 10.2 (10)	1.05	LGE
<i>L. latinasus</i>	Several	Several	31.20 ± 1.7	33.00 ± 1.9	1.06*	Heyer 1978
<i>L. latinasus</i>	Argentina	-	30.30 ± 0.9 (7)	32.20 ± 2.2 (6)	1.06 [▲]	Ponssa and Barrionuevo 2012
<i>L. latinasus</i>	Argentina	30°00'10.83" S 57°22'31.61" W	30.43 ± 1.67 (21)	32.59 ± 1.65 (19)	1.07	R. Cajade and J.M. Piñeiro, unpublished data
<i>L. latinasus</i>	Argentina	27°26' S, 58°44' W	28.35 ± 1.62 (60)	29.30 ± 1.92 (50)	1.03*	Schaefer 2007
<i>L. latinasus</i>	Argentina	27°3' S, 58°45' W	27.76 ± 2.2 (43)	28.50 ± 2.4 (27)	1.02	Duré and Kehr 2004
<i>L. latinasus</i>	Argentina	27°25'53.2" S, 58°44'44.8" W	32.38 ± 2.94 (34)	33.02 ± 3.15 (17)	1.02	Present study
<i>L. latrans</i>	Argentina	Several	65.41 ± 28.84 (94)	63.59 ± 26.72 (89)	-1.03	López et al. 2017
<i>L. mystacinus</i> #	Several	Several	53.00 ± 4.6	56.50 ± 2.7	1.07*	Heyer 1978
<i>L. mystacinus</i> #	Brazil	16°13'50" S, 48°04'49" W	55.80 ± 2.2 (17)	60.80 ± 5.5 (18)	1.09 [▲]	De-Carvalho et al. 2008
<i>L. mystacinus</i> #	Brazil	-	52.90 ± 2.8 (7)	57.90 ± 3.1 (6)	1.09*	Oliveira Filho and Giarretta 2008
<i>L. plaumanni</i>	Argentina	26°13'15.6" S, 53°49'16.2" W	38.64 ± 2.57 (27)	41.78 ± 2.42 (9)	1.08*	LGE
<i>L. podicipinus</i>	Brazil	19°34' S, 57°00' W	35.19 ± 1.34 (21)	39.47 ± 2.13 (36)	1.12*	Prado et al. 2000
<i>L. podicipinus</i>	Brazil	19°34' S, 57°00' W	32.20 ± 3.4 (55)	38.00 ± 3.7 (53)	1.18 [▲]	Rodrigues et al. 2004

Breed fast, die young: demography of a poorly known fossorial frog from the xeric Neotropics

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Manuscript received: 30 January 2017

Accepted: 12 June 2017 by EDGAR LEHR

Abstract. We successfully used skeletochronology to provide the first detailed demographic data regarding *Ceratophrys stolzmanni*, a cryptic, fossorial amphibian inhabiting the xeric Neotropics. We observed a female-biased sexual size dimorphism, but no differences in age parameters between the two sexes. Growth rate is accelerated during the first year of life, both before and after metamorphosis, followed by a rapid sexual maturation and a short lifespan. Both males and females reached sexual maturity before they were one year old, the mean age was two years, and longevity was low for both sexes, with only 2% of the tested individuals, all females, reaching the age of four years. We discuss the implications of the observed aging and growth patterns for the long-term survival and conservation of the species, comparing our results to other tropical species.

Key words. Amphibia, Anura, Ceratophryidae, age, size, longevity, sexual dimorphism, Pacific dry forest.

Introduction

Neotropical seasonally dry forests are currently recognized as one of the most threatened ecosystems worldwide (ESPINOSA et al. 2012). In Ecuador, this type of habitat has been strongly impacted by human activity with more than 87% of the original area being transformed, mostly for agriculture and cattle farming (RON et al. 2011). Considering its low representation in the National Protected Area System (0.3%), this ecosystem should be a priority for conservation (BANDA et al. 2016). Neotropical dry forests are endemism hotspots (LINARES-PALOMINO et al. 2009), and although plant and bird diversity are relatively well studied, hardly any information is available for other groups (ESCRIBANO-ÁVILA 2016).

Effective conservation requires understanding species' natural history and identifying factors that influence extinction risks and the ability to recover after perturbations (SELWOOD et al. 2015). Life history traits, like longevity, age

at first reproduction, and body size are amongst the most important parameters for the study of populations and a prerequisite for designing effective conservation strategies (BIEK et al. 2002). Age- and size-related parameters determine the fitness of individuals and as such are under strong selective pressure (STEARNS 1992). While measuring size is a straightforward affair in most amphibian species, reliably estimating age by means of capture-mark-recapture techniques will require time and financial investment, which are not always available. As an alternative, skeletochronology is currently being used for robust estimation of age in amphibians and reptiles (SINSCH 2015), with a large body of data accumulating. However, this knowledge is biased towards temperate species. Data concerning tropical amphibians is still scarce (SINSCH 2015), with even baseline information lacking for whole families (BIEK et al. 2002) and even though the diversity of adaptation and degree of threat are much higher in tropical environments. Skeletochronology uses cross sections of long bones to reveal in-

dividual growth patterns in organisms that have their growth cycles synchronized with environmental seasonality, such as temperature or rainfall regimes (SMIRINA 1994; SINSCH et al. 2007). Therefore, periods of arrested growth are marked in the hard tissue of these organisms, which through staining appear as intensely coloured, dense lines (i.e., lines of arrested growth, LAGs) (CASTANET & SMIRINA 1990). The formation of LAGs is considered to be genetically controlled and correspond to seasonal periods of inactivity (hibernation or aestivation) (SINSCH 2015). Besides being a reliable tool, skeletochronology has also the advantage of not requiring the sacrifice of animals, since bones from clipped phalanges can be used to obtain accurate age estimates.

The frogs belonging to the South American family Ceratophryidae have attracted a lot of interest, especially in the pet trade, due to their peculiar shapes and voracious appetite, but most scientific studies have focused on phylogeny and morphologic adaptations (WILD 1997, FABREZI 2011, FAIVOVICH et al. 2014), whereas little information is available regarding their ecology and life-history (DUELLMAN & LIZANA 1994, SCHALK et al. 2014, FABREZI et al. 2016). One of the least known species of this family is the Pacific horned toad – *Ceratophrys stolzmanni*, a fossorial frog inhabiting tropical xeric lowland forests in coastal Ecuador and Peru. There is a general lack of information about its life history, with observations being limited to a small number of individuals in captivity (ORTIZ et al. 2013) and mostly dealing with reproductive aspects. Because of its restricted distribution and high pressures on its native forest habitat through logging and expansion of agriculture, *C. stolzmanni* is currently assessed as a vulnerable species by the IUCN (ANGULO et al. 2004). It is active only during the rainy season when heavy rains will trigger explosive breeding. Larval development is fast, with metamorphosis occurring as quickly as two weeks after oviposition (SZÉKELY et al. 2017). Over the course of the dry season, individuals remain buried in the ground, forming a protective cocoon that reduces water loss and so protects them from dehydration (FAIVOVICH et al. 2014).

To understand the natural history of this species and support the conservation of its habitat, we here present the first data regarding age and growth parameters for a population inhabiting the dry forests of southern Ecuador. Because the extreme seasonal variation in the precipitation regime imposes a cessation of activity on this species, we believe that *C. stolzmanni* is an adequate species for studying the expression of growth marks by means of skeletochronology.

Materials and methods

Study area

Our study took place in Arenillas Ecological Reserve, located in El Oro Province, southwestern Ecuador (03°34' S, 80°08' E, 40 m above sea level). The area has been under military protection for the last 60 years, after which,

in 2001, it was incorporated into the Ecuadorian National Protected Area System, and in 2005 was declared an Important Bird and Biodiversity Area (code ECO35, BirdLife International 2016). The total area occupied by Arenillas Ecological Reserve has been decreasing continuously, from 17.083 ha in 1994 to 14.282 ha in 2001, and 10.277 ha in 2012 (CAMACHO & ULLAURI 2013). This loss was caused by illegal logging and the ongoing expansion of shrimp farms and cultivated land within the officially protected area. The reserve protects one of the last remnants of original Pacific dry forests, an ecosystem with low deciduous trees and a dense herbaceous understorey (ESPINOSA et al. 2016). The climate is characterized by a dry season from June to December (less than 10 mm of monthly precipitation for at least four months) and a rainy season, from January to May. Total annual precipitation averages 667 mm, with large fluctuations between years. The mean annual temperature is 25°C, with a 3.4°C variation between the coldest and warmest months (ESPINOSA et al. 2016). Nine species of amphibians, representing five families, have been reported from the reserve (SZÉKELY et al. 2016).

Data collection

A total of 153 adult individuals (79 males and 74 females) and 92 freshly metamorphosed individuals were sampled in 2015 (January through April). They were caught along transects in the forest; for each individual we recorded the following measurements: body mass using a My Weigh 300Z portable scale (0.1 g precision), snout-vent length (SVL) and head width using a Dial-Max calliper (0.1 mm precision). The males were recognized by the presence of secondary sexual characters such as dark coloration on throat and nuptial pads on forelimbs. We collected the third digit of the right forelimb from all adults and from five freshly metamorphosed froglets. All individuals were released at their capture sites thereafter, and the bone samples were preserved in 96% ethanol; tissues that were not used for skeletochronology (skin and muscle) were deposited at the Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Quito, Ecuador (QCAZ 66291–62491).

Skeletochronology

Our tissue preparation protocol followed the method established by CASTANET & SMIRINA (1990) with minor modifications. We removed skin and soft tissues, decalcified the bones in 5% nitric acid for 2 to 18 min, depending on sizes, and afterwards left them to soak in tap water overnight. We embedded the bones in TissueTek embedding medium for cryotomy, cut 12–16 µm cross sections with a Tehsys CR 3000 cryotome, stained these with Ehrlich's hematoxylin for four hours, and finally washed them in distilled water for three minutes. Sections with the smallest marrow cavity and the thickest cortical bone were mounted on slides using Aquatex (aqueous mounting agent for microscopy,

Merck Millipore) and photographed them using an Olympus E-620 microscope-mounted camera (Olympus CX 31 microscope) with Quick Photo Micro 2.3 software. Three independent observers (US, DC and FS) counted the lines of arrested growth (LAGs) in 3–5 sections per individual. Faintly stained bone marks, situated in close proximity of the annual LAG were considered double/multiple lines and counted as one year (LECLAIR et al. 2005, SINSCH et al. 2007).

The parameters analysed follow the definition by LESKOVAR et al. (2006): (1) age at sexual maturity as the minimum estimated age of breeding individuals; (2) size at sexual maturity as the average SVL of individuals with the minimum reproductive age; (3) longevity as the maximum age; (4) potential reproductive lifespan as the difference between longevity and age at sexual maturity; (5) median lifespan as the mean of age distribution. Since the distance between consecutive LAGs is a good indicator of growth, the abrupt rapprochement of LAGs was regarded as the attainment of sexual maturity (SMIRINA 1994).

Data analysis

Analyses were carried out in R environment (R Core Team 2016), with the significance level set at 0.05. Size-related parameters were normally distributed (body mass after ln-transformation; Shapiro-Wilk test, $P > 0.05$) and conformed to the assumption of homoscedasticity (Levene's test, $P > 0.05$). To detect differences between sexes, we used the Wilcoxon-Mann-Whitney test for age distribution, and Welch's t-test for morphometric variables. To test the effect of age on size (SVL, body mass), we used a General Linear Model (GLM, Gaussian distribution) with age and sex as predictors.

We used the packages FSA (OGLE 2014) and nlstools (BATY et al. 2015) to compute von Bertalanffy's growth model (VON BERTALANFFY 1938) following the formula modified by BEVERTON & HOLT (2012):

$$SVL_t = SVL_{\max} \times (1 - e^{-k \times (t - t_0)}),$$

where SVL_t is the expected or average SVL at the 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. We fitted von Bertalanffy's growth model and estimated growth parameters (VBGP) by non-linear least squares regression. Two estimated VBGP were considered significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap.

We propose an alternative method to estimate juvenile post-metamorphic growth by analysing the bone growth pattern observed in cross sections. The attainment of sexual maturity is associated with reduced growth rates, corresponding to LAG spacing being reduced in the periosteal bone. In *C. stolzmanni*, juvenile post-metamorphic growth is most evident in the periosteal bone, bounded between the line of metamorphosis and the first LAG (which, in

this case, corresponds to the time when sexual maturity is reached). With ImageJ software v. 1.50i (RASBAND 1997–2016), we measured two parameters in those cross-sections of the diaphysis where the line of metamorphosis was evident: (1) the distance between the line of metamorphosis and the first LAG, at the widest growth area in the periosteal bone, as a proxy of terrestrial growth during the first year of life (TG), and (2) the longest diameter of the first LAG (D). Using these two parameters, we computed a relative growth index: $RGI = (2 \times TG)/D$, which allowed us to test if growth before sexual maturity was a source for the observed sexual size dimorphism in *C. stolzmanni*.

Results

Body size and sexual size dimorphism

At metamorphosis, juveniles had on average 34.4 mm (SVL), and 4.14 g (body mass), but with large individual variation (Table 1). For adults, significant differences were found between the two sexes (Table 1) in that females were overall larger and heavier than males (Welch's t-test, SVL: $t_{142.18} = -8.84$, $P < 0.001$; mass: $t_{145.6} = -10.63$, $P < 0.001$). In the same age group, females were on average between 6 and 11% longer and between 32 and 57% heavier than the males, with differences increasing with age. For both sexes, body mass and SVL were significantly correlated (Pearson correlation – male: $r^2 = 0.67$, d.f. = 76, $P < 0.001$; female $r^2 = 0.63$, d.f. = 72, $P < 0.001$).

