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Dipartimento di Scienze Ecologiche e Biologiche

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Ecologia e Gestione Sostenibile delle Risorse Ambientali – XXXIV ciclo

An integrative approach to the conservation of the endangered Apennine yellow-bellied toad in southern Italy

(BIO/07)

Tesi di dottorato di:

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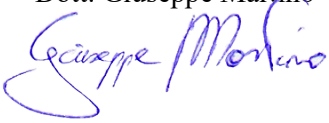

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1. Introduction

1.1 State of the art

Biodiversity is facing with the sixth mass extinction (Barnosky et al., 2011; McCallum 2015; Shivanna 2020), and it is widely acknowledged that amphibians are among the most endangered groups of animals (Beebee and Griffiths 2005; Cushman 2006; Scheele et al., 2019). According to the last IUCN assessment (IUCN 2021), more than one third of all amphibian species are threatened with extinction, while nearly half are experiencing regional or local decline due to habitat reduction, pollution, climate change and emerging diseases, such as the pandemic chytridiomycosis. It has been recently estimated that amphibians are the group of vertebrates whose species on the brink of extinction have the largest number of populations consisting of fewer than 250 individuals (see Fig. 1; Ceballos et al., 2020), with an estimated extinction rate overcoming the extinction rate of all the other vertebrates (Stuart et al. 2004, McCallum 2007). Such a sever loss of biodiversity claims for concrete and effective conservation actions (Crandall et al., 2000; Moritz 2002; Hendry et al., 2010; Eizaguirre and Baltazar-Soares 2014 and references therein).

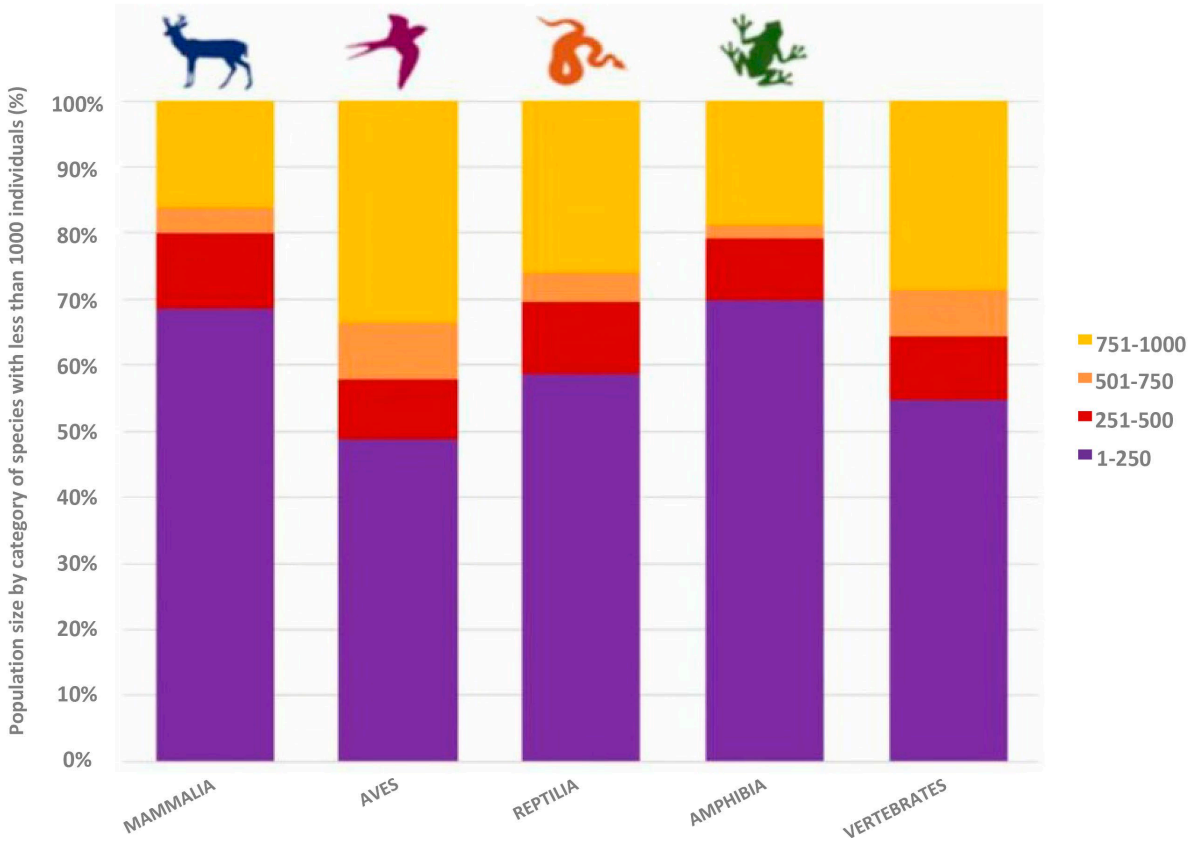

Figure 1. Population size of terrestrial vertebrate species on the brink (i.e., with under 1,000 individuals). Most of these species are especially close to extinction because they consist of fewer than 250 individuals. In most cases, those few individuals are scattered through several small populations (Ceballos et al., 2020).

Traditionally, conservation policies have been addressed to the preservation of endangered habitat or to the protection of species diversity, with special reference to declining species. Yet, conservation exercises have rarely accounted for the differential contribution of single populations (i.e. a group of organisms of one species that interbreed and live in the same place at the same time) to the species conservation (Mace and Purvis, 2008; Laikre, 2010; Mimura et al., 2017). Indeed, being populations the unit of evolution, distinct populations have usually differentiated gene pools and different levels of genetic diversity, which are intimately linked to the evolutionary potential of population to face with environmental changes (Frankham, 2010). Accordingly, population with higher levels of genetic diversity are less likely to be affected by the detrimental consequences of habitat reduction/fragmentation and population decline, like inbreeding depression and genetic drift. Furthermore, high genetic diversity is more likely to warrant adaptation to rapid environmental changes, like climate changes and/or disease outbreaks (Frankham, 2010; Allendorf et al., 2012; Lanfear, et al., 2014). Thus, the identification of the populations with higher levels of genetic diversity should represent a conservation priority, as it might provide species with better chances to survive in the face of extinction threats. Nevertheless, in spite of its extreme value and the increased genetic data availability, population genetic diversity has been largely ignored in the context of biodiversity management strategies, highlighting a worrying gap between conservation biology research and conservation biology policies (Pérez-Espona and ConGRESS Consortium, 2017; Vernesi et al., 2008).

Another aspect that has been to date little or not considered in conservation policies is the contribution of phenotypic diversity and plasticity to the conservation of populations. The role of plasticity in phenotypic adaptation to rapid environmental changes has been recently attentioned by evolutionary and ecological research (Fox et al., 2019). In fact, the process of adaptation to novel environmental conditions can also occur via the expression of phenotypic plasticity, i.e. the ability of one genotype to express varying phenotypes when exposed to different environmental conditions (Chevin et al., 2010a,b). By acting at the level of the individual, plasticity is a rapid-response mechanism that enable organisms to adapt and survive in a rapidly changing world (but see Oostra et al., 2018). Therefore, the identification of inter-individual variation within populations in suites of phenotypic traits of adaptive value, like performance traits (i.e. the ability to found out an ecologically relevant task), stress tolerance, anti-predatory behaviour can play a role in identifying populations for successful conservation programs. Yet, integrative approaches accounting for both genetic and phenotypic diversity in a conservation framework are scarce – if not missing at all.

