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PRIMARY RESEARCH PAPER

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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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*

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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

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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 (Metcalfe & 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 amplectant 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 $\sim 18‰$ 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 $20 \pm 2\ °C$, 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.*
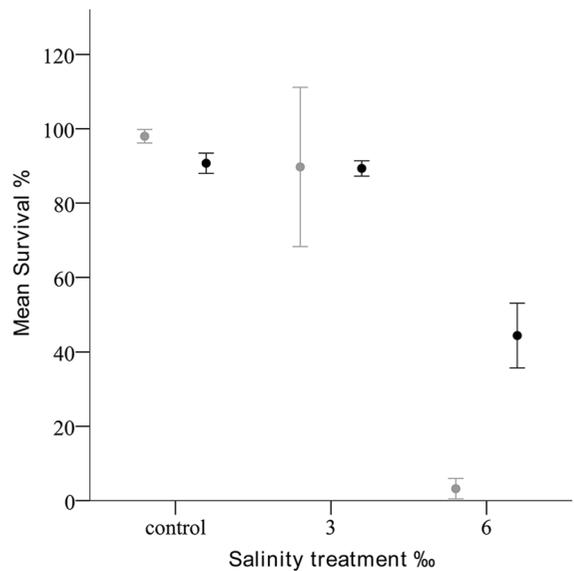
Fig. 2 Mean survival (%) of *P. fuscus* (*grey*) and *P. syriacus* (*black*) to Gosner stage 25, under various salinity levels. *Error bars* represent 95% confidence intervals

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. cultripes* 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)

Sample size is given in parentheses for total length measurements

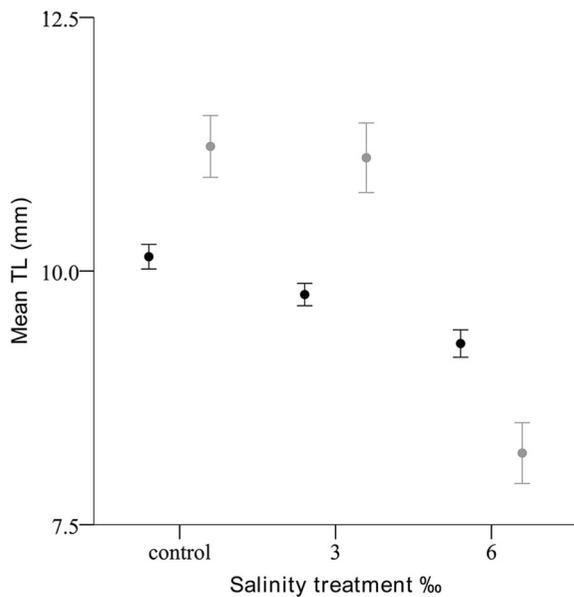| Species	Treatment (‰)	Survival rate (%)	Total length (mm)
P. fuscus	0.5 (control)	97.9 ± 1.5	11.2 ± 1.1 (56)
	3 (moderate)	89.7 ± 17.3	11.1 ± 1.2 (48)
	6 (high)	3.2 ± 2.2	8.2 ± 0.9 (36)
	9 (extreme)	0	–
P. syriacus	0.5 (control)	90.7 ± 4.7	10.1 ± 0.9 (198)
	3 (moderate)	89.3 ± 3.6	9.8 ± 0.9 (245)
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

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.

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