Age structure and age at first reproduction

We found cross-sections in which LAGs were clearly marked in 152 (99%) adult individuals (Fig. 1). The only individual that showed poorly expressed LAGs was excluded from the analysis. The metamorphosis line was evident in 73% of adults and in two of the five juveniles. Also, sections of 59.4% individuals showed double or/and multiple LAGs, of which 91% showed a multiple first LAG, while only 26% showed a multiple LAG in their second year of life, and 2% had a multiple LAG in the third year. *Ceratophrys stolzmanni* usually breeds in February–March, and metamorphosis occurs in March–April, depending on climatic conditions. Because adult samples were collected before and during the reproduction period (January–March), the precise age of the individuals is slightly (1–3 months) lower than the number of LAGs. For example, individuals in the 1-year old age group (showing one LAG), are 9–11 months old.

The age structure of the studied *C. stolzmanni* population is illustrated in Fig. 2. The youngest breeding individuals were 1 year old (both males and females). Average SVL and body mass at first reproduction were 60.7 mm and 21.6 g for males and 64.4 mm and 28.4 g for females (Table 1). There were no significant differences in the mean age between the two sexes (Wilcoxon-Mann-Whitney test, $n_{\text{males}} = 78$, $n_{\text{females}} = 74$, $U = 2609$, $P = 0.46$). The median

Table 1. Morphometric variables of *Ceratophrys stolzmanni* according to sex and age. Parameters for which males are significantly different from females of same age (Welch's t test, corrected with the Bonferroni method for multiple comparisons) are marked with asterisks: ** – $P < 0.01$, *** – $P < 0.001$.

Number of LAGs		n	SVL mean $\pm$ SD (range) (mm)	Head width mean $\pm$ SD (range) (mm)	Body mass mean $\pm$ SD (range) (g)
0	at metamorphosis	92	34.45 $\pm$ 4.85 (23.8–47.9)	18.17 $\pm$ 2.71 (11.7–24.3)	4.14 $\pm$ 1.89 (1.2–11.9)
1	male	14	60.67 $\pm$ 4.69 (53.9–70.4)	30.08 $\pm$ 1.84 (27.7–33.6)	21.58 $\pm$ 5.14 (16–32.1)
	female	7	64.41 $\pm$ 7.0 (55–75.9)	31.42 $\pm$ 3.4 (28.3–36.8)	28.38 $\pm$ 8.55 (18.7–40.8)
2	male	49	59.22 $\pm$ 3.65 *** (52.6–69.1)	29.22 $\pm$ 1.67 *** (24.9–33.3)	21.87 $\pm$ 4.71 *** (14.1–36.7)
	female	51	65.44 $\pm$ 4.33 (56.8–77.5)	31.66 $\pm$ 2.36 (27.7–37)	31.05 $\pm$ 6.55 (17.9–43.8)
3	male	15	59.5 $\pm$ 2.67*** (53–63.1)	29.06 $\pm$ 1.9 ** (25.5–31.6)	21.06 $\pm$ 3.07 *** (14.6–27)
	female	13	66.32 $\pm$ 3.51 (61.9–72.2)	31.16 $\pm$ 1.43 (28.1–34)	33.16 $\pm$ 5.48 (24.8–40.4)
4	female	3	63.33 $\pm$ 3.85 (59–66.4)	30.53 $\pm$ 1.85 (28.4–31.7)	30.26 $\pm$ 5.56 (24.1–34.9)

value was 2 years for both sexes. Longevity was 4 years in 3 females (2% of tested individuals). Potential reproductive lifespan was 2 years for males, and 3 years for females.

On average, after metamorphosis, during their first year of life, males were able to increase their mass 5.21 times and SVL 1.76 times, while females increased their mass 6.85 times and SVL 1.87 times. Size could not be significantly predicted by age (GLM–SVL: $F_{(1,150)} = 3798.7$, $P = 0.748$; mass: $F_{(1,150)} = 11.142$, $P = 0.373$); age classes overlapped in size and variation amongst the one year old cohort was the largest.

Table 2. Statistics for von Bertalanffy growth parameters in *Ceratophrys stolzmanni* males and females. SE = standard error, CI = confidence interval, SVL_{max} = asymptotic body size, k = growth coefficient.

Sex	Parameter	Estimate	SE	CI 95%	P
Males	SVL_{max}	59.32	0.401	58.52–60.12	<0.001
	k	5.79	13.12	-20.37–31.97	0.66
Females	SVL_{max}	65.67	0.668	64.34–67.00	<0.001
	k	2.17	0.548	1.08–3.27	<0.001

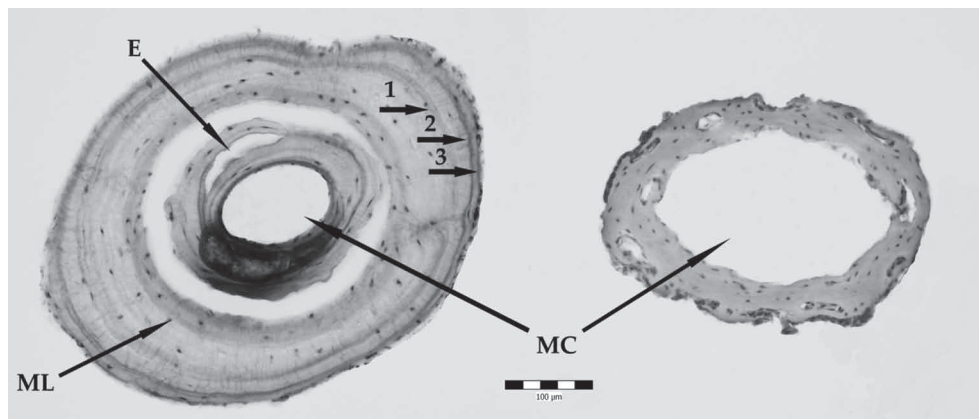


Figure 1. Cross-sections through *Ceratophrys stolzmanni* phalanges: left. three-year old male; right. freshly metamorphosed juvenile. LAGs are indicated by black arrows and numbers. ML – metamorphosis line; MC – marrow cavity; E – endosteum. LAG 1 is triplicated, LAG 2 is duplicated. LAG rapprochement is visible after LAG 1, indicating the attainment of sexual maturity.

Table 3. Life-expectancy of tropical amphibians (studies carried out at latitudes between 23°27' N and 23°27' S) living in xeric or arid environments that impose the cessation of activity. Min. age - minimum age reported for sexually mature individuals and longevity, in years.

Family	Species	Sex	Min. age	Longevity	Location	Source
Bufonidae	<i>Bufo pentoni</i>	M	2	4	Senegal	(BARBAULT et al. 1979)
		F	2	4		
Bufonidae	<i>Nimbaphrynoides occidentalis</i>	M	0.3	2	Liberia	(CASTANET et al. 2000)
		F	0.3	5		
Microhylidae	<i>Scaphiophryne gottlebei</i>	M	<1	2	Madagascar	(GUARINO et al. 2010)
		F	1	2		
Mantellidae	<i>Boophis occidentalis</i>	M	4	11	Madagascar	(ANDREONE et al. 2002)
Mantellidae	<i>Mantella expectata</i>	M	1	3	Madagascar	(GUARINO et al. 2010)
		F	1	3		

Growth

von Bertalanffy models fitted growth in *C. stolzmanni* in both sexes (residual standard error, males: 3.118 on 71 degrees of freedom, females: 3.912 on 68 degrees of freedom), but the growth rate coefficient could not be estimated accurately in males (Table 2). The asymptotic average SVL was significantly higher in females, while the growth pattern followed a more abrupt decreasing trajectory in males compared to females (Fig. 3). We computed the RGI in 33 males and 32 females, and found no significant differences between the two sexes (Welch's t-test, $t_{60,23} = -1.882$, $P = 0.065$).

Discussion

We provide the first detailed morphometric and demographic data for a population of the Pacific horned frog, showing that the skeletochronological method can successfully be used to determine the age in this species. Like other tropical species living in xeric environments (Table 3), Pacific horned frogs grow fast during their first year until

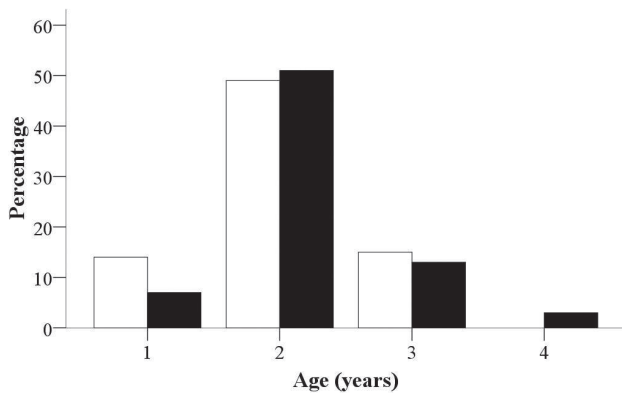


Figure 2. Age structure of *Ceratophrys stolzmanni* in Arenillas Ecological Reserve, Ecuador, determined by skeletochronology. Open bars – males, full bars – females.

reaching sexual maturity at a young age. The growth rate of adults is marked by a sharp decrease once sexual maturity has been reached, and lifespan is short. Tropical amphibians tend to have shorter lifespans than those inhabiting colder habitats, higher altitudes or latitudes (SMIRINA 1994, MORRISON & HERO 2003); also, longevity is generally correlated with age at maturity (GUARINO et al. 2003). In other species of the family Ceratophryidae from semi-arid subtropical South American dry Chaco, reported values of longevity (based only on a small number of individuals per species) are: *Lepidobatrachus llanensis*, six years; *Lepidobatrachus laevis*, seven years; *Chacophrys pierotti*, six years; and *Ceratophrys cranwelli*, 14 years (FABREZI & QUINZIO 2008). However, no data regarding the age structure of populations are available for comparison.

The sampled *C. stolzmanni* population consisted predominantly of young individuals, with the majority being two years old, and only three females having lived to an age of four years. Since our observations were not restricted to the breeding season, the fact that we sampled fewer 1-year

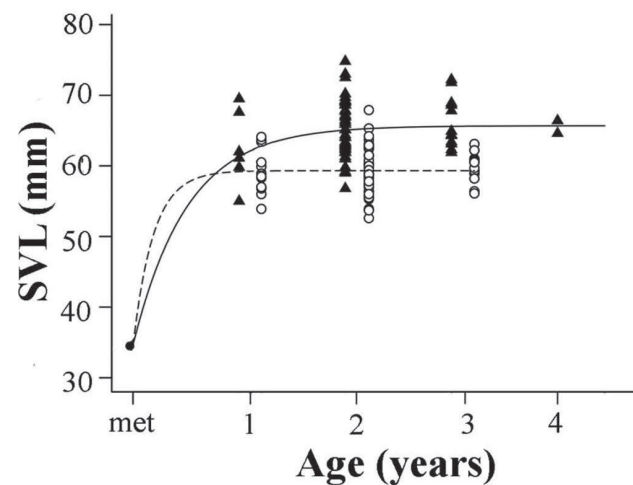


Figure 3. Growth described by von Bertalanffy's model in *Ceratophrys stolzmanni* males (open circles, dotted line) and females (full triangles, continuous line). met – metamorphosis, full circle – average size at metamorphosis.

olds than 2-year olds might reflect either a lower detectability of this age group, or a fluctuation in survival rates between the years.

Growth is accelerated during the first year, both before (SZÉKELY et al. 2017) and after metamorphosis. In the 2–3 weeks of larval development, the tadpoles of *C. stolzmanni* show one of the fastest growth rates recorded for any anuran species (for a comparison, see RICHTER-BOIX et al. 2011). Our data suggest that they may have reached 57 and 53% of their adult SVL (males and females, respectively) at metamorphosis. The rapid development and accelerated growth rate in larval stages seem to be a characteristic of Ceratophryidae, and are considered a response to life in unpredictable environments with long periods of inactivity due to extreme dryness (FABREZI 2011). Both males and females reach sexual maturity in their first year of life, after which growth is greatly decelerated, probably because of a large investment into reproduction. The rapid onset of sexual maturity is regarded as an adaptive response to high annual mortality rates (METCALFE & MONAGHAN 2003) since it is more advantageous to breed early and at a small size than to delay maturity and run an increased risk of not breeding at all.

Variation in size per age class was large, and different age classes overlapped for all tested morphometric values, rendering size measurements unreliable for estimating age in this species. Large variations in size at the same age can be explained by different individual growth rates before maturity – both before and after metamorphosis. Data obtained from skeletochronological and mark-recapture studies showed that size is a poor predictor for age in amphibians (HALLIDAY & VERRELL 1988) because the growth rate before maturity has a much larger impact on size than age. Female Pacific horned frogs are larger than males, which is the case in the vast majority of amphibians (SHINE 1979). In species where fecundity is correlated with size, this is considered to be the result of different pressures on the two sexes, with the selection for larger size being stronger on females (SHINE 1989). In some cases, sexual size dimorphism is caused by differences in age at first reproduction (MONNET & CHERRY 2002). Since sexual maturity was reached by both male and female *C. stolzmanni* before the age of one year, we infer that their dimorphism is determined by different growth rates. This assumption is also supported by von Bertalanffy's model estimates of asymptotic average snout–vent length, which was significantly higher in females. Our study suggests that von Bertalanffy models using skeletochronological data are of limited use in species that grow fast and are short-lived, i.e., the age class width of one year is too wide to adequately represent growth rates. RGI indicated that both males and females experienced the same growth rates from metamorphosis to sexual maturity (i.e., at one year old), which is corroborated by the lack of a significant sexual size dimorphism in the one-year age class. Our results indicate that growth rates after reaching sexual maturity shape the growth models and sexual size differences in *C. stolzmanni*.