The Apennine yellow-bellied toad *Bombina pachypus* (Bonaparte, 1838) is an appropriate biological system to test integrative approaches in a conservation framework. *B. pachypus* is an

endemic species for the Italian peninsula showing strong population decline through the most of its range. The causes of decline have been identified in a series of contributing factors including climate change, habitat loss, and chytridiomycosis outbreaks, caused by the Chytrid fungus *Batrachochytrium dendrobatidis* (Andreone et al. 2009; Barbieri et al. 2004; Stagni et al., 2004; Mirabile et al., 2009; Canestrelli et al., 2013). Interestingly, the population decline is apparently not affecting the populations inhabiting the southernmost portion of its range, i.e. the Aspromonte region. The Aspromonte region is a mountain region which acted as Pleistocene glacial refugium for temperate species and has been identified as hotspot of intraspecific genetic diversity for many species, including *B. pachypus* (Canestrelli et al., 2006a). Preliminary investigations linked the high level of genetic diversity of *B. pachypus* populations from this area with a higher resistance to the chytridiomycosis outbreak (Canestrelli et al., 2013). However, a comprehensive framework accounting for environmental/climatic factors and the genetic and phenotypic features of *B. pachypus* populations is still missing.

1.1 Aims of the research

The aim of this research is to investigate the characteristics and the conservation status of the *B. pachypus* populations from the Aspromonte region, in order to understand factors and processes explaining the resistance of this population to the decline. To achieve this goal we developed an integrative approach accounting for environmental/climatic factors and both genetic and phenotypic features of *B. pachypus* populations. In particular, this research aimed to answer the following questions:

1. What is the current distribution and conservation status of *B. pachypus* populations within the Aspromonte region?
2. What is the occurrence pattern of the pathogen *B. dendrobatidis* among *B. pachypus* populations?
3. What is the fine-scale geographic structure of genetic variation of *B. pachypus* populations in this area, acknowledged as its genetic diversity hotspot?
4. Is there inter-individual variation in adaptive phenotypic traits?

To answer these questions, we (i) surveyed the whole region to assess whether occurrence pattern of populations has remained stable over time, in the whole area or in parts of it; (ii) assessed the genetic structure of populations, quantified their levels of genetic diversity, and compared them with those of populations sampled in the same area in the past; (iii) performed an analysis of current potential risk factors by focusing on the present-day occurrence of the chytrid pathogen *B. dendrobatidis*, as well as by estimating variation in bioclimatic suitability of the

hotspot area, from current to future climatic scenarios; (iv) explored the pattern of inter-individual variation in a phenotypic trait of indisputable adaptive value, the deimatic display kown as *unken-reflex,* performed by this species under predator attack. This trait, to date considered a stereotyped response to predator can be treated as a proxy of the way these toads cope with environmental challenges. Results from this study provide novel insights into the ecology, evolution and conservation of *B. pachypus* populations.

This study was developed in three distinct, but intimately linked, experimental phases. The first phase of the study, described in Chapter 2, consisted in characterizing the geographic structure of genetic variation of *B. pachypus* populations from the Calabrian mountain system, in order to define the boundaries of the Aspromonte population. Thanks to the results from this study we were also able to define the proper genetic diversty hotspot of *B. pachypus* in the Aspromonte region. Furthermore, during this phase I collected evidences on the demographic trend and epidemiological status of the Aspromonte population. In the second phase of the study, described in Chapter 3, I performed an intensive field survey to map the fine scale distribution of *B. pachypus*, in order to define the effective conservation status of this species within its hotspot of biodiversity. During this stage, I brought together species occurrences from a long-term survey started before my PhD. In this frame, we collected occurrence data also for three other endemic amphibian species which are showing local or regional decline within the Italian peninsula. These data provided a comparative framework to evaluate the conservation status of the Amphibian fauna in the Aspromonte region. Finally, the third phase described in Chapters 4 and 5, was dedicated to the study of the interindividual variation in a phenotypic trait of high adaptive value: the anti-predatory deimatic behaviour named *unken-reflex*, which consists in suddenly showing aposematic ventral coloration after the predator attack is perceived. Results from these studies are thus discussed in the frames of conservation and ecological research.

1.2 The study species *Bombina pachypus*

The Appennine yellow-bellied toad, *Bombina pachypus* (Bonbaparte, 1838), is a small amphibian belonging to the family Bombinatoridae (Anura). It is endemic to the Italian peninsula, where it shows a fragmented distribution from the Ligurian Apennine to the Aspromonte massif (Caputo et al., 1993; Mangini et al., 2002; Lanza et al., 2007) (Fig. 2). Although its distribution spans from the sea level up to 1800 m a.s.l. (Mazzotti et al., 1999; Sperone et al., 2006), this species prefers hilly and sub-mountain humid habitats. *B. pachypus* is a heliophilous and moderately thermophilic amphibian, breeding in small and shallow humid habitat, such as seasonal pools, marshes, ponds, springs, and lateral ponds of small rivers and

streams (Fig. 3). This species is also used to colonize artificial water collections, such as drinking troughs for animals, irrigation tanks and troughs (Canessa et al., 2013). It prefers temporary water bodies that have limited canopy coverage and high insolation, allowing for high water temperatures and rapid larval development (Hartel et al., 2007). *B. pachypus* has diurnal activity, and is active from March to October, depending on latitude and altitude (Guarino et al., 1998; Sperone et al., 2006). The reproductive activity begins in spring and lasts throughout the summer; wintering starts from late autumn on ground, usually under stones and leaves at short distance from the breeding site (Di Cerbo & Ferri, 2000).

B. pachypus is protected by the European Union, being mentioned in Appendix II of the Bern Convention and in Annexes II and IV of the Habitat Directive (92/43/EEC). It is also classified as Endangered species (EN) by the IUCN Red List (Andreone et al., 2009) and the Red List of Italian Vertebrates (Rondinini et al., 2013).

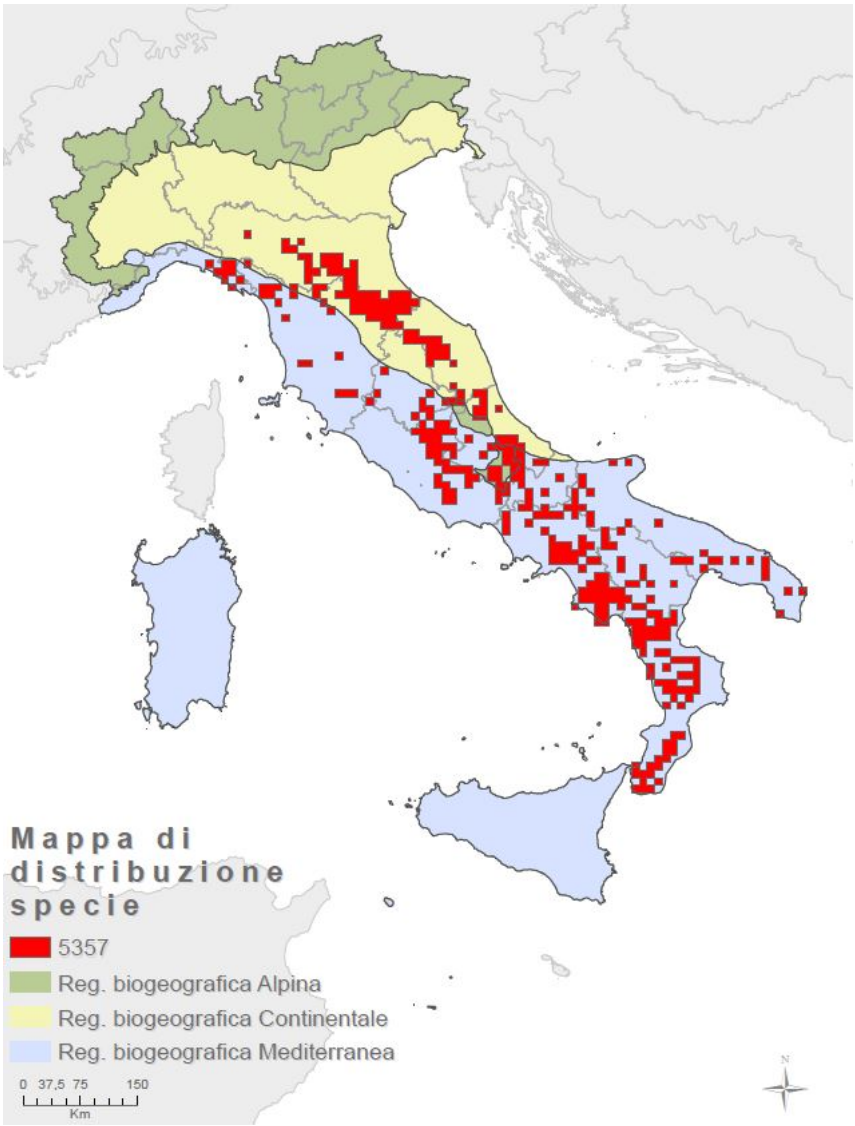

Figure 2. Distribution map of *Bombina pachypus*. Image from the 4th National Report ex. art 17 Habitat Directive (92/43/CEE) period 2013-2018.