As in other species (SINSCH et al. 2007), a relatively large proportion of individuals formed double and/or multiple LAGs. The presence of these lines is usually attributed to a decrease in growth rate, which can be caused by a variety of environmental and internal factors (CASTANET & SMIRINA 1990). In the case of *C. stolzmanni*, we suspect that the presence of double/multiple LAGs may be the reflection of unfavourable periods during the active season that restricted activity and thus growth (e.g., intervals with low precipitation during the rainy season, or excessive growth of grass vegetation that can impede movement and foraging).

The short reproductive lifespan of the species has important conservation implications, because a persistent drought of 2–3 consecutive years with low recruitment would endanger the survival of the whole population (MARSH & TRENHAM 2001). A recent study emphasizes the vulnerability of the Southern Region of Ecuador, and especially of the dry-forests in this area, to the predicted future climate scenarios (EGUIGUREN-VELEPUCHA et al. 2016). Prolonged droughts associated with El Niño/La Niña oscillations have been shown to affect population densities especially in short-lived tropical amphibians (e.g., *Eleutherodactylus coqui*; STEWART 1995), and have been proposed as the main cause for the disappearance of other species (POUNDS & CRUMP 1994); their influence is far from being understood, however.

The endangered Tumbes-Piura dry forest ecoregion was chosen as one of the 66 priority ecoregions for the conservation of Neotropical amphibians (LOYOLA et al. 2008). Conversion of tropical dry forest into agricultural land was demonstrated to negatively impact on amphibian assemblages (SUAZO-ORTUÑO et al. 2008), and the consequences can be exacerbated by climate change. We highlight the urgent need for effective conservation of Arenillas Ecological Reserve, as increased anthropogenic pressures and land-use modification will probably lead to further fragmentation of the Pacific horned toad habitat and reduce opportunities for successful reproduction and recolonization, so that the risk of catastrophic declines would be augmented in species with short lifespans and few reproductive opportunities, such as *C. stolzmanni*.

Acknowledgements

Our research was carried out under research permit MAE-DNB-CM-2015-0016 obtained from the Ministerio del Ambiente de Ecuador. Export permits for the bone samples were granted by the Pontificia Universidad Católica del Ecuador, Museo de Zoología, QCAZ (Autorización de Exportación Científica no. 334/2015). The skeletochronological procedure was approved by the Ethics Committee of the Faculty of Natural and Agricultural Sciences, Ovidius University, Constanța. P. SZÉKELY and D. COGĂLNICEANU received funding from the Secretaría de Educación Superior, Ciencia, Tecnología e Innovación, Ecuador (SENESCYT), through the Prometeo Project. The collaboration between Ovidius University Constanța and University of Liège was supported by Wallonie-Bruxelles International (W.B.I.) and CCCDI-UEFISCDI – project no. 105 BM/2017. Special thanks are due to the staff of Arenillas Forest Reserve for their support during our study.

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Coping with Aridity: Life History of *Chacophrys pierottii*, a Fossorial Anuran of Gran Chaco

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Abstract. We studied size, age, and growth in *Chacophrys pierottii*, a fossorial amphibian inhabiting the arid region of Gran Chaco, in northern Argentina. We provide the first detailed demographic data for this cryptic species using skeletochronology. We observed female-biased sexual size dimorphism but no differences in age parameters between the two sexes. *Chacophrys pierottii* has a short life span of up to 5 a, both males and females reaching sexual maturity after 1 a. Our results suggest that the rapid growth to maturity followed by a relatively short reproductive lifespan is a response to the constraints posed by arid environments. The life history parameters in the studied population of *C. pierottii* are in line with the observed patterns of other anurans inhabiting xeric habitats. Our study provides conservation managers with basic data required for the accurate assessment of the species status and the adoption of proper conservation strategies.

Keywords. Age; Amphibia; Ceratophryidae; Sexual dimorphism; Skeletochronology.

INTRODUCTION

The Neotropics have a high amphibian species diversity, housing almost half the known species (Bolaños et al., 2008), but our knowledge of this group is at best mediocre (Duellman, 1999). Amphibians are the most threatened group of terrestrial vertebrates (Baillie et al., 2010; Blaustein et al., 2010; Hof et al., 2011), with 39% of the species in the Neotropics under threat, representing 60% of all globally threatened amphibians (Bolaños et al., 2008). Life history traits, like body size, mean lifespan, longevity, and age at first reproduction are among the most important parameters for the study of amphibian populations (Allen et al., 2017; Campbell et al., 2017) and a prerequisite for designing effective conservation strategies (Biek et al., 2002; Vukov et al., 2015; Greenberg et al., 2017). Because age- and size-related parameters determine the fitness of individuals, they are under strong selective pressure (Stearns, 1992) and, as such, are finely tuned to environmental conditions (Caruso et al., 2014; Cayuela et al., 2016; Liao et al., 2016). In most amphibian species, obtaining data on body size is usually straightforward, but the reliable estimation of age by means of capture-mark-recapture techniques is extremely labour intensive (Sinsch, 2015). As an alternative, skeletochronology is frequently used for age estimation in amphibians and reptiles (Sinsch, 2015) and has recently been

used increasingly in tropical species (Sinsch and Dehling, 2017). In addition to providing reliable age estimates, skeletochronology has the advantage of not requiring euthanasia of the studied animals, since a single phalanx is sufficient (Castanet and Smirina, 1990).

Amphibians are particularly sensitive to both high temperatures and low humidity (Navas et al., 2008), so in order to colonize and survive in arid habitats, a fossorial life-style has evolved several times in different anuran families (Jorgensen and Reilly, 2013). Amphibians inhabiting arid habitats are under higher threat due to climate-change induced aridization and increasing temperatures (Duarte et al., 2012), so knowledge of their life history adaptations is required for adequate conservation measures.

Chacophrys pierottii (Vellard, 1948), the “*escuercito chaqueño*” or “Chaco horned frog,” is a poorly known fossorial anuran of the family Ceratophryidae distributed across the arid and semi-arid sub-regions of the Gran Chaco ecoregion (northern Argentina), western Paraguay, and southern Bolivia (Frost, 2016). The Gran Chaco (Olson et al., 2001) is the second-largest ecoregion in South America, after Amazonia, and includes the largest seasonally dry forests in the Neotropics (Bucher, 1982). This environment is characterized by a strongly seasonal rainfall regime, with more than 80% of precipitation concentrated between October and April (Bucher, 1980) when the highest anuran activity occurs (Perotti, 1997).

How to cite this article: Marangoni F, Stănescu F, Courtis A, Piñeiro J.M., Ingaramo M.R., Cajade R., Cogălniceanu D. 2018. Coping with aridity: life history of *Chacophrys pierottii*, a fossorial anuran of Gran Chaco. *South American Journal of Herpetology* 13:230–237. <http://doi.org/10.2994/SAJH-D-17-00070.1>

One of the highest rates of global deforestation occurs in the Gran Chaco (Hoyos et al., 2013), comparable to Amazonia (Hansen et al., 2013), to generate land suitable for monoculture (mainly soy) or dedicated pastures for livestock, thus causing the loss of a large number of species (Nori et al., 2016). Since *Chacophrys pierottii* leads a secretive, nocturnal, fossorial lifestyle with short, explosive reproductive events in ephemeral ponds (Bucher, 1982), little is known about its natural history. The information currently available is limited to larval stages (e.g., Faivovich and Carrizo 1992; Wild, 1999; Quinzio et al., 2006; Fabrezi, 2011; Fabrezi et al., 2016), diet and foraging (Pueta and Perotti, 2013; Schalk et al., 2014), occasional predation events (Pereyra and Akmentins, 2013), male vocalization (Lescano, 2011), geographic distribution (e.g., Rosset, 2001; Sanabria et al., 2012), and phylogeny (e.g., Fabrezi and Quinzio, 2008; Faivovich et al., 2014; Fabrezi et al., 2016). The goal of this study was to provide fundamental demographic data on body size- and age-related parameters in adult *C. pierottii* in order to better understand the life history adaptations that allow amphibians to cope with arid environments, and thus support their conservation.

MATERIALS AND METHODS

We performed nocturnal field surveys on 6–8 December 2013 on a 10 km dirt road between the localities Fuerte Esperanza and Misión Nueva Pompeya, Chaco province, Argentina (Ruta Provincial 61, between 24°56'27.99"S, 61°29'26.69"W and 25°1'41.53"S, 61°31'25.55"W; 150 m above sea level), following a heavy storm. A more detailed description of the study area was presented in Stănescu et al. (2016). We captured a total of 26 *Chacophrys pierottii* adult individuals (7 males and 19 females) on the road, between 21:00–01:00 h, during a reproductive migration event towards the temporary ponds formed on the sides of the road. Males were recognized by the presence of either an enlarged vocal sac or vocal slits in the floor of the mouth. For each individual, we measured snout-vent length (SVL) and head width (HW) using a digital caliper (0.1 mm precision) and body mass (BM) using an electronic scale (0.01 g precision). We clipped the third digit of the right forearm and preserved it individually in 70% ethanol for age determination by skeletochronology. Of the 26 individuals captured, 19 were preserved and deposited in the collection of Instituto de Biología Subtropical (Universidad Nacional de Misiones, Consejo Nacional de Investigaciones Científicas y Técnicas; LGE) under voucher numbers LGE 7709–7727, while the remaining 7 were released at the capture site. We assessed sexual size dimorphism (SSD) for each body measurement since it is a key aspect in our understanding of the evolution of life history traits and mating systems (Kupfer, 2007). We

computed the sexual dimorphism index (SDI) following Lovich and Gibbons (1992): $SDI = \frac{\text{mean size}_{\text{larger sex}}}{\text{mean size}_{\text{smaller sex}}}$ with the result arbitrarily defined as positive when females were larger than males, and negative when males were larger than females.

Skeletochronology is a non-lethal, widely-used method to estimate age in amphibians by counting the number of lines of arrested growth (LAGs) in cross sections of phalanges (Sinsch et al., 2015). The growth periods appear as broad bands of tissue separated by narrower lines, or annuli, that mark periods of reduced growth (Halliday and Verrel, 1988). We followed the standard methods in skeletochronology (e.g., Smirina, 1972; Castanet and Smirina, 1990), with minor modifications proposed by Marangoni (2006). Clipped digits were washed in water for 30 min, decalcified in 5% nitric acid for 1–3 h, dehydrated, paraffin-embedded, sectioned using a rotation microtome (Arcano RMT-30) at 14–16 μm , and stained with Harris haematoxylin. For each studied individual we selected 5–10 cross-sections with the smallest diameter of the medullar cavity and the thickest periosteal bone between the line of medullar cavity and the outer line of periosteum (Rozenblut and Ogielska, 2005) and mounted them on microscope slides with a 50% glycerin and 50% alcohol solution. Afterwards, we took digital images of these cross-sections using a high-resolution camera (Sony SSC-DC50AP) attached to a microscope (Olympus BX50) and the two best digital images per individual were selected for further analysis. Cross-sections were observed and measured using Image-Pro Plus version 4.5 (Media Cybernetics 1993–2001), and calibrated using a standard ocular micrometre. Two independent observers (FM and AC) recorded the presence/absence of the line of metamorphosis (LM) and counted the LAGs. LM is a fine line within the first-year growth zone, separating larval tissue (usually stained darker) from the post-metamorphic bone, as indicated by Rozenblut and Ogielska (2005) and Sinsch and Dehling (2017). The presence of LM indicates that no resorption occurred. In those individuals with no remnant LM, we estimated the degree of resorption by osteometrical analysis following Sagor et al. (1998). Thus, we computed a frequency distribution with the diameters of the innermost and second LAGs and, when the diameter of the innermost LAGs exceeded the group mean by 2 SD or more, we considered that the first LAG was eroded. We distinguished annual growth marks (i.e., LAGs *sensu stricto*) from non-annual ones (i.e., irregular interruptions during short periods of inactivity) using the method described by Sinsch et al. (2007). Annuli (*sensu* Peabody, 1958) were easily distinguished from actual LAGs because they always stained more weakly than true LAGs and were often broader, as previously described by Leclair et al. (2005) and Sinsch et al. (2007) in temperate species.

We computed the following age-related parameters: mean lifespan (i.e., mean of age distribution), age of sexu-

Table 1. Descriptive statistics (mean $\pm$ SD) of the variables measured: body mass (BM), snout-vent length (SVL), head width (HW), and bone diameter (BD), of male and female *Chacophrys pierottii* from the Arid Chaco of Argentina. The differences between males and females were compared using ANOVA, P values indicated. The sexual dimorphism index (SDI) was computed according to Lovich and Gibbons (1992); n = sample size.

Size variables measured	Females ($n = 19$)	Males ($n = 7$)	ANOVA	SDI
Body mass (g)	26.37 $\pm$ 7.18	16.29 $\pm$ 2.15	$P < 0.01$	1.62
	12.33–41.26	14.16–19.47		
Snout-vent length (mm)	59.14 $\pm$ 4.16	51.44 $\pm$ 2.33	$P < 0.001$	1.15
	48.96–65.57	47.89–55.07		
Head width (mm)	24.04 $\pm$ 1.32	20.67 $\pm$ 1.44	$P < 0.0001$	1.16
	21.94–26.40	19.16–23.11		
Bone diameter (μm)	280.88 $\pm$ 48.88	251.03 $\pm$ 66.44	$P < 0.01$	1.12
	211.44–380.86	163.41–364.74		

al maturity (i.e., minimum age), longevity (i.e., maximum age) and potential reproductive lifespan (i.e., the difference between maximum and minimum age). We considered the distance between two LAGs to be an indicator of individual growth at a given age, and a pattern of decreasing intervals between LAGs after a few years is thought to indicate the onset of sexual maturity, with resources being reallocated from growth to reproduction (Smirina, 1994). Therefore, we additionally inferred the age of sexual maturity by observing the bone growth pattern in the cross-sections. We measured the longest and shortest perpendicular axes of bone diameter (BD) to the nearest μm from two diaphyseal sections per individual, following the method of Hemelaar (1985). We determined the average diameter of the diaphysis by computing the square root of the product of the two axis measurements.

We first tested the data for normality and homoscedasticity using Shapiro-Wilk and Levene tests respectively and then chose statistical tests accordingly. We used a significance level of $\alpha = 0.05$. All tests were performed using the statistical package Statistica 6.0 (Statsoft Inc., 2001). We computed von Bertalanffy's growth model (Bertalanffy, 1938) following Beverton and Holt (1957): $\text{SVL}_t = \text{SVL}_{\text{max}} \times (1 - e^{-k \times (t - t_0)})$, 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. We considered SVL at metamorphosis to be 35 mm from Quinzio et al. (2006). We fitted von Bertalanffy growth model by nonlinear least squares regression. This analysis was performed in R version 3.0.3 (R Core Team, 2014) with the packages FSA (Ogle, 2016) and nlstools (Baty et al., 2015).

RESULTS

Males and females showed significant differences regarding SVL, BM, HW and BD (MANOVA Wilk's $\lambda = 0.236$, $F_{4,21} = 8.924$, $P < 0.01$; Table 1). Univariate ANOVAs showed that females were significantly larger than males in all measured body size parameters, and

SDI showed the highest value for body mass (Table 1). All processed samples corresponding to 26 individuals (7 males and 19 females) showed well-defined LAGs allowing for age determination (Fig. 1). The line of metamorphosis was visible in 90% ($n = 24$) of the samples. Endosteal resorption was observed only in four cases but did not prevent age estimation. Females were younger than males, but no significant difference in mean lifespan was found between sexes (ANOVA, $F_{1,24} = 1.751$, $P = 0.198$; Table 2). The minimum age observed in the sample was 2 a in females and 3 a in males, while longevity was 5 a in both sexes (Fig. 1). We successfully inferred the age of sexual maturity from the bone growth pattern in 65% of the individuals (12 females, 5 males). Thus, the growth pattern indicated that sexual maturity was attained after the first year of life in both males in females. Based on the minimum age observed in the sample, the potential reproductive lifespan was three years in females and two years in males, but according to the growth pattern observed in the sections it was four years in both sexes. The relation between age and SVL fitted von Bertalanffy's growth model only in females ($\text{SVL}_{\text{max}} = 62.15 \text{ mm} \pm 3.38 \text{ SE}$, CI 95% = 54.98–69.33; $k = 0.57 \pm 0.26 \text{ SE}$, CI 95% = 0.02–1.14; Fig. 2). The predicted asymptotic average SVL (SVL_{max}) was slightly larger than the measured average values but smaller than the SVL of the largest female. The model could not be applied in males due to the small sample size.

Table 2. Age related parameters (reported in years) in the studied population of *Chacophrys pierottii*. PRL 1 = potential reproductive lifespan according to the minimum age observed in the samples; PRL 2 = potential reproductive lifespan according to the inferred age of sexual maturity from the bone growth pattern.

Age related parameters (a)	Females ($n = 19$)	Males ($n = 7$)
Mean age $\pm$ SD	3.74 $\pm$ 0.93	4.29 $\pm$ 0.95
Minimum age	2	3
Inferred age of sexual maturity	1	1
Maximum age	5	5
PRL 1	3	2
PRL 2	4	4

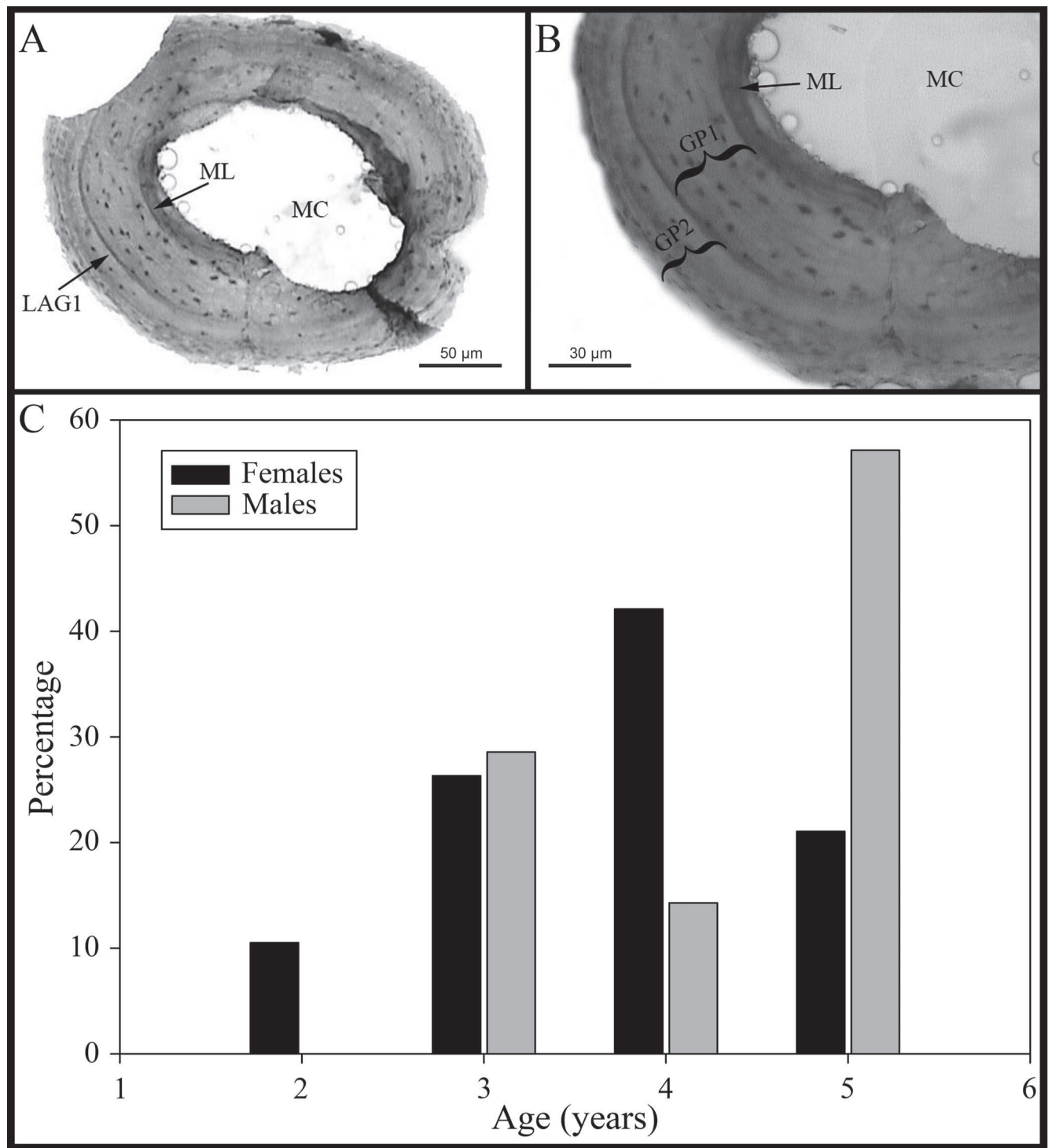


Figure 1. Cross-section through the phalanx diaphysis and age distribution of *Chacophrys pierottii*. **(A)** One line of arrested growth (LAG1) is visible in the periosteal bone (indicated by arrow), the medullar cavity (MC), metamorphosis line (ML). **(B)** Two distinct growth periods (GP) are visible, one between metamorphosis and the first LAG (GP1), and another between the first period of arrested growth and the moment of capture (GP2). **(C)** Age distribution in the studied population of *C. pierottii* from Arid Chaco, Argentina.

DISCUSSION

Our study provides the first demographic data including size and age-related parameters in adult *Chacophrys*

pierottii. We found a strong sexual size dimorphism, females being significantly larger than males. This is the most common sexual dimorphism among amphibians, being reported in about 90% of anuran species (Shine,

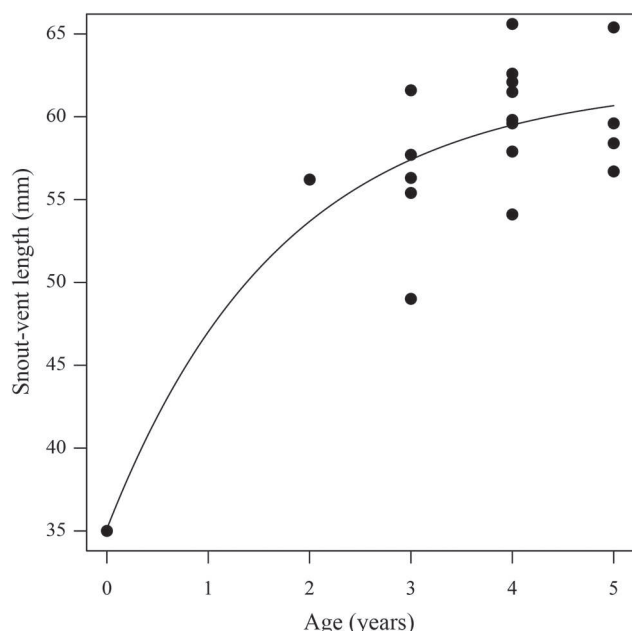


Figure 2. Growth model (Bertalanffy, 1938) of *Chacophrys pierottii* females ($n = 19$), considering snout-vent length and time to reach that length (age in years).

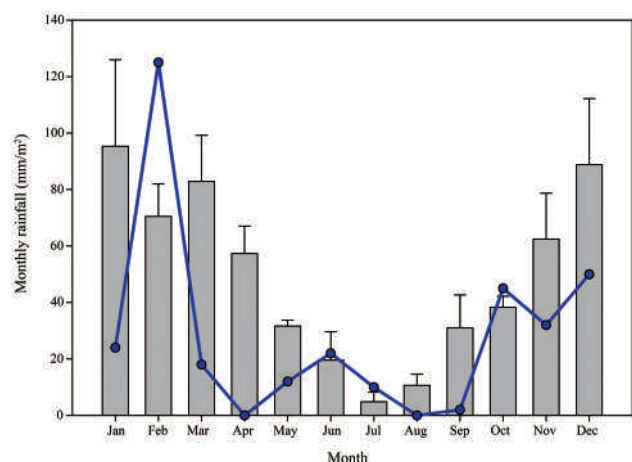


Figure 3. Average monthly rainfall 1967–2016 (grey bars) versus monthly rainfall in 2013 (blue line with dots) recorded at “Estación Experimental Agropecuaria Ingeniero Juárez,” Instituto Nacional de Tecnología Agropecuaria, Argentina.

1979; Kupfer, 2007). There are several proposed causal factors (Halliday and Tejedo, 1995; Monnet and Cherry, 2002), such as the capacity of larger females to produce larger clutches and larger eggs (Crump, 1974; Kuramoto, 1978), restrictions on the growth of males because of the energy demand linked to reproductive activity (e.g., demands of acoustic advertisement, parental care and/or not feed while calling; Woolbright, 1989), and sex-specific differences in mortality rate caused by differential predation pressure between sexes (Howard, 1981).

The presence of well-expressed growth marks in the bone tissue of *Chacophrys pierottii*, which is subjected to

a highly seasonal subtropical climate in the Arid Chaco, supports the hypothesis that LAG formation has a general intrinsic (i.e., genetic) control which is synchronized with, and reinforced by the natural seasonal cycles (e.g., Sinsch et al., 2007; Beşer et al., 2017; Kumbar and Lad, 2017; López et al., 2017; Sinsch and Dehling, 2017). The presence of LAGs in this species was previously reported by Fabrezi and Quinzio (2008) in two males. We successfully applied the skeletochronological method for the first time in this enigmatic species at the population level, which allowed us to provide new insights into its life history. Thus, we found that *C. pierottii* from the Arid Chaco are relatively short-lived (i.e., 5 a), females and males having a maximum of four reproductive events during adulthood. The age of sexual maturity inferred from the bone growth pattern indicates that both males and females are able to breed after their first year of life. However, the youngest males and females in our sample were respectively three and two years old. An explanation for the lack of one-year-old individuals in our sample, apart from the small sample size in males, could be that individuals might skip breeding during extremely arid years when resources are scarce, as shown by Muths et al. (2010, 2013) in temperate toads inhabiting harsh environments with limited resources and short growth periods. On the other hand, lack of reproductive success (i.e., failure to metamorphose and/or mass mortality of juveniles) during the previous reproductive period, could have led to the same result. Both hypotheses are supported by rainfall data obtained from “Estación Experimental Agropecuaria Ingeniero Juárez”, Instituto Nacional de Tecnología Agropecuaria (INTA), located about 70 km north from our study site. The data showed that annual rainfall was only 340 mm/a in 2013, compared to 810 mm/a in 2012; the average annual rainfall in this area during the last 43 a (1967–2016) was 641 mm/a (min–max: 340–1,010 mm/a), being 2013 the most arid year during this given period of time (Fig. 3). The total rainfall during the activity season corresponding to October–December 2012 and January–April 2013, when the 2013 cohort of juveniles should have hatched and metamorphosed, was only 310 mm compared to 625.5 mm during the previous activity season, October–December 2011 and January–April 2012.