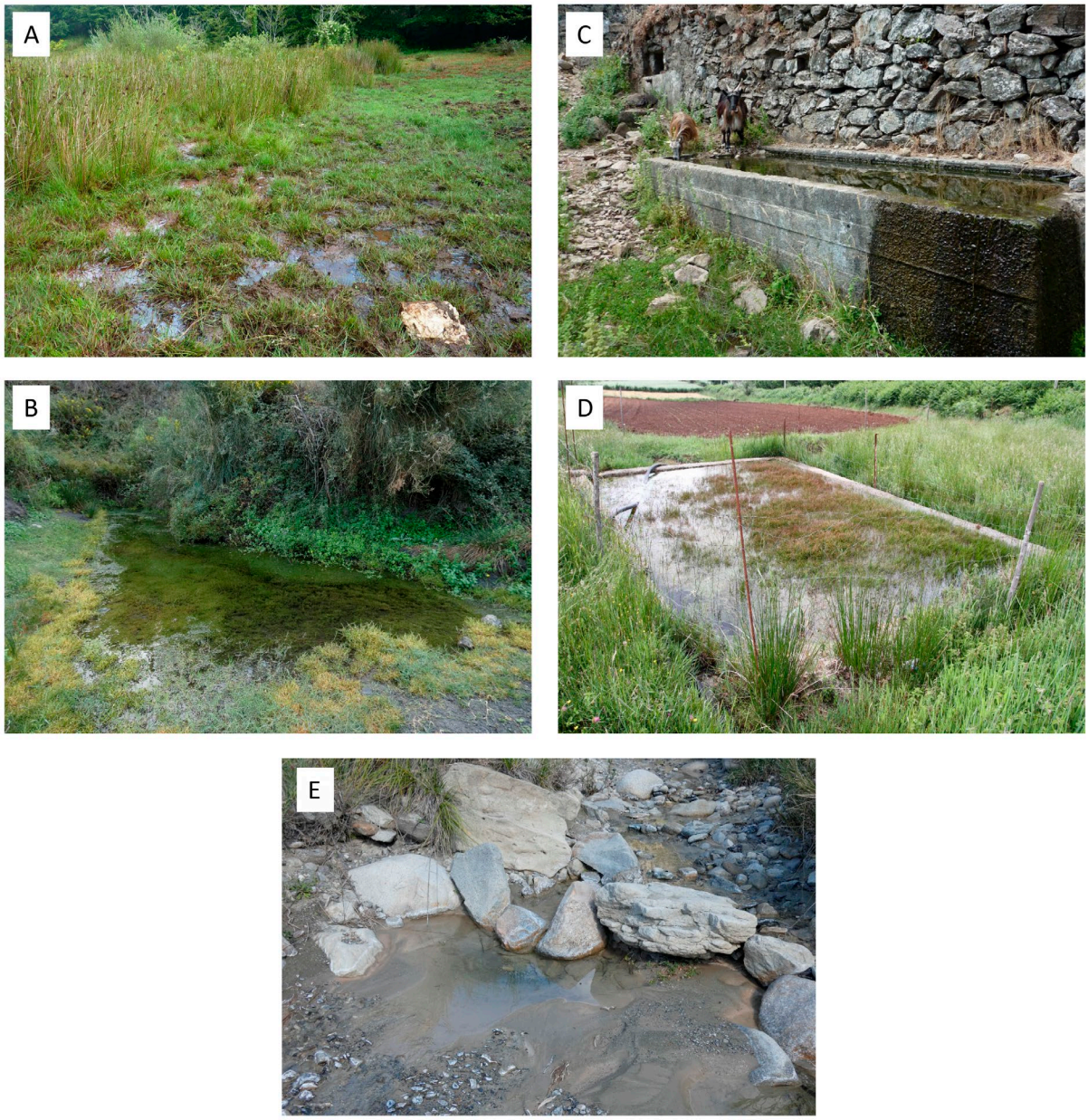

Figure 3. The main habitats of the *Bombina pachypus*: **A** - Bog; **B** - Spring with poddle; **C -** Drinking troughs; **D -** Irrigated tanks; **E -** lateral ponds of small streams.

2. Drilling down hotspots of intraspecific diversity to bring them into on-ground conservation of threatened species.

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Abstract

Unprecedented rates of biodiversity loss raise the urgency for preserving species ability to cope with ongoing global changes. An approach in this direction is to target intra-specific hotspots of genetic diversity as conservation priorities. However, these hotspots are often identified by sampling at a spatial resolution too coarse to be useful in practical management of threatened species, hindering the long-appealed dialog between conservation stakeholders and conservation genetic researchers. Here, we investigated the spatial and temporal variation in species presence, genetic diversity, as well as potential risk factors, within a previously identified hotspot of genetic diversity for the endangered Apennine yellow bellied toad *Bombina pachypus*. Our results show that this hotspot is neither a geographically homogeneous nor a temporally stable unit. Over a time-window spanning 10-40 years since previous assessments, *B. pachypus* populations declined in large portions of its hotspot, and their genetic diversity levels decreased. Considering the demographic trend, genetic and epidemiological data, and models of current and future climatic suitability, populations at the extreme south of the hotspot area still qualify for urgent in-situ conservation actions, whereas northern populations would be better managed through a mix of in-situ and ex-situ actions. Our results emphasize that identifying hotspot of genetic diversity, albeit an essential step, does not suffice to warrant on-ground conservation of threatened species. Hotspots should be analysed at finer geographic and temporal scales, to provide conservation stakeholders with key knowledge to best define conservation priorities, and to optimize resource allocation to alternative management practices.

Keywords: Evolutionary potential, amphibian declines, genetic diversity hotspots, management strategies, *Bombina pachypus*, southern Italy, *Batrachochytrium dendrobatidis*, species distribution models.

2.1 Introduction

Unprecedented rates of biodiversity loss raise the urgency for preserving species ability to cope with ongoing global changes (Crandall et al., 2000; Moritz, 2002; Hendry et al., 2010; Eizaguirre and Baltazar-Soares, 2014 and references therein). However, conservation policies have traditionally been oriented towards the protection of species diversity and habitats, rarely considering the evolutionary potential of individual populations within species (Mace and Purvis, 2008; Laikre, 2010; Mimura et al., 2017). A direct approach in this direction, which has been largely debated for decades, is to directly target populations with high levels of genetic diversity (Frankham, 2010). In fact, owing to the intimate links between genetic diversity and effective population size, these populations are, at the same time, less likely to be affected by the detrimental consequences of inbreeding depression and genetic drift, and more likely to warrant evolvability and adaptive potential (Frankham, 2010; Allendorf et al., 2012; Lanfear, et al., 2014).When different populations, all with relatively high levels of genetic diversity, are concentrated in the same area it is possible to define an intra-specific hotspot of genetic diversity (Hewitt, 2000, 2004; Petit et al., 2003; Hampe and Petit, 2005;), clearly representing a conservation priority. Importantly, as pointed out by Pérez-Espona and colleagues (2017), genetic diversity data useful to detect hotspots, are now available for a large amount of taxa, at least within some intensively studied areas. However, in spite of both their extreme value and data availability, hotspots of genetic diversity have been largely ignored in the context of biodiversity management strategies, highlighting an existing gap between conservation geneticists' achievements and conservation stakeholders' priorities (Pérez-Espona and ConGRESS Consortium, 2017; Vernesi et al., 2008).