Similar life history patterns, with rapidly attained sexual maturity and short lifespan, were reported for other anurans inhabiting xeric environments which impose harsh constraints in terms of limited water availability and short breeding and growth seasons: *Sclerophrys pentoni* (Anderson, 1893) (Francillon et al., 1984), *Pelophylax saharicus* (Boulenger in Hartert, 1913) (Esteban et al., 1999; Bellakhal et al., 2008), *Incilius alvarius* (Girard in Baird, 1859), *Anaxyrus cognatus* (Say in James, 1823), *Anaxyrus punctatus* (Baird and Girard, 1852), and *Scaphiopus couchii* Baird, 1854 (Sullivan and Fernandez, 1999), *Mantella expectata* Busse and Böhme, 1992 and *Scaphiophryne gottle-*

bei Busse and Böhme, 1992 (Guarino et al., 2010), *Dermatonotus muelleri* (Boettger, 1885) (Stănescu et al., 2016), and *Ceratophrys stolzmanni* Steindachner, 1882 (Székely et al., 2018). Previous studies emphasize additional adaptations to xeric environments in *Chacophrys pierottii*: rapid larval development of 15–18 days and accelerated growth rates to metamorphosis, similar to *Lepidobatrachus laevis* Budgett, 1899 and *L. llanensis* Reig and Cej, 1963, other two inhabitants of the Arid Chaco (Fabrezi and Quinzio, 2008; Fabrezi, 2011; Zeng et al., 2014), or to *Ceratophrys stolzmanni*, an inhabitant of the Pacific dry forests of Ecuador (Székely et al., 2017). Quinzio et al. (2006) reported an average SVL at metamorphosis of 35 mm at the end of January, while Pueta and Perotti (2013) found that juveniles had an average SVL of 37.1 mm in February; according to these independent observations, an average growth rate of 2 mm/month can be inferred during the activity season in juvenile individuals. Thus, provided that a minimum average growth rate of 2 mm/month can be maintained during the activity season following metamorphosis (i.e., March–April and October–December), juveniles should be able to attain a minimum average SVL of 47 and 49 mm in December and January, respectively, when breeding events were observed. According to the observed bone growth patterns in cross sections, most of the growth occurs during the first year of life, decreasing abruptly after this age, when presumably sexual maturity occurs. This is consistent with the minimum SVL of sexually mature individuals in our sample—47.9 mm.

Although *Chacophrys pierottii* is categorized as “Least Concern” according to IUCN (Aquino et al., 2004) and “Not Threatened” according to the categorization of Argentinean amphibian fauna (Vaira et al., 2012), the species is threatened by the illegal pet-trade, being collected during explosive breeding events (Aquino et al., 2004), and habitat destruction, which is mainly caused by deforestation (Aquino et al., 2004; Hoyos et al., 2013). The short reproductive lifespan of the species has important conservation implications, because persistent droughts with low recruitment over consecutive years would endanger the survival of whole populations (Marsh and Trenham, 2001). A similar situation was reported for another Neotropical fossorial species, *Ceratophrys stolzmanni*, which inhabits seasonally dry forests in Ecuador (Székely et al., 2018). Thus, our study provides much-needed basic data that will support more accurate assessments regarding the conservation status of this species and implicitly, appropriate conservation strategies.

ACKNOWLEDGMENTS

This research received support from the Ministerio de Ciencia, Tecnología e Innovación Productiva (MINCyT), Universidad Nacional de Misiones (UNaM)

(grant RO/12/06, FM) and the Romanian National Authority for Scientific Research CNCS- UEFISCDI (grant PN-II-CAPACITĂȚI-Modulul III-Cooperări Bilaterale 732/23.07.2013, and partially grant PN-II-ID-PCE-2011-3-0173, DC). The collecting permit was granted by Dirección de Fauna y Áreas Naturales Protegidas of the Chaco province. AC, FM, JMP, MRI and RC are very grateful for the continuous support of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The meteorological data was kindly provided by “Estación Experimental Agropecuaria Ingeniero Juárez” - Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. We are very grateful to Ivana Renko for invaluable help during fieldwork and to Christopher M. Schalk and Diana Székely for helpful comments on earlier versions of the manuscript.

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The impact of salinity on early developmental stages in two sympatric spadefoot toads and implications for amphibian conservation in coastal areas

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Received: 7 August 2016 / Revised: 3 December 2016 / Accepted: 9 December 2016 / Published online: 19 December 2016
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Abstract Salinity tolerance is critical during the early ontogeny of amphibians, shaping future population size, health and dispersal in a certain area. We focused our research on two related anurans with similar ecological niches—*Pelobates fuscus* and *P. syriacus*—inhabiting the western Black Sea coast, at the limits of their ranges. We hypothesize that their differences in salinity tolerance are shaping the actual range limits in coastal areas, within the sympatry zone. We quantified experimentally the impact of salinity (range 0–9‰) during early ontogeny to ask if salinity can modulate their coexistence, by affecting differently reproductive success and fitness. Exposure to salinity from egg to developmental stage Gosner 25

caused mild to severe malformations and affected survival and size in both species, but the impact was lower in *P. syriacus* compared to *P. fuscus* when exposed to salt concentrations of 6‰. Embryos of either species did not survive the 9‰ salinity concentration. We expect that increases in salinization up to 6‰ could severely reduce the range of *P. fuscus*, but not *P. syriacus*, in coastal areas. These results are highly relevant for the conservation of *P. fuscus*, which is already declining across Europe.

Keywords Amphibia · Embryos · Breeding ponds · Danube Delta · *Pelobates syriacus* · *P. fuscus*

Handling editor: Lee B. Kats

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Introduction

Salinization is increasingly recognized as a major and widespread threat affecting freshwater communities (Williams, 1999, 2001; Halse et al., 2003; Hart et al., 2003; Kaushal et al., 2005; Herbert et al., 2015; Cañedo-Argüelles et al., 2016; Oliveira et al., 2016). Water salinity increase can have both natural and anthropogenic origins; for example, sea level rise and increased storm frequencies are expected consequences of climate change, with the potential to increase salinisation in coastal habitats (Craft et al., 2008; Oude et al., 2010; Church et al., 2013), while aquatic habitats located in the proximity of large irrigated agricultural areas and/or important road

infrastructures, where salt is used as defrosting agent during winter, are prone mostly to anthropogenic salinisation (Williams, 2001; Karraker et al., 2008; Findlay & Kelly, 2011). Amphibians are particularly sensitive to changes in the water chemistry due to their complex life-cycles with aquatic stages of various lengths, and highly permeable tegument, making them excellent bioindicators of water quality. Furthermore, amphibians are already the most endangered group of vertebrates worldwide, affected by a variety of threats ranging from habitat destruction to viral and fungal diseases (e.g. Stuart et al., 2004; Baillie et al., 2010; Blaustein et al., 2010; Hof et al., 2011). Previous research has shown that water salinity is a major limiting factor, restraining distribution and affecting development, behaviour, fitness and ultimately survival in pond-breeding amphibians (Gómez-Mestre & Tejedo, 2003; Gómez-Mestre et al., 2004; Sanzo & Hecnar, 2006; Smith et al., 2007; Denoël et al., 2010; Alexander et al., 2012; Bernabò et al., 2013; Hopkins & Brodie, 2015; Kearny et al., 2016). The physiological mechanisms that underlie osmoregulation vary among species and between ontogenetic stages (Gordon & Tucker, 1965; Degani & Nevo, 1986; Gómez-Mestre & Tejedo, 2003), and there is evidence suggesting that tolerance to salinity is lower during early ontogeny (i.e. up to developmental stage Gosner 25; Gosner, 1960) compared to postmetamorphic stages (Beebee, 1985; Boutilier et al., 1992; Hopkins & Brodie, 2015). Salinity is particularly a major stressor during the early development of amphibians since exposure to salinity induces energetically demanding osmoregulation processes, at the expense of resources normally required for growth and development (Wu & Gómez-Mestre, 2012; Burraco & Gómez-Mestre, 2016; Kearny et al., 2016). Since stress experienced during early life stages has been proven to have long lasting effects (Metcalf & Monaghan, 2001; Wu & Gómez-Mestre, 2012), salinity tolerance during early life stages is highly relevant for the permanence of pond-breeding anurans in a given area, and thus for any conservation effort concerning these taxa.

Exposure to increased salinity can alter amphibian community structure and species richness by excluding salt-sensitive species (Collins & Russell, 2009). Moreover, salinity alone, or in synergy with other environmental factors, has the potential to modulate interspecific interactions, being thus especially

relevant in syntopic populations of closely related species, with low niche differentiation (Gómez-Mestre & Tejedo, 2002). We studied the impact of salinity during the early ontogeny of two closely related pond-breeding anurans with narrow, similar ecological niches—*Pelobates fuscus* (Laurenti, 1768) and *P. syriacus* (Boettger, 1889)—occurring in syntopic populations, at the limits of their distribution ranges along the western Black Sea coast. Previous studies focusing on the distribution patterns of these species (e.g. Tarkhnishvili et al., 2009; Iosif et al., 2014) suggested that the range limits in the area of sympatry might be shaped by the competition between the two related species, rather than by abiotic factors. However, these studies did not take water salinity into account. Research regarding salinity tolerance in *P. fuscus* (Stănescu et al., 2013) and *P. syriacus* (Degani & Nevo, 1986; Shpun et al., 1993) suggested that these two species have different salinity tolerance thresholds, but no comparative study has been done to date and only allotopic populations were tested. The sympatry zone along the western Black Sea coast overlaps with the large wetlands of Danube Delta, a highly dynamic and unpredictable aquatic environment, composed of a mosaic of aquatic habitats with varying salinity levels. Therefore, we hypothesized that water salinity is an important mediator, determining the distribution patterns and coexistence of the two species within this particular sympatry zone. Since the geographic range of *P. syriacus* covers large tracts of arid lands, often located along coastal areas (Tarkhnishvili et al., 2009; Munwes et al., 2010), and *P. syriacus* is more abundant than *P. fuscus* in the study area (Stănescu et al., 2016; unpublished data), we expected that the salinity of the breeding habitats would have a higher impact on the reproductive success and fitness in *P. fuscus*, and thus indirectly modulate the coexistence of the two species. We tested our hypothesis experimentally by quantifying the impact of salinity on survival and size at Gosner stage 25, a critical developmental stage marking the transition from larval stages to free-swimming and feeding tadpoles. The previous studies regarding salinity tolerance in *P. fuscus* (Stănescu et al., 2013) and *P. syriacus* (Degani & Nevo, 1986; Shpun et al., 1993) were conducted on tadpoles, juveniles and/or adults from inland, allotopic populations and none considered the early developmental stages of either species, when embryos and larvae are restricted at the

oviposition sites due to their limited mobility; furthermore, the methodologies used vary greatly, making comparisons less pertinent. In our study, we used a simple, standardized protocol to provide comparable results for the two species.

Materials and methods

Study species. The spadefoot toads *P. fuscus* and *P. syriacus* are burrowing anurans with similar life histories, distributed across Europe and Western Asia (Fig. 1). They both require a strict protection regime throughout their native range within the European Union, under the Habitats Directive, 92/43/EEC (EU, 1992). Although the adults are explosive breeders, the aquatic stage of *Pelobates* species is the longest among European amphibians, lasting between 3 and 6 months (Cogălniceanu et al., 2000; Munwes et al., 2010), thus making them highly vulnerable to risks related to pond desiccation and changes of the water chemistry. The ranges of *P. fuscus* and *P. syriacus* overlap along the lower course of Danube River and the western coast of the Black Sea, both reaching here their southern (*P. fuscus*) and northern (*P. syriacus*) range limits (Džukić et al., 2008; Cogălniceanu et al., 2013). *P. fuscus* suffered multiple dramatic local declines across Europe during the last three decades

(Nyström et al., 2002; Eggert et al., 2006), being already extinct in Switzerland (Agasyan et al., 2009), and its southern and western range forecasted to contract under future climate scenarios (Araújo et al., 2006). Both species breed in temporary and/or permanent ponds, apparently sharing the same ecological niches; the morphology of their spawn and tadpoles is almost identical, making them very difficult to differentiate directly in the field within their sympatry zone (Cogălniceanu et al., 2000; Sidorovska et al., 2002). Therefore, assessing the impact of salinity on the reproductive success in the aquatic habitats within the sympatry zone is challenging to perform during usual fieldwork.

Sampling and study area. We sampled two temporary ponds (*P. fuscus*—Vadu: 44°25′51.96″N, 28°44′7.08″E; 1 m b.s.l and *P. syriacus*—Sinoe: 44°37′14.52″N, 28°48′40.32″E; 2 m b.s.l.) along the Black Sea coast, Romania. Both ponds (less than 20 km apart) are used by the two species for egg-laying, and the choice of these locations depended on the availability of amplexant pairs, since spawn cannot be assigned to species based on morphology only. The two sampling sites are very similar in conditions (including salinity range) and are part of the same complex wetland system, located at the southern extremity of Danube Delta Biosphere Reserve, which predominantly consists of interconnected lagoon

Fig. 1 Global distribution range of *P. fuscus* (orange shade) and *P. syriacus* (green shade) (source of the distribution data: <http://www.iucnredlist.org/>, accessed 01 August 2016). The sympatry area is marked in red and the study area is indicated on the map by a black arrow



systems, and low, flat areas vulnerable to erosion and sea level rise. Permanent and temporary aquatic habitats covered by reed and mace are crossed by natural levees—partly salinized, flat sand dunes covered by steppe vegetation. The sampled breeding ponds are separated from the Black Sea by sand levees and a lagoon. We consider that the studied sites and populations are representative for the northern half of the Romanian Black Sea coastal area and wetland system, where there is no geographic or men-made barrier that would separate populations.

The maximum salinity of the Black Sea surface layer is $\sim 18\text{‰}$ in the central area and decreases towards the shore, where seawater mixes with freshwater from its tributaries (e.g. Danube River) (Stewart et al., 2007; Grinevetsky et al., 2015). We tested the water salinity of available aquatic habitats in the study area during March–June (i.e. the time period corresponding to the aquatic stages of the studied species) during four consecutive years, 2013–2016, using an Oakton SalT-estr 11 salinometer. Salinity of the available aquatic habitats ranged from 0.4 to over 10‰. Water salinity of the sampling sites varied largely within the same pond, both during and across the years (Vadu: 0.7–1.5‰; Sinoe: 0.4–1.7‰), which is expected since the wetland system is highly dependent on Danube River water flow.

Experimental design. Amplectant pairs were captured in early spring, during the breeding season (March–April), placed individually in plastic boxes until egg deposition, and released afterwards. We collected the freshly deposited clutches and introduced them in the experiment within maximum 12 h after deposition (Gosner stages 1–5). We used six clutches from *P. fuscus* (five replicates) and 15 from *P. syriacus* (14 replicates). Since the clutch size varies between species, the number of replicates used for *P. fuscus* was smaller, limited by the number of eggs available. In both cases, we extracted random portions of 15–30 eggs from each clutch and mixed them in 1 l plastic boxes filled with 800 ml of saline solution, such as each box contained a portion of eggs from each of the clutches.

We tested three salinity concentrations, within the range found in the available aquatic habitats in the study area: 3‰ (moderate), 6‰ (high) and 9‰ (extreme), and aged tap water 0.5‰, as control. We obtained the desired salinity concentrations by mixing aged tap water and Ocean Fish marine salt for ornamental aquariums. We conducted the experiment in the laboratory, at $20 \pm 2\text{ °C}$, with photoperiod following the natural

cycle. We checked daily each experimental recipient and removed dead embryos and when the case, decomposing jelly, thus keeping the water clean. We maintained a stable water level and salinity concentration by adding aged tap water to compensate for evaporation. We considered the experiment completed when all larvae reached Gosner stage 25 (i.e. external gills resorbed, free-swimming larvae with obvious mouthparts, able to feed independently). The entire experiment ended after 14 days. We checked for deformities induced by salinity: we considered severe deformities those deviations of the body–tail angle within 45° – 90° interval and mild, those between 10° and 45° . In addition to body–tail deformities, we also observed lesions of the tail (i.e. broken blood vessels) and tail fin (i.e. fringed edges) in individuals of both species raised in the high salinity treatment, but these were difficult to properly quantify and were not included in further analyses.

Random subsamples of the surviving larvae at Gosner stage 25 from each replicate were pooled for each treatment and photographed in white plastic trays alongside a plastic ruler in 1 cm water column. The total length (TL) was measured from photographs using ImageJ software version 1.46r.

Part of the resulting larvae were released to their breeding ponds, while part were introduced in a subsequent experimental study (not included in this paper). Larvae from the saline solutions were gradually acclimated to freshwater and released afterwards.

Data analysis. We expressed survival as the percent of surviving larvae at Gosner stage 25 from the initial number of eggs introduced in the experiment, in each replicate. We checked all data for normality using Shapiro–Wilk test. Since our data was not normally distributed we used Mann–Whitney test to check for differences in survival between the two species, for each treatment and control. We used Kruskal–Wallis tests followed by Dunn–Bonferroni pairwise comparisons post hoc test, to check for differences in TL across the treatments and control, for each species. We performed the statistical analyses using PASW Statistics for Windows, version 18.0 (Chicago: SPSS Inc 2009). The significance level was set at $\alpha = 0.05$.

Results

No embryos survived in the extreme salinity treatment (9‰). Both species showed similar survival rates

under moderate salinity concentration ($z = -1.58$, $P = 0.13$). We found significant differences between the species' survival in both control ($z = -3.15$, $P < 0.001$) and the high salinity treatment ($z = -3.2$, $P < 0.001$). *P. fuscus* larvae had a significantly lower survival in the high salinity treatment compared to *P. syriacus*, but a significantly higher survival in the control (Table 1; Fig. 2).

Salinity significantly affected the TL of larvae at Gosner stage 25 in both species: Kruskal–Wallis: *P. fuscus*, $\chi^2 = 71.5$, $P < 0.001$; *P. syriacus*: $\chi^2 = 66.5$, $P < 0.001$ (Fig. 3). Post hoc pairwise comparisons showed that larvae of both species attained significantly smaller TLs at Gosner stage 25 in the high salinity treatments, compared to the moderate salinity treatments (Dunn–Bonferroni: $P < 0.001$). There was no significant difference in the TL of larvae raised in control compared to moderate salinity concentration in *P. fuscus* (Dunn–Bonferroni: $P = 1.000$), while in *P. syriacus*, larvae raised in control were significantly longer compared to larvae from the moderate treatment (Dunn–Bonferroni: $P < 0.001$).

All *P. fuscus* larvae raised in high salinity concentration suffered mild (body–tail angle 10° – 45°) to severe (body–tail angle 45° – 90°) deformities and died within 5 days after attaining Gosner stage 25. In contrast, less than 5% of *P. syriacus* larvae from 6‰ treatment presented mild deformities.

Discussion

Our study showed that exposure to salinity has a significant negative impact on both *P. fuscus* and *P. syriacus* during early ontogeny, but the two species differ in their tolerance to salinity, *P. syriacus* showing higher tolerance and survival rates compared to *P.*

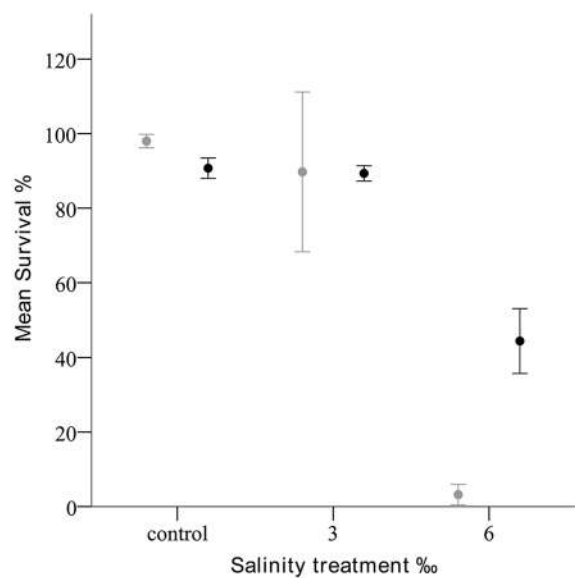


Fig. 2 Mean survival (%) of *P. fuscus* (grey) and *P. syriacus* (black) to Gosner stage 25, under various salinity levels. Error bars represent 95% confidence intervals

fuscus, when exposed to salt concentrations of 6‰. Overall, this agrees with previous studies that showed that salinity tolerance varies greatly among the members of genus *Pelobates*. Thus, *P. syriacus* tadpoles and juveniles are osmoconforming in saline solutions (i.e. NaCl) up to ~6‰ (Degani & Nevo, 1986), while adults can be acclimated to saline solutions up to ~13‰ (Shpun et al., 1993); size and time to metamorphosis in *P. fuscus* tadpoles are not affected by salinity concentrations of 4‰ (Stănescu et al., 2013). *Pelobates varaldii* tadpoles were observed exclusively in freshwater habitats (El Hamoumi et al., 2007; Escoriza, 2013), while in contrast, *P. cultripipes* tadpoles were found in natural habitats with water salinity up to 10‰ (Thirion, 2014). The

Table 1 Survival rates (%) and total length at Gosner stage 25 in *P. fuscus* and *P. syriacus* raised under various salinity concentrations (mean ± SD)

Species	Treatment (‰)	Survival rate (%)	Total length (mm)
<i>P. fuscus</i>	0.5 (control)	97.9 ± 1.5	11.2 ± 1.1 (56)
	3 (moderate)	89.7 ± 17.3	11.1 ± 1.2 (48)
	6 (high)	3.2 ± 2.2	8.2 ± 0.9 (36)
	9 (extreme)	0	–
<i>P. syriacus</i>	0.5 (control)	90.7 ± 4.7	10.1 ± 0.9 (198)
	3 (moderate)	89.3 ± 3.6	9.8 ± 0.9 (245)
	6 (high)	44.4 ± 14.4	9.3 ± 0.9 (157)
	9 (extreme)	0	–

Sample size is given in parentheses for total length measurements

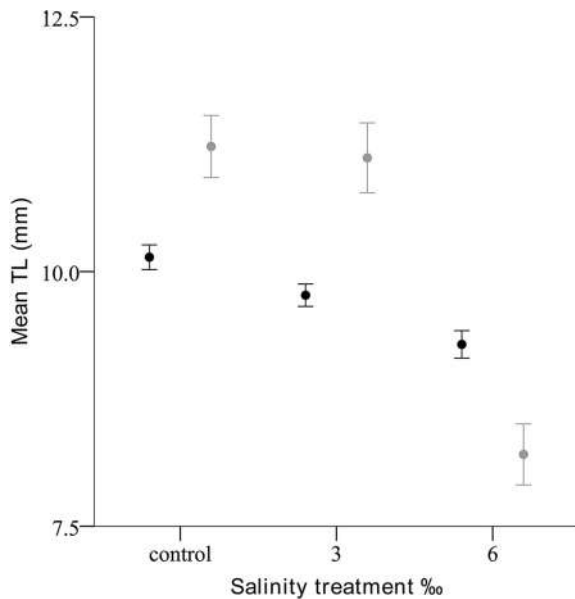


Fig. 3 Mean total length (mm) of *P. fuscus* (grey) and *P. syriacus* (black) larvae at Gosner stage 25, raised under various salinity levels. Error bars represent 92% confidence intervals

tolerance thresholds in our study are similar to those found in tadpoles of both species (Degani & Nevo, 1986; Stănescu et al., 2013), and approximately two times lower compared to those found in adult *P. syriacus* (Shpun et al., 1993). We suggest that the higher salinity tolerance allows *P. syriacus* to take advantage of a wider range of breeding habitats in vulnerable coastal areas, and critically sensitive life-history stages of this species (i.e. eggs and immobile embryos) have better chances to withstand saline water intrusions compared to *P. fuscus*. This is also supported by the geographic range of the two species: northern distribution in mostly humid areas for *P. fuscus*, and southern distribution in mostly coastal arid regions for *P. syriacus*. Therefore, we expect that even slight increases in salinization (up to 6‰) could reduce the range of *P. fuscus*, but not *P. syriacus* in coastal areas.

Summarizing, the tolerance thresholds to salinity varies within Pelobatidae as it follows: *P. varaldii* appears to be the most sensitive, followed by *P. fuscus* and *P. syriacus*, while *P. cultripes* appears the most tolerant to salinity among the four species. However, the tolerance to salinity has not been yet tested experimentally in *P. varaldii*, while in *P. fuscus* and *P. syriacus*, we lack hard evidence from their natural habitats. Tolerance to salinity varies on the course of ontogeny, early developmental stages being more

sensitive than postmetamorphic stages. Such an example is evident in *P. syriacus*, where adults can tolerate saline solutions up to 13‰ (Shpun et al., 1993), while larvae, tadpoles and juveniles can only cope with salinity concentrations up to of 6‰ (Degani & Nevo, 1986; present study). A similar situation was observed in *P. cultripes* by Thirion (2014), where a salinity concentration of 6‰ stopped development in early stages, but tadpoles were found in natural habitats with salinity concentrations up to 10‰. Another aspect to be considered is the variation of tolerance to salinity along salinity gradients. This is why our results may not necessarily reflect tolerance levels in all populations of the two species, and might be the result of local adaptation to fluctuating salinity levels in coastal areas (Gómez-Mestre & Tejedo, 2004).

Previous studies showed that tolerance to salinity varies largely within amphibians (see review by Hopkins & Brodie, 2015). Few species of anurans are able to survive in extremely saline environments, such as *Fejervarya cancrivora*—40‰ (Uchiyama & Yoshizawa, 1992), *Epidalea calamita*—23‰ (Gómez-Mestre & Tejedo, 2003), *Osteopilus septentrionalis*—12‰ (Brown & Walls, 2013), but most experience deleterious effects at exposure to concentrations over 9‰. As such, the two *Pelobates* species from our study appear to be rather sensitive to salt in their early developmental stages, both experiencing higher than 50% mortality rates when raised in 6‰ saline solutions. Even in sublethal dose, increased salinity had a significant negative impact on larvae size and morphology at Gosner stage 25 in both species. The fact that exposure to salinity induces malformations in amphibian larvae has been previously documented in other species (e.g. Karraker, 2007; Karraker et al., 2008, 2010). While the frequency of malformations in *P. syriacus* is below the 5% threshold, and can thus be considered as normal (Johnson et al., 2010), the impact caused to *P. fuscus* larvae is significant and supports the hypothesis of different tolerance thresholds in the two species. Malformations caused by exposure to salt hinder swimming in later aquatic stages, impeding orientation, food acquiring and predator avoidance (Sanzo & Hecnar, 2006). In addition, increased salinity resulted in reduced body size, starting at 3‰ for *P. syriacus* and 6‰ for *P. fuscus*, which is expected to have a negative effect on future individual fitness and

survival. Larger tadpoles swim faster and are more difficult to grasp by predators than smaller ones, and when more sizes are available, only small individuals are eaten (Richards & Bull, 1990). Since tadpoles alter their behaviour in response to perceived risk, smaller individuals will have reduced activity in the presence of a larger spectrum of predators (Jara & Perotti, 2010), which in turn can cause lower foraging rate and avoidance of potentially nutritious food resources, slower growth rates, delayed metamorphosis or metamorphosis at a smaller size, all translating in reduced overall fitness and survival (Berven, 1990; Morey & Reznick, 2001; Altwegg & Reyer, 2003; Tarvin et al., 2015).

Salinity tolerance alone may not be enough for spawn survival in coastal areas. Oviposition site selection provides a critical advantage for reproductive success, and previous studies have shown that adult amphibians of several species tend to actively choose their oviposition habitats, by assessing various environmental cues such as: the presence of aquatic predators and/or pesticides (Takahashi, 2007), hydroperiod and/or the presence of conspecifics (Crump, 1991), pH and water depth (Sridhar & Bickford, 2015). However, there are only few studies regarding breeding site selection in response to salinity in amphibians (Viertel, 1999; Haramura, 2008) and broad generalizations cannot be made yet (see review by Hopkins & Brodie, 2015). For example, Haramura (2008) showed that females *Buergeria japonica* avoided depositing their spawn in water with salinity greater than 1‰, but the oviposition site may have been chosen based on additional cues, other than salinity concentration (see also Haramura, 2011). Oviposition site selection was not assessed yet in relation with salinity in any of the *Pelobates* species. However, it may not be necessarily an advantage in unpredictable environments, where the salinity of the breeding habitats can fluctuate within short periods of time.