Traditionally, hotspots have been identified over large areas (e.g., considering the entire range of a species) using the analytical tools and the sampling scheme of phylogeography (Hewitt, 2011), providing therefore a spatial resolution that can hardly be considered in practical conservation exercises (Cañadas and Vázquez, 2014). Consequently, important questions for on-ground management practices, still remain largely unexplored. Can a hotspot be considered as a homogeneous regional unit for management purposes? Are all populations 'created' equal within hotspot areas? Or, in order to achieve optimal resource allocation and maximum chance of success, should populations be prioritized as well? Answering these open questions could have major practical implications for the apportionment of financial budgets among alternative management practices, as well as for the design of protected areas.

Here, we explore the importance of addressing these questions, in a collaborative framework between academic researchers and conservation authorities (governmental, protected area

managers). To this aim, we analysed spatial and temporal patterns of variation in species presence, genetic diversity, as well as potential risk factors, within a previously identified hotspot of genetic diversity for the endangered Apennine yellow bellied toad *Bombina pachypus*. Indeed, substantial previous knowledge available for this species makes it especially suitable to reach the study aim. The Apennine yellow-bellied toad is an amphibian endemic to the Italian peninsula, whose hotspot of genetic diversity has been identified at the southernmost portion of its range (Canestrelli et al., 2006a). Extensive population declines in the last decades have raised severe concern for the conservation status of this species (Barbieri et al., 2004), that is now listed as endangered on the Red List of IUCN (Andreone et al., 2009). Previous studies have suggested the potential conservation value of the hotspot area for this species, showing that: i) *B. pachypus* populations are facing a dramatic demographic decline in the whole range, except for this area (Barbieri et al., 2004); ii) genetic diversity levels in this area are by far higher than in the rest of the range (Canestrelli et al., 2006a); and iii) populations from this area are reported as viable despite the long-term co-occurrence of the 'killer fungus' *Batrachochytrium dendrobatidis* (Canestrelli et al., 2013), considered one of the main drivers of the decline (Stagni et al., 2004).

In this paper, we investigated whether the hotspot area can be considered - and managed - as a single and temporally stable geographic unit, or if substantial variation exists within this area in parameters of key importance for the conservation of *B. pachypus* populations. To achieve this goal, we integrated previous knowledge with the results of three experimental steps. First, we surveyed the whole region to assess whether occurrence pattern of populations has remained stable over time, in the whole area or in parts of it. Second, we assessed the genetic structure of populations, quantified their levels of genetic diversity, and compared them with those of populations sampled in the same area in the past. Third, we performed an analysis of current potential risk factors by focusing on the present-day occurrence of the chytrid pathogen *B. dendrobatidis*, as well as by estimating variation in bioclimatic suitability of the hotspot area, from current to future climatic scenarios.

2.2 Material and Methods

Species presence data

Barbieri and colleagues (2004) indicated southern Italy (Calabria administrative region) as the only area within the distribution range where the yellow bellied toad was not facing demographic declines at the end of 1990s. In order to confirm this finding and/or to characterize spatial and temporal patterns of variation, we carried out extensive field surveys within this region. Field activities were carried out from April to October, corresponding with the reproductive season of the species, from 2013 to 2016. Field activities and sampling procedures were approved by the Italian Ministry of the Environment (protocol numbers: 0042634/PNM, and 0007727/PNM).

We screened peer-reviewed and grey literature, and museum collections for records of presence of the toad in Calabria. We retained only 56 records with detailed information about location and year of observation and we divided the region into 4 areas where most of the records are concentrated: Catena Costiera, Sila, Serre and Aspromonte (Figure 1). The boundaries of these 4 areas were defined arbitrarily, although roughly based on mountain ranges.

Each site of historical presence was visited three times during the whole campaign. We considered the species as currently present in a site when at least one evidence of its presence (either adults, or tadpoles, or clutches) was observed in at least one visit. Field research was also opportunistically extended to other 80 non-reported but potentially suitable sites in the surroundings, in order to search for new records.

Samples for subsequent laboratory procedures were collected from the adult individuals encountered during the last visit at each site. In order to limit the stress associated to handling procedures for the individual toad, each individual was subjected to a single sampling procedure (i.e. either toe-clipping or skin-swabbing; see below).

Genetic diversity and population structure

Population genetic structure and diversity were assessed by genotyping 130 individuals from 22 sites, ranging over the whole region (Table 1 and Figure 1). Individuals from 15 of these sites ("new" samples in Table 1) were sampled by toe-clipping after anaesthetization in a 0.1% solution of MS222 (3-aminobenzoic acid ethyl ester). Toe-clipping is a permanent marking procedure, allowing to avoid re-sampling DNA from the same individual in case of multiple sampling session, as is the case in the present study. In principle, this procedure could also be used to estimate population size, using mark-recapture experimental designs. However, we did not use it for this additional purpose, as multiple captures of the same individual were never

observed during this study. All individuals were immediately released at the collection sites, while tissue samples were stored in 95% alcohol. Tissue samples from the remaining 7 sites ("old" samples in Table 1) were collected during previous studies carried out from 1978 to 2006 (see Canestrelli et al., 2006a, 2013 and references therein), and were stored in 95% alcohol.

Genomic DNA was extracted using the ZR Genomic DNA™ - Tissue MiniPrep kit (Zymoresearch) following manufacturer's instructions. We initially tested 11 microsatellite loci that previously proved to amplify in the sister species *B. variegata*: Bv11.7, Bv32.7, B13, B14, F22, 1A, 5F, 8A, 9H, 10F, 12F (Nürnberger et al., 2003; Stuckas and Tiedemann 2006; Hauswaldt et al., 2007). We excluded from the analyses the loci Bv32.7 (failed the amplification step for our samples), B13 (yielded inconsistent reactions), B14 (yielding missing data in 26% of the individuals), and 12F (monomorphic in preliminary trials based on 30 randomly selected individuals). PCR conditions for the 7 loci analysed in this study, along with fluorescent dyes used to label forward primers and multiplex assembly are shown in Supplementary material. Fragment analysis of PCR products was performed by Macrogen Inc. on an ABI 3730xl Genetic Analyser (Applied Biosystems) with a 400HD size standard.

Allele calling was performed with GeneMapper® 4.1 checking electropherograms by eye. Microsatellite dataset was then analysed with Micro-Checker 2.2.3 (Van Oosterhout et al., 2004) to test for the presence of null alleles, large alleles drop-out or scoring errors. We used Genepop 4.5.1 (Raymond and Rousset, 1995; Rousset, 2008) to test for departures from Hardy-Weinberg equilibrium and for genotypic linkage disequilibrium. Population genetic diversity was estimated as expected heterozigosity (He) and allelic richness (Ar) using Genetix 4.05.2 (Belkhir et al., 1996) and Fstat 2.9.3.2 (Goudet, 2001), respectively. These analyses were carried out excluding sites where we sampled less than four individuals.