The importance of salinity to amphibian conservation is even greater under the predicted climate change scenarios, with higher levels of disturbance of the hydrological cycle and changes in the water chemistry expected in the future. In this context, amphibian conservation in coastal lowlands will face additional challenges due to extreme weather events such as severe drought, floods, saltwater intrusion and habitat loss caused by sea level rise (Nicholls et al., 1999;

Craft et al., 2008; Erwin, 2009; Junk et al., 2013; Osland et al., 2016). A recent study (Oliveira et al., 2016) showed that sea level rise has the potential, alone or in synergy with other climate-change processes, to impact significantly the amphibian communities inhabiting lowland coastal areas, resulting in species range shifts, including the spread of invasive species and pathogens, habitat fragmentation, habitat loss, population declines and ultimately, species losses. The Black Sea coastal lowlands are no exception, since sea level rise correlated to global climate changes has been documented for this region (Stanev & Peneva, 2001; Tsimplis et al., 2004). In the light of our results and predicted scenarios regarding the contraction range of *P. fuscus* (Araújo et al., 2006), we consider that conservation efforts should focus on populations inhabiting the southern edge of its distribution range, along the Black Sea coast. Our results confirm that salinity is a limiting factor for amphibians' distribution, and increases in salinity levels (whether natural or anthropogenic) will result in loss of habitat, range contraction and changes in amphibian communities. The methodology used is simple and allows comparisons between a wide range of species that differ in their larval development time and, by focusing on the most sensitive and vulnerable stage in the complex life-cycle of amphibians, it provides good estimates on the impact of increased salinity on their survival.

Acknowledgements The Romanian National Authority for Scientific Research CNCS–UEFISCDI supported our research through Grant PN-II-ID-PCE-2011-3-0173. We are grateful to Danube Delta Biosphere Reserve Administration and the Ethics Committee from Ovidius University of Constanta for providing all the necessary permits and supporting our research in the study area. We thank our colleagues Elena Buhaciuc, Iosif Ruben and Monica Bogdan for help during fieldwork. We are grateful to our reviewers for their constructive comments and recommendations.

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Published by the British
Herpetological Society

Life history traits of a Neotropical microhylid (*Dermatonotus muelleri*, Boettger 1885) from the Arid Chaco, Argentina

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We explored age-related parameters and growth patterns in a population of *Dermatonotus muelleri* inhabiting the Arid Chaco, at the southern limit of the species' distribution range using skeletochronology. In addition, we studied sexual size dimorphism and female reproductive investment. Males and females attained sexual maturity at a similar age (2 years) and both had a low reproductive lifespan (3 years). Females were significantly larger than males and had a higher reproductive investment compared to other anurans. The growth rate coefficient (k) was similar in males (1.14) and females (1.07), indicating that the sexual size dimorphism in this species is caused by differentiated growth before sexual maturity. Overall, we suggest that the short reproductive lifespan justifies the high reproductive investment in the studied population.

Key words: age, explosive breeder, growth, reproductive investment, sexual size dimorphism

INTRODUCTION

Amphibians are suffering a severe worldwide decline, with nearly one-third (32.5%) of the species threatened (Stuart et al., 2004), mostly due to habitat loss and fragmentation (Gibbons et al., 2000; Cushman, 2006; Gardner et al., 2007). Almost half of all known amphibian species occur in the Neotropical realm, of which 38% are considered to be globally threatened (Bolaños et al., 2008). While the Neotropics is considered the global epicentre of catastrophic decline for amphibians, there is still a high rate of species descriptions from this area, which tends only to add new species to those already under severe decline. Sound conservation measures in this region are hampered by the limited knowledge on the life history and habitat requirements of the species of concern.

Demographic life-history traits are essential for understanding population dynamics and plasticity in response to environmental variability (Caswell, 1983). Age-related parameters (e.g., age at first reproduction, reproductive lifespan), fecundity and reproductive effort are some of the major life-history traits tackled by the life history theory (Stearns, 2000) and they are important components of anuran reproductive strategies (Duellman & Trueb, 1994). Skeletochronology, along with 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). Age is estimated from bone crosscuts, based on the presence of lines of arrested growth (LAGs), cyclic and annular bone growth mainly caused by the seasonal variations in temperature and/or rainfall (Smirina, 1994; Sinsch et al., 2007). Although most of the skeletochronological studies focused on amphibians from temperate areas, there is an increasing interest towards amphibians from tropical and subtropical regions, where growth is subjected to periodicity through wet/dry seasons (e.g., Guarino, et al., 1998; Khonsue et al., 2000; Kumbar & Pancharatna, 2001; Kumbar et al., 2002; Lai et al., 2005; Lindquist et al., 2012; Cajade et al., 2013). Furthermore, there is evidence that well-expressed growth marks may occur in tropical and subtropical amphibians in which growth is not constrained by environmental conditions, supporting the hypothesis that LAG formation is ultimately caused by a general genetic control (e.g., Castanet et al., 1993; Marangoni et al., 2009; 2012).

The Great Chaco ecoregion, the second largest in South America after Amazonia, includes the largest seasonally dry forests on the continent (Bucher, 1982). It is mainly a wooded region, strongly affected by extensive livestock raising, extractive forestry and poorly planned agricultural expansion (Nature Conservancy et al., 2005; Nori et al., 2013). Deforestation rates in this region are among the highest in the world (Hoyos, 2013), and Chaco is considered one of the highest diversity regions for herpetofauna in Argentina (Vaira et al., 2012).

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Dermatonotus muelleri is a neotropical microhylid widely distributed in central and southern Chaco, from Brazil (Maranhão to São Paulo) through south-eastern Bolivia, north-western Paraguay and northern Argentina, with natural populations threatened by habitat destruction and pet trade in Argentina and Paraguay (Colli et al., 2004). It is a nocturnal anuran, highly specialised to burrowing and preying on termites; it aestivates in subterranean chambers and emerges during the wet season, to feed and reproduce (Nomura et al., 2009; Nomura & Rossa-Feres, 2011). Although breeding is explosive and several reproductive events may occur during the wet season, females mate only once, while males are polygynous (Nomura, 2003). Larval development is fast and metamorphosis is completed within 22–26 days from egg deposition (Fabrezi et al., 2012).

The aim of our study was to explore life history traits of *D. muelleri*. To the best of our knowledge, the present study is the first to investigate age and growth traits in this species (see however Marangoni et al., 2009). We determined (i) age structure parameters, (ii) growth patterns, (iii) sexual size dimorphism and (iv) female reproductive investment in a population at the southern limit of the species' distribution range.

MATERIALS AND METHODS

Study area and sampling

The study area is located within the dry forests (Arid Chaco) of the western Great Chaco ecoregion, between the localities Fuerte Esperanza and Misión Nueva Pompeya (Chaco Province), northern Argentina. Natural habitats are represented by xerophytic forests of *Schinopsis quebracho-colorado*, associated with *Aspidosperma quebracho-blanco* and *Prosopis* spp., alternating with open grasslands and cacti, including tree-cacti (Cabrera & Willink, 1980). The climate is characterised by extreme seasonal temperature variations, ranging from 40°C in summer to occasional winter frost; annual mean temperatures range from 24 to 25.5°C. The precipitation regime is strongly seasonal, with over 80% of the rainfall concentrated between October and March, while the driest months are July and August (Bucher, 1980; Savaria-Toledo, 1993).

We performed nocturnal field surveys on a 10 km dirt-road segment (Ruta Provincial no. 61: 24°56'27.99"S, 61°29'26.69"W; 25° 1'41.53"S, 61°31'25.55"W; 150 m.a.s.l), between 6–8 December 2013, following a heavy storm. In addition to *D. muelleri*, we recorded the presence of the following 21 species in the study area: Bufonidae (1 sp.) - *Rhinella schneideri*; Ceratophryidae (4 sp.) - *Ceratophrys cranwelli*, *Chacophrys pierottii*, *Lepidobatrachus laevis*, *L. llanensis*; Hylidae (9 sp.) - *Dendropsophus nanus*, *D. sanborni*, *Hypsiboas raniceps*, *Phyllomedusa sauvagii*, *Pseudis platensis*, *Scinax acuminatus*, *S. fuscovarius*, *S. nasicus*, *Trachycephalus typhonius*; Leiuperidae (1 sp.) - *Physalaemus biligonigerus*; Leptodactylidae (5 sp.) - *Leptodactylus bufonius*, *L. fuscus*, *L. laticeps*, *L. latinasus*, *L. mystacinus*; Microhylidae (1 sp.) - *Elachistocleis bicolor*.

We sampled 43 adult individuals (25 males, 18 females) of *D. muelleri* from temporary ponds along the road, during one explosive reproductive event. We determined sex based on the presence of the nuptial pads and dark vocal sac in males, or egg masses that could be visualised through the skin of females. We measured snout-vent length (SVL) and head-width (HW) with digital callipers at 0.1 mm precision and body mass (BM) with a portable electronic balance at 0.01 g precision. We clipped and stored the longest toe of the right forearm in 70% alcohol for age assessment. Ten females and 11 males were preserved for genetics and morphological studies and deposited in the herpetological Collection of Instituto de Biología Subtropical (National Research Council CONICET and Universidad Nacional de Misiones), Posadas, Misiones province, under acronyms LGE 7688–7708. The remaining 22 individuals were released at the capture site. The sampling procedures complied with all relevant regulations and the necessary permits were obtained.

Skeletochronology

We followed the skeletochronological method of Castanet & Smirina (1990) with minor modifications. We decalcified the penultimate phalanges for 8 hours in 5% nitric acid, washed and kept them in distilled water overnight. We cut 14 µm thick cross-sections using a Tehsys 3000CR cryotome, stained them for 20–30 minutes in Ehrlich's haematoxylin and then washed with distilled water for 1 hour. 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 v. 2.3 software). Three independent observers (FS, FM and DC) counted the lines of arrested growth (LAGs) in 2–3 sections per individual.

Reproductive investment

We determined the ovarian mass (OM) as the difference between the body mass before and after ovary removal, in nine of the preserved females (acronyms LGE 7689, 7691–7693, 7696–699, 7705). The ovarian complement (OC) represents the total number of mature ova from each gravid female and is considered a measure of their fertility or reproductive potential (Crump, 1974; Basso, 1990). We removed and weighed approximately 10% of each ovary and counted the mature ova under a Nikon C-DS magnifying glass. Mature ova had well-defined black and yellow poles and pronounced larger size, consistent with the post-vitellogenesis class (Crump, 1974). We photographed a random sample of about 200 ova from each ovary with a digital Nikon Coolpix S10 camera, mounted on a Nikon C-DS magnifying glass. We measured the longest and shortest perpendicular axes of 100 ova per sample to the nearest 0.01 mm using Image-Pro Plus v. 1.1 (Media Cybernetics, 1993–94). We determined mature ovum size (OS) by square rooting the product of the two axis measurements.

Data analysis

All statistical analyses were performed in R environment, v. 3.0.3 (R Core Team, 2014). We used the following packages: stats (R Core Team, 2014), car (Fox & Weisberg, 2011), psych (Revelle, 2014), FSA (Ogle, 2014) and nlstools (Baty et al., 2014). We tested all data for normality and homoscedasticity using Shapiro-Wilk and Levene tests and chose the statistic tests accordingly.

Age-related parameters

We computed the following age-related parameters: 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). Since growth rate decreases after reaching sexual maturity, it was possible to determine the age of maturation by observing this pattern in a given individual (Smirina, 1994).

We used a two-sample Kolmogorov-Smirnov and Mann-Whitney test to check for differences in the shape of age distribution and median age between males and females. We used Spearman correlations to analyse the associations between age and size parameters.

Growth patterns

We computed von Bertalanffy growth model (von Bertalanffy, 1938) following Beverton & Holt (1957): $SVL_t = SVL_{max} \times (1 - e^{-k \times (t - t_0)})$, 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.

We used measurements of SVL at metamorphosis and from 1-year juveniles (19.11 and 52.84 mm respectively) provided by Marangoni et al. (2009). We fitted a von Bertalanffy growth model and estimated growth parameters (VBGP) by nonlinear least squares regression. Two estimated VBGP were considered significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap.

Sexual size dimorphism

We checked for significant differences in size parameters (i.e. SVL, BM, HW) between sexes using Student's t -test and alternatively, Welch's t -test. We used Pearson product-moment correlation coefficient adjusted for small sample sizes (r_{adj}) to analyse the associations between these parameters.

We computed a sexual dimorphism index (SDI) with the results arbitrarily defined as positive when females are larger than males and negative in the converse situation (Lovich & Gibbons, 1992):

$$SDI = \frac{\text{mean size}_{\text{larger sex}}}{\text{mean size}_{\text{smaller sex}}}$$

Female reproductive investment

We estimated the ovarian size factor (OSF) which correlates the number and size of mature ova to body length, following Duellman & Crump (1974): $OSF = (OC \times OS) / SVL$. Finally, we estimated the reproductive effort (RE) following Prado & Haddad (2000): $RE = (OM / BM) \times 100$. We used Spearman (ρ) and Pearson product-moment correlation coefficient adjusted for small sample sizes (r_{adj}) to analyse the associations between size and reproductive parameters.

RESULTS

Age-related parameters

We were able to estimate age in all individuals and they all showed various degrees of endosteal resorption and double LAGs (Fig. 1). We were able to infer age at sexual maturity from the periosteal growth pattern in 61% of the females at 2 years, and in 44% and 12% of the males at 2 and 3 years respectively. The youngest age class estimated in our sample was 2 years in females (6%) and 3 years in males (60%). We thus considered that both sexes attain sexual maturity at the same age: 2 years. Mean lifespan did not differ significantly between males (mean±SD=3.48±0.65 years) and females (mean±SD=3.39±0.69 years) (Mann-Whitney $U=213$, $p=0.75$). Longevity and the potential reproductive lifespan (i.e. considering the estimated age at sexual maturity of 2 years) were 5 and 3 years respectively, in both sexes. There were no significant differences in the shape of age distribution between sexes (Kolmogorov-Smirnov $Z=0.180$, $p=1$) (Fig. 2). 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. Age was not significantly correlated to size (i.e. SVL, BM, HW) in males or females.