Genetic population structure was inferred by means of the Bayesian clustering algorithm implemented in Tess 2.3.1 (Chen et al., 2007), with the admixture model, under the conditional autoregressive model (CAR), and the geographical coordinates of individuals as prior information. Tess was used as clustering method since it has been shown to perform better than similar methods when the number loci is limited and/or when genetic structure is shallow (Chen et al., 2007; François and Durand, 2010). We performed a set of preliminary analyses with 20000 runs, discarding the first 5000 as burn-in, and with 10 replicates for each value of K from 2 to 10 to test model performance. For the final analysis we ran 100 replicates for each value of K from 2 to 6, with 100000 steps and discarding the first 50000 as burn-in. The spatial interaction parameter was set to the default value (0.6) and the option of update this parameter was activated. The clustering model that best fitted the data was inferred by the deviance information criterion (DIC), averaging the DIC values over the 100 replicates for each K and selecting the K

value at which the average DIC reached a plateau. The 10 runs with the lowest DIC values for the inferred K were finally selected, and the estimated admixture proportions were averaged over them using Clumpp 1.1.2 (Jakobsson and Rosenberg, 2007).

The extent of genetic differentiation between the inferred clusters was analysed by estimating pairwise Fst values (Weir & Cockerham, 1984), by means Fstat. Individuals of mixed ancestry were assigned to the cluster with the highest admixture proportion. The statistical significance of the estimated Fst values was assessed through 3000 permutations, and the nominal 5% level of significance was adjusted for multiple comparisons, using the Bonferroni correction.

Pathogen assay

Skin swabs were collected from 105 individuals collected in 15 sites (Figure 1). Genomic DNA was extracted from swabs following the protocol of Boyle and colleagues (2004) as modified by Zampiglia and colleagues (2013). The molecular diagnostic assay was conducted in 25 µl reaction volumes using a nested PCR protocol as developed by Goka and colleagues (2009). The assay was performed once for each sample and it included a positive (DNA extracted from *B. dendrobatidis* zoospores JEL423, kindly provided by Prof. Joyce Longcore) and a negative (DNA-free distilled water) control. PCR products were loaded on a 1% agarose gel and checked for the diagnostic band at approximately 300 bp size to be considered as *B. dendrobatidis* positive. A geographic representative subset of samples (20%) was analysed in duplicate.

Changes in bioclimatic suitability

We calibrated species distribution models (SDM) with a dataset of 361 occurrences covering the entire distribution range of the species and a set of bioclimatic variables. The occurrences were obtained by pooling the data collected during the field campaign (this study) and different data sources: Stoch (2000-2005), Global Biodiversity Information Facility (www.GBIF.org), Observado (www.observation.org) and Canestrelli and colleagues (2006a). The dataset was filtered to remove duplicated records and data georeferenced with uncertainty. To limit spatial autocorrelation, we thinned the raw occurrences using the spThin R package (Aiello-Lammens et al., 2015), obtaining five alternative calibration subsets with 182 occurrence data (see Supplementary material for details). Considering the same data sources available for the presence, we collected all data available for the same study area for species of reptiles and amphibians. We collected a total of 7614 records that we used in model calibration as background points in order to limit the effects of the existing sampling bias (Ranc et al., 2017).

The bioclimatic variables for current climate were obtained from Hijmans and colleagues (2005). Following a variance inflation factor (VIF) analysis on the original 18 bioclimatic variables, we considered in the analyses only the following 7 variables with VIF < 5: annual mean temperature, mean diurnal range, temperature seasonality, mean temperature of wettest quarter, mean temperature of driest quarter, precipitation of wettest month, and precipitation seasonality. For the future climate, we considered the same 7 variables calculated under three general circulation models (CCSM4, MIROC-ESM, MPI-ESM-P) and two emission scenarios (RCP2.6 and RCP8.5) adopted by the IPCC5 (Intergovernmental Panel on Climate Change).

Using an ensemble forecasting approach (Araújo and New, 2007), we calibrated the SDM considering the current climate, the five thinned occurrence sets, the set of background points, and the following five algorithms as implemented in the biomod2 R packages (Thuiller et al., 2009): generalized linear model (GLM); generalized additive model (GAM); generalized boosted models (GBM); multivariate adaptive regression spline (MARS), and maximum entropy (MAXENT).

For model evaluation, we considered again five sets of points, each including the 179 occurrence points excluded through the thinning procedure from the calibration datasets and 7488 random background points (maintaining the same prevalence as in the calibration dataset). For each model, we measured the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Models with AUC value greater than 0.7 (Swets, 1988) were projected under the current and future climate over the entire peninsular Italy. Then, for each projection we obtained a final consensus SDM, calculated as the weighted average of all available models (weights for each model based on the respective AUC score, as in Marmion et al., 2009).

2.3 Results

Species presence in space and time

We found evidence of presence of *B. pachypus* in 28 localities out of the original 136 (21%), including 11 sites of historical presence (out of the original 56) and 17 new records. The highest number of historical sites with confirmed presence was found in the Aspromonte (8 out of 19; 42%), followed by the Serre (2 out of 6; 33%) and the Catena Costiera (1 out of 8; 13%). No presence was confirmed for the Sila (0 presences out of the original 23). The 17 new records of presences were unevenly distributed among regions, with 15 new sites found in the Aspromonte, 2 sites found in the Catena Costiera, and no new site found in Sila and Serre regions. The Aspromonte region was also the area with the highest number of individual observed per site/day, with an average of 21.6 individuals (s.d. 18.3) compared to 3.5 individuals (s.d. 1.9) observed on average in the other regions.

Genetic diversity and population structure

The number of alleles observed at each microsatellite locus was as follows: 1A = 11, 8A = 13, 5F = 12, 10F = 12, 9H = 25, Bv11 = 3 and F22 = 9. All loci were used for downstream analyses, since none of them showed evidence of null alleles in more than one sampling site. Missing data accounted to 3.7% over the whole dataset. No evidence of genotypic linkage disequilibrium between loci was observed, and no departures from the Hardy-Weinberg equilibrium was detected after the Bonferroni correction was applied (Rice, 1989).

Expected heterozigosity ranged from 0.45 (site 18) to 0.78 (site 8), while allelic richness ranged from 2.58 (site 18) to 4.16 (site 11; Figure 1C). Estimates of both the expected heterozigosity and the allelic richness for each sampled site are shown in Table 1. Among the 'old' samples, the highest values of both heterozigosity and allelic richness were observed within Serre region, whereas among the 'new' samples, samples with the highest values of genetic diversity were observed within Aspromonte region. Although the location and size of our samples prevented us from carrying out quantitative temporal comparisons, it is worth noting that 'old' samples systematically yielded higher values of genetic diversity when compared with the nearest 'new' samples from the same area (see Table 1).

Bayesian clustering analysis of population structure revealed the presence of three main population clusters within the study area. Pie-charts displaying the contribution of the three clusters to the genetic pool of each site and bar-plots indicating individual admixture proportions are shown in Figure 1B. The three clusters showed a clear geographic structure along the north-south axis: one was restricted to the north, in Catena Costiera and northern Sila, one had a central

distribution, ranging from southern Sila to Serre, and the third one was restricted to the south, in Aspromonte. Evidence of admixture between clusters was observed in some individuals, particularly from sites located in intermediate areas (sites 5 and 12). The northern cluster was the most genetically differentiated, with pairwise Fst values of 0.11 and 0.18 with the central and the southern clusters, respectively (both $P < 0.01$). Instead, the extent of differentiation was lower between the central and southern the clusters, with a Fst value of 0.05 ($P < 0.01$).

Occurrence of Batrachochytrium dendrobatidis

The diagnostic tests for *B. dendrobatidis* occurrence yielded positive results for the presence of the pathogen on the skin of 3 individuals from 2 sites in the Catena Costiera. Instead, and in contrast to previous assessments (Canestrelli et al., 2013), no positive tests were observed for individuals sampled in the Serre and Aspromonte (Figure 1D). All the analyses carried out in duplicate yielded fully consistent results.

Species distribution modelling

We calibrated 25 models (5 presence-background sets for 5 modelling techniques) with, on average, good model performance (mean AUC = 0.80; s.d. AUC = 0.04). GBM was the model with the highest AUC values (mean AUC = 0.86) and the GLM with the lowest AUC (mean AUC = 0.76). Temperature was by far the most important bioclimatic factor for explaining the distribution of the yellow-bellied toad, with mean temperature of the driest quarter, mean temperature of the wettest quarter, and annual mean temperature being the most important variables, followed by the two precipitation variables (Table 2).

Regardless of the climate scenario considered, the future climate suitability is predicted to decrease substantially (Figure 2), especially in the north and central Apennines, where bioclimatic suitability drops dramatically in some areas. A much higher climatic stability in time is predicted for the southern part of the distribution range, where however the models predict a future range shift towards higher elevations (Figure 2).

2.4 Discussion

Identifying hotspots of intraspecific genetic diversity is now increasingly seen as a key step towards conservation practices effective in the long-term (Thomassen et al., 2011; Brooks et al., 2015). For the first time, however, our results show that this fundamental step does not suffice, and that finer spatial and temporal scales of analysis, which are characteristic of monitoring programs (Brodersen and Seehausen, 2014; Mimura et al., 2017), should be adopted in order to provide conservation stakeholders with important knowledge to best define conservation priorities and management practices.

The hotspot of intraspecific genetic diversity of *B. pachypus* is not a homogeneous geographic unit, either in terms of genetic diversity, or in terms of species presence and risk factors' distribution. Moreover, our results provide evidence of geographically structured temporal changes in the analysed features. In the next sections we will discuss these spatio-temporal patterns of variation, and how they could help to identify priorities in the context of both short-term and long-term conservation programs.

Spatial and temporal patterns of variation

Over less than 15 years since the last assessment of species presence (Barbieri et al., 2004), patterns of *B. pachypus* occurrence in southern Italy have dramatically changed, although with marked differences among sub-regions. At the extreme of these differences are the Sila and the Aspromonte massifs. Throughout the Sila region we completely failed to identify sites of current *B. pachypus* presence, in spite of the particular sampling effort we devoted to this area (41% of the inspected sites of historical presence were located within this area). While this lack of evidence is not a conclusive argument that the species completely disappeared from the area, it clearly testifies to a dramatic decline in the number of sites of occurrence. On the other hand, in the Aspromonte region several sites of historical presence were confirmed, and new ones identified. Together with the higher number of individuals per site observed in this area than elsewhere, the Aspromonte region has clearly appeared as the least affected by population declines. Further indication of diffused declines comes also from the observed temporal decreases in genetic diversity estimates, suggesting widespread bottlenecks in population size throughout the hotspot area in southern Italy (Figure 1C).

These findings rise worrisome questions for the conservation of this endangered toad. Why did *B. pachypus* start to decline even in southern Italy? And why is there a geographic structure in this trend? First of all, our results allow us to exclude some of the most commonly invoked answers to these and similar questions. Habitat degradation is probably not a factor in this trend.

The Aspromonte and Sila regions are largely covered by national parks, while the Serre region falls within a regional park. Also, in most instances, no evidence of alteration of the physical habitat or of the ecological community was observed at the sites visited. Thus, while a role in species decline at single sites cannot be excluded (Barbieri et al., 2004), habitat degradation can hardly explain the general pattern of declines throughout the study area. Likewise, bioclimatic factors are unlikely to explain the pattern, at least as the single factor. Indeed, the Sila massif, where no site of presence was found, does not suffer of a limited bioclimatic suitability, as compared with neighbouring areas, and most historical sites of presence are located exactly within the area of bioclimatic optimum. Finally, not even *B. dendrobatidis* can be reliably invoked as the 'lone-killer' (Pounds and Coloma, 2008 and references therein). As a matter of fact, *B. dendrobatidis* was widespread in the area (including the Aspromonte) at least since the early '80s (Canestrelli et al., 2013), that is, well before the observed declines begun. Nonetheless, its role in the observed declines in conjunction with other factors cannot be excluded. Indeed, contrary to results of the previous assessment, *B. dendrobatidis* occurrence was confirmed in the northern but not in the southern portion of the study area.

These results lead to at least two non-mutually-excluding hypotheses, useful to attempt answering the above questions. First, the overall reduction of the chytrid geographic distribution [and its absence from sites that are very close to those where high chytrid prevalence was reported until 2003] might be the outcome of epidemic dynamics related to environmental changes. In fact, experimental evidence showed chytrid pathogenicity to decrease at increasing temperatures, with possible implications even at microgeographic scales (Greenspan et al., 2017a), as well as increased host vulnerability to climate change driven by infection (Greenspan et al., 2017b). Second, geographic differences in the mechanisms of resistance of the amphibian host might be implicated (Luquet et al., 2012). In this regard, it is worth recalling that geographic patterns of variation in *B. pachypus* presence and chytrid distribution, are congruent with the geographic structure of the genetic variation among *B. pachypus* populations. Indeed, all the most abundant populations, currently free from chytrid infection, belong to the southern cluster confined to the Aspromonte area. Although not conclusive in disentangling these two scenarios, our results set the stage for future experimental efforts along this road, based on testable research hypotheses.

An interesting corollary to the first scenario comes from bioclimatic suitability predicted for future scenarios in the Aspromonte region. Indeed, *B. pachypus* climate optimum in the Aspromonte massif was predicted to shift upward in elevation in the near future, possibly leading to a selective scenario with two contrasting forces acting on populations, *B. pachypus* being on the horns of a dilemma. Sites of current presence will likely lose their bioclimatic suitability in

the near future, but in a direction predicted to favour the toad host over the chytrid pathogenicity (i.e. increasing environmental temperatures). On the other hand, colonizing sites at higher elevation might allow *B. pachypus* to continue matching its bioclimatic optimum, but at the expense of more favourable climatic conditions for its chytrid pathogen. Intriguingly, although speculative for the time being, this scenario might be experimentally evaluated through monitoring environmental variables, epidemiological parameters, and *B. pachypus* performance traits, among populations from the Aspromonte area. Since this is also the only area where the species appeared relatively abundant, such a monitoring and research effort might be one of the last options available to shed light on the mechanisms behind its decline.

Geographic structure of conservation targets

A thorough understanding of mechanisms underlying species' decline is an essential step towards better informed conservation efforts, but the observed spatial patterns and temporal variations clearly call for timely actions, aimed at preventing further populations' disappearance in the short-term. We see four major implications of our results, useful to identify priorities and to optimize resource allocation for such a 'short-term' program.

First, and contrary with what was previously inferred, not all populations within the hotspot area should be considered as at high priority for species' conservation programs in situ. Indeed, in most of this area, declines appeared at an advanced stage (if not ultimate). Since an optimal target for in situ actions should be maximizing the chance of species' persistence, Aspromonte populations appear the ones whose demographic, genetic, and epidemiological features, make them suitable for in situ actions, including stringent protection and monitoring of breeding sites.

Second, in those areas (Catena Costiera and Serre) where the species is still present, but where data suggest strong negative demographic trends, pilot experiments of captive-breeding with mixed stocks (individuals of both local and Aspromonte origin) should be implemented. Under the hypothesis of better resistance performance of Aspromonte populations to the chytrid pathogen, such experiments might favour assisted flows of beneficial alleles within local populations (Jones, 2013). If successful under controlled (semi-natural) experimental conditions, these experiments might provide material for subsequent reintroduction programs aimed at species' recovery.