Growth patterns

The relation between age and SVL fitted von Bertalanffy's growth model in both sexes (Fig. 3). The asymptotic average snout-vent length was significantly higher in females, while the growth rate coefficient was similar in both sexes (Table 1).

Sexual size dimorphism

Females were significantly larger than males in all three parameters measured: SVL Student's t -test: $t_{41}=5.549$, $p<0.001$; BM Welch's t -test: $t_{23.4}=11.390$, $p<0.001$; HW Student's t -test: $t_{41}=3.916$, $p<0.001$. The sexual dimorphism index was 1.08 for SVL, 1.07 for HW, 1.60 for BM. The sexual dimorphism index was much higher for BM since we used data from females captured

Table 1. von Bertalanffy growth pattern statistics in *D. muelleri* males (n=25) and females (n=18).

Sex	Parameter	Estimate	SD	CI 95%
Males	SVL _{max}	71.37	5.25	69.62–73.83
	k	1.14	0.98	0.83–1.60
Females	SVL _{max}	77.64	7.68	74.69–82.01
	k	1.07	0.96	0.73–1.60

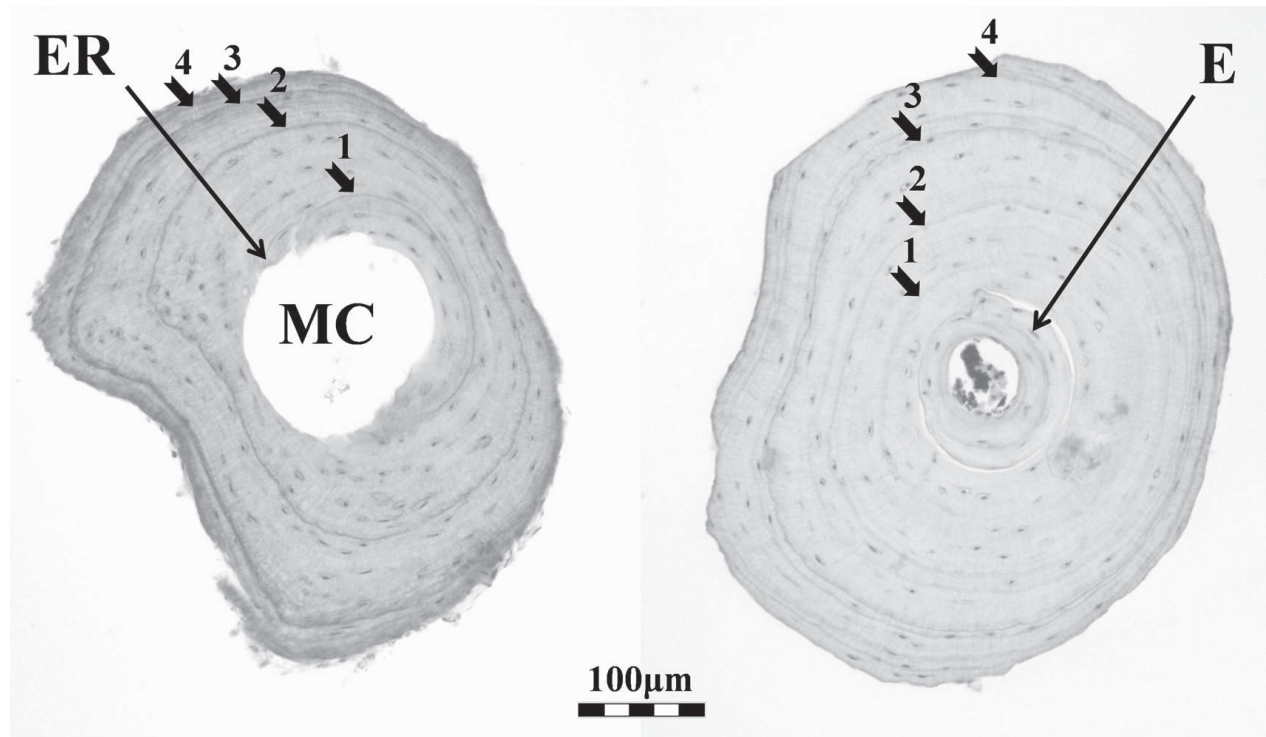


Fig. 1. Phalanx cross-sections in *D. muelleri*: 4-year old male (left) and female (right). LAGs are indicated by black arrows and numbers. MC - marrow cavity, ER - endosteal resorption, E - endosteum.

Table 2. Descriptive statistics of size measurements in *D. muelleri* males ($n=25$) and females ($n=18$): body mass (BM), snout-vent length (SVL) and head width (HW).

Sex	Parameter	Mean	SD	Min	Max
Males	BM (g)	32.55	3.41	25.94	41.79
	SVL (mm)	70.20	2.92	64.61	74.96
	HW (mm)	14.49	0.67	13.47	16.03
Females	BM (g)	52.06	6.67	38.91	62.79
	SVL (mm)	75.86	3.78	66.08	81.94
	HW (mm)	15.41	0.86	13.81	17.35

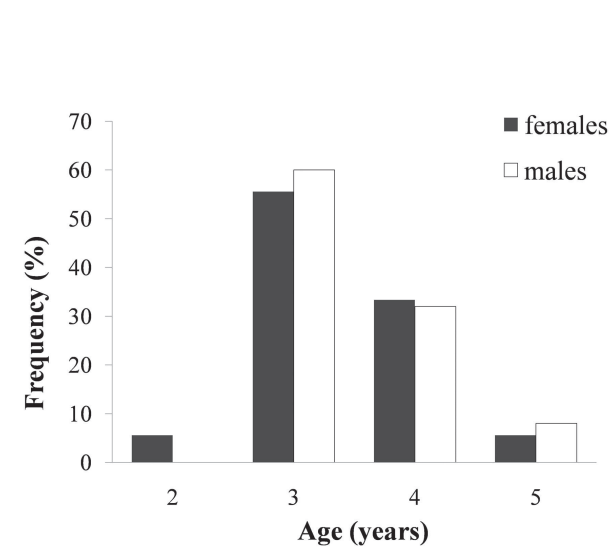


Fig. 2. Age structure of the studied *D. muelleri* population.

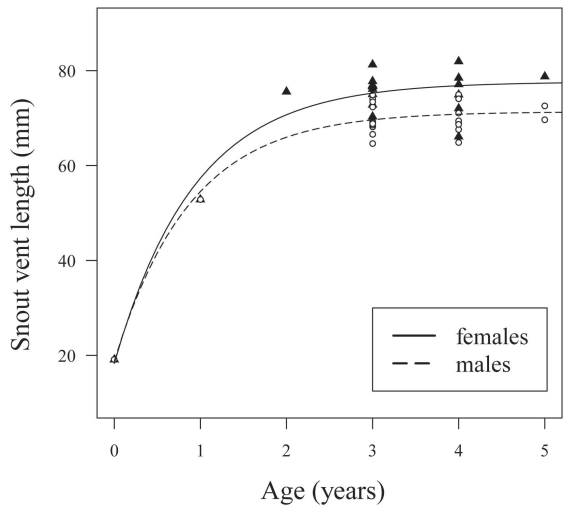


Fig. 3. Growth described by von Bertalanffy's model in *D. muelleri* males (white circles) and females (black triangles).

just before oviposition. Descriptive statistics of body size parameters are summarized in Table 2. We found significant associations between SVL and BM ($r_{adj}=0.508$, $p<0.01$), SVL and HW ($r_{adj}=0.570$, $p<0.01$), and BM and HW ($r_{adj}=0.250$, $p<0.05$) in females. We found significant associations only between SVL and BM ($r_{adj}=0.138$, $p<0.05$) in males.

Female reproductive investment

We computed the reproductive parameters in nine of the ten collected females (mean±SD): 15.85±1.94 g (OM), 10611±1784 ova (OC), 1.36±0.06 mm (OS), 218.8±23.5 (OSF) and 30.18±3.18 % (RE). OC was negatively correlated with OS (Spearman $\rho=-0.683$, $p<0.05$), RE was negatively correlated to SVL ($r_{adj}=0.450$, $p<0.05$), while OM was positively correlated to SVL ($r_{adj}=0.492$, $p<0.05$).

DISCUSSION

The studied population of *D. muelleri* from Arid Chaco showed a female-biased SSD, despite similar age-related parameters (i.e. mean lifespan, age at sexual maturity, reproductive lifespan and longevity) and growth rates. Because of the relatively short reproductive lifespan, females invest up to a third of their body mass in reproduction. The age and growth parameters are similar to those found in desert anurans (i.e. early sexual maturity and short lifespan, Esteban et al., 1999; Sullivan & Fernandez, 1999) as adaptive responses to the harsh environmental conditions. However, the estimated

growth parameters should be considered with caution considering the relatively small sample size.

Female-biased SSD is the most common pattern recorded in amphibians and the main proximate determinants proposed to explain this pattern are sexual selection, differences in age related parameters, growth and survival rates (Shine, 1979; Halliday & Verell, 1988; Hemelaar, 1988; Shine, 1990; Monnet & Cherry, 2002; Kupfer, 2007; Hasumi, 2010; Sinsch et al., 2010). Our results suit best the hypothesis that SSD might stem from differences in growth before sexual maturity (Halliday & Verell, 1988; Shine, 1990). Larger females had larger ovarian masses that were positively correlated to body size. Negative correlations were found between the number and the size of mature ova, and between the reproductive effort and body size. The only study providing some information related to the reproductive investment in *D. muelleri* was done by Perotti (1997), who found a similar ovarian complement and a high ovarian size factor in two females from Salta province, Argentina (Table 3). Previous studies suggest that the reproductive effort is correlated to female size both within and between taxa (Crump, 1974; Perotti, 1997; Prado & Haddad, 2005). However, *D. muelleri* females showed a high reproductive effort compared to other species within or even below its size class (Table 3).

The high reproductive investment (i.e. ovarian complement, reproductive effort and ovarian size factor) observed in *D. muelleri* can also be explained in the terms of r-selection (Pianka, 1970), where in a variable

Table 3. Reproductive investment in representatives from four anuran families in the Neotropics: Bufonidae (*Rhinella marina*), Hylidae (*Hypsiboas raniceps*, *Phrynohyas venulosa*, *Pseudis paradoxa*), Microhylidae (*Chiasmocleis bassleri*, *C. mehelyi*, *C. ventrimaculata*, *Dermatonotus muelleri*, *Elachistocleis cf. bicolor*, *Hamptophryne boliviana*) and Leptodactylidae (*Leptodacylus chaquensis*). RE: reproductive effort; OC: ovarian complement expressed as the total number of mature ovarian ova; OSF: the ovarian size factor. Values are expressed as mean±SD. *Minimum and maximum sample sizes used by authors when measuring the variables.

Species (n)	SVL (mm)	RE (%)	OC	OSF	Data source
<i>R. marina</i> (5)	132.6	-	8598	97.26	Crump, 1974
<i>H. raniceps</i> (6–34)*	60.2±4.4	11.9±6.0	1991±533	-	Prado & Haddad, 2005
<i>P. venulosa</i> (1)	83.2	53	10985	101.33	Perotti, 1994
<i>P. venulosa</i> (3–10)*	77.4±6.0	8.2±1.3	3981±271	-	Prado & Haddad, 2005
<i>P. paradoxa</i> (7–17)*	57.5±8.0	5.5±3.1	1834±1	-	Prado & Haddad, 2005
<i>C. bassleri</i> (3)	27.3	-	212	7.77	Crump, 1974
<i>C. mehelyi</i> (3)	23.8±0.6	-	217±33	-	Prado & Haddad, 2005
<i>C. ventrimaculata</i> (1)	22.0	-	210	9.55	Crump, 1974
<i>D. muelleri</i> (2)	74.2	-	10991	109.68	Perotti, 1997
<i>D. muelleri</i> (9)	65.7±4.2	30.18±3.18	10611±1784	219±24	<i>Present study</i>
<i>E. cf. bicolor</i> (3–13)*	26.1±1.5	18.0±4.5	478±279	-	Prado & Haddad, 2005
<i>H. boliviana</i> (2)	42.0	-	1788	42.33	Crump, 1974
<i>L. chaquensis</i> (3)	76.4	20.89	14649	116.21	Perotti, 1994
<i>L. chaquensis</i> (26–50)*	71.3±4.5	16.0±2.9	4936±1720	-	Prado & Haddad, 2005

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. Overall, we suggest 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.

ACKNOWLEDGEMENTS

This research received support from the Ministerio de Ciencia, Tecnología e Innovación Productiva (MINCyT) and Universidad Nacional de Misiones (UNaM) (grant RO/12/06, FM) and from the Romanian National Authority for Scientific Research CNCS-UEFISCDI (grant PN-II-CAPACITĂȚI-Modulul III-Cooperări Bilaterale 732/23.07.2013, DC), and partially (grant PN-II-ID-PCE-2011-3-0173, DC). The collecting permit was granted by Dirección de Fauna y Áreas Naturales Protegidas of the Chaco province. The skeletochronological procedure was approved by the Ethics Committee of the Faculty of Natural and Agricultural Sciences, Ovidius University, Constanța. FM and IR are very grateful for the continuous support of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) IBS-Universidad Nacional de Misiones. We are grateful to R. Cajade for invaluable help during fieldwork. We thank L. Rozyłowicz, R. Băncilă and R. Iosif for their useful comments on earlier versions of the manuscript.

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Accepted: 7 April 2015