Third, our results showed a drastic reduction of the geographic occurrence of the chytrid pathogen on its *B. pachypus* host, if compared to what was observed in the recent past (Canestrelli et al., 2013), but they do not imply pathogen decline in the area, since we did not analyse the whole range of its potential hosts in southern Italy. Thus, monitoring its occurrence

in situ both in *B. pachypus* and other potential hosts (Simoncelli et al., 2005; Zampiglia et al., 2013), as well as within environmental matrices, will be a mandatory step of in situ actions, as will be also a proper prophylaxis in ex situ programs.

Fourth, but not least, our results showed a possible drastic reduction of habitat suitability expected by 2070-2100, along with an upward range shift. Conservation plans should consider this expected trend, and should plan actions accordingly. In particular, captive-breeding programs should be designed to assess species performance along elevation clines. Useful approaches in this regard might be the assessment of comparative performances (with and without the chytrid pathogen) in experimental climate chambers, or the use of replicated semi-natural settings located along environmental clines. Such approaches might also provide data of extreme value to help identifying priority sites for future restocking or reintroduction programs of this species, both in southern Italy and elsewhere. Finally, such approaches might provide the necessary information to address the possible role of the Sila region in *B. pachypus* conservation strategies. That is, whether, under the changing bioclimatic scenarios, this area might become a bioclimatic refugium for *B. pachypus*, and so a major area for reintroduction programs, or if the apparent disappearance of the species from this area is just a 'canary in mine' of what will soon happen throughout the region.

2.5 Figures and tables

Table 1. Geographic location, sample size, sampling year, and estimates of genetic diversity, for the 22 populations of *Bombina pachypus* sampled for the analysis of genetic variation in southern Italy. Sampling areas are defined according to Figure 1. H_E: unbiased expected heterozygosity; A_r: allelic richness.

	Site	Area	Lat. N	Long. E	Classification	Samplingyear	Sample size	H_E (s.e.)	A_r
1	Monte Cocuzzo (1981)	Catena Costiera	39°14'N	16° 10'E	Old	1981	9	0.62 (0.04)	3.72
2	Monte Cocuzzo (2014)	Catena Costiera	39°14'N	16° 07'E	New	2014	4	0.57 (0.06)	3.43
3	Belmonte Calabro	Catena Costiera	39°11'N	16° 06'E	New	2015	3	-	-
4	Cellara	Sila	39° 07'N	16°12'E	New	2015	2	-	-
5	Macchia Longa	Sila	39°22'N	16°36'E	Old	1981	9	0.62 (0.04)	3.77
6	Taverna	Sila	39° 01'N	16°35'E	Old	2003	2	-	-
7	Girifalco	Serre	38°49'N	16°29'E	Old	1978	9	0.68 (0.03)	3.99
8	Cardinale	Serre	38°44'N	16°24'E	Old	1978	4	0.78 (0.03)	4.14
9	Lacina	Serre	38°34'N	16°23'E	New	2013	5	0.63 (0.04)	3.43
10	Serra San Bruno	Serre	38°34'N	16°19'E	New	2013	1	-	-
11	Stilo	Serre	38°30'N	16°25'E	Old	2003	8	0.76 (0.02)	4.16
12	Zomaro	Aspromonte	38°17'N	16° 06'E	New	2013-2015	8	0.65 (0.03)	3.75
13	Delianuova	Aspromonte	38°14'N	15°53'E	New	2013	2	-	-
14	Melia	Aspromonte	38°13'N	15°43'E	New	2015	8	0.53 (0.04)	2.93
15	Gambarie	Aspromonte	38° 10'N	15°50'E	Old	1981	9	0.67 (0.04)	3.96
16	Rifugio Giardini	Aspromonte	38° 09'N	15°55'E	New	2015	5	0.61 (0.05)	3.41
17	Rifugio Canovai	Aspromonte	38° 07'N	15°57'E	New	2015	5	0.56 (0.05)	2.89
18	Samo	Aspromonte	38° 05'N	16° 00'E	New	2015	8	0.45 (0.04)	2.58
19	Cardeto	Aspromonte	38° 04'N	15°44'E	New	2015	6	0.50 (0.05)	2.63
20	Mosorrofa	Aspromonte	38° 05'N	15°43'E	New	2015	7	0.49 (0.05)	2.79
21	Condofuri	Aspromonte	37°59'N	15°52'E	New	2015	9	0.56 (0.04)	3.17
22	Montebello Ionico	Aspromonte	37°58'N	15°44'E	New	2016	7	0.46 (0.05)	2.84

Table 2. Variable importance for the different algorithms. Values in the table are the average across all modeling runs. A value of 0 assumes no influence of the variable on the model; the higher the value, the more influence the variable has on the model (maximum = 1; Thuiller, Lafourcade, Engler and Araújo, 2009). Climatic variables defined in Hijmans and colleagues 2005.

	GLM	GBM	GAM	MARS	MAXENT
Annual Mean Temperature	0.2776	0.0398	0.638	0.3724	0.0526
Mean Diurnal Range	0.0564	0.1744	0.046	0.0518	0.0574
Temperature Seasonality	0.0892	0.1094	0.235	0.1472	0.1038
Mean Temperature of Wettest Quarter	0.2074	0.2478	0.2064	0.1662	0.2264
Mean Temperature of the Driest Quarter	0.1822	0.2006	0.6012	0.3826	0.2162
Precipitation of the Wettest Month	0.1612	0.0892	0.145	0.137	0.1118
Precipitation Seasonality	0.0966	0.037	0.213	0.1814	0.1444

Figure 1. Spatial and temporal patterns of variation in species presence, genetic diversity, and chytrid occurrence, for the Apennine yellow-bellied toad in southern Italy (Calabria region). Sources of historical data and samples are reported within the main text. A: Sites of past and current presence of *Bombina pachypus* in the area; dashed shapes show the four subregions considered in this study. B: Results of the Bayesian clustering analysis used to assess the population genetic structure of *B. pachypus* in the area; admixture proportions of each sampled individual for the 3 genetic clusters identified as the best clustering option by the method implemented in TESS. C: Geographic variation in estimates of genetic diversity (A_r: allelic richness), among populations of *B. pachypus* sampled either for the present study or in the past. D: Past and current presence of the chytrid pathogen *Batrachochytrium dendrobatidis* among the sampled populations of *B. pachypus* from the study area.

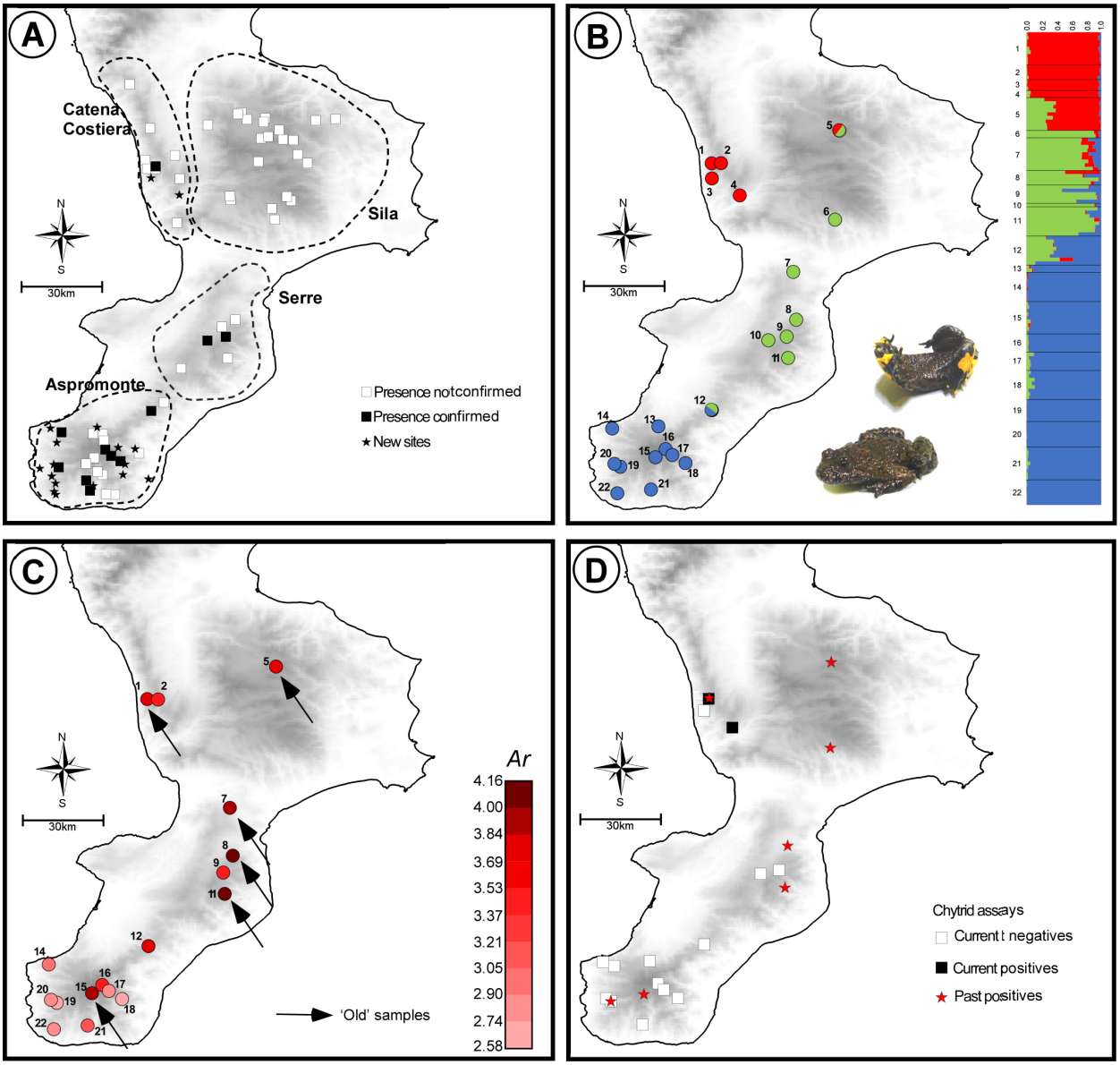
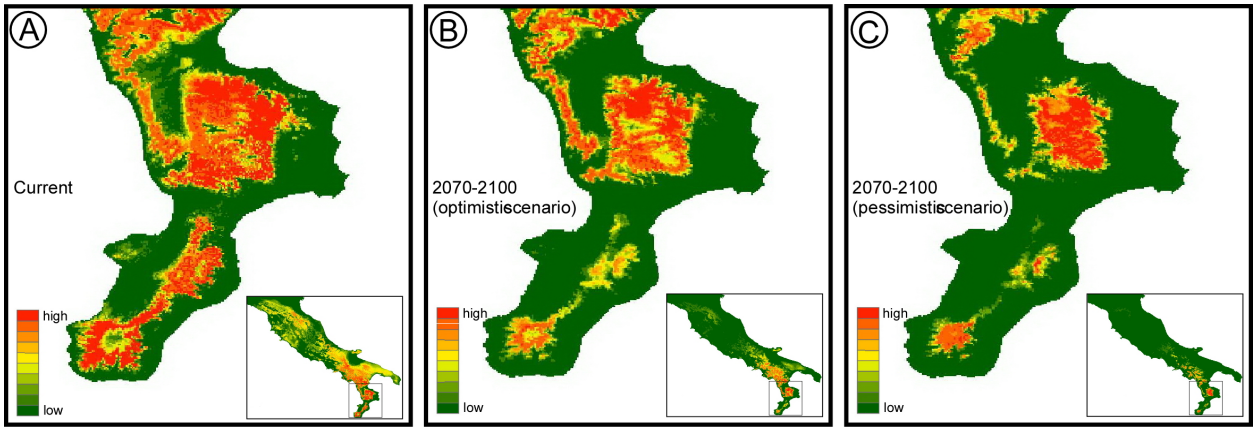


Figure 2. Species distribution model estimated for *Bombina pachypus* under current bioclimatic conditions (A), and its projection to the near future (2070-2100), under optimistic (B) and pessimistic (C) scenarios of greenhouse gas emission (RCP2.6 and RCP8.5 respectively).



3. Ecology and conservation status of endangered amphibians within a Mediterranean hotspot of biodiversity

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Abstract

Amphibian biodiversity loss in recent years has exceeded that of all other groups of vertebrates. In this context, biodiversity hotspots represent priority targets for conservation in amphibian populations. However, little information is available on the distribution and conservation status of amphibian species within most biodiversity hotspots. Here, we characterized the distribution and conservation status of four endangered amphibians (*Bombina pachypus*, *Salamandra salamandra gigliolii*, *Salamandrina terdigitata*, and *Rana italica*) in the Aspromonte Mountain region, a biodiversity hotspot in southern Italy where the conservation status of amphibians is almost unexplored. We conducted an intensive field survey of 507 potential breeding sites spanning over 2.326 km^2. We found that all four species were widespread in the study area. We observed 337 species occurrences: 63 for *S. s. gigliolii*, 29 for *S. terdigitata*, 84 for *B. pachypus*, and 161 for *R. italica*. Species distribution analysis revealed that *S. s. gigliolii* and *R. italica* populations had an extended and homogenous distribution. Conversely, *S. terdigitata* showed a dispersed pattern, with long distances between breeding sites, and *B. pachypus* an aggregated pattern, associated with the availability of suitable artificial habitats. On the other hand, we reported a decrease in *B. pachypus* occupancy in its natural habitats, which was related to a negative trend of populations. Overall, our results provide an encouraging framework for the conservation of amphibian populations in this area, but highlight the low coverage of endangered amphibian populations in protected areas, claiming for a reassessment of conservation policies and spatial conservation planning for the Aspromonte region

Keywords: Apennine yellow-bellied toad, amphibian decline, biodiversity conservation, biodiversity hotspot, fire salamander, Italian peninsula, Italian stream frog, spectacled salamander

3.1 Introduction

Amphibians are declining worldwide owing to habitat degradation, pollution, climate change, and emerging diseases (Blaustein and Kiesecker 2002; Collins and Storfer 2003; Stuart et al., 2004; Collins 2010; Blaustein et al., 2011; Catenazzi 2015; Scheele et al., 2019). Nearly half of all amphibian species are experiencing regional or local decline, and approximately one-third are threatened by extinction. In Europe, 23% of amphibian species are included in the at threat categories of the International Union for Conservation of Nature European Red List (Temple and Cox 2009; Sindaco 2016). Despite the extinction rate in amphibians exceeding that of any other group of vertebrates (Stuart et al., 2004; McCallum 2007), the conservation community has made little progress in halting or reversing these trends (Grant et al., 2016).

The Italian Peninsula is one of the most important biodiversity hotspots in the Palearctic region and hosts a highly diverse amphibian fauna, comprising about half of all amphibian species in Europe (Sindaco et al., 2006; Temple and Cox 2009; Rondinini et al., 2013), as well as many endemic evolutionary lineages. A generalized decline has been detected in the Italian populations of many amphibians, including some endemic taxa such as the Apennine yellow-bellied (*Bombina pachypus*), the Italian stream frog (*Rana italica*), the fire salamander (*Salamandra salamandra gigliolii*) and the spectacled salamander (*Salamandrina terdigitata* and *S. perspicillata*) (Barbieri et al., 2004; Lanza et al., 2007; Andreone et al., 2009). Despite the widespread decline of amphibians in Italy (Barbieri et al., 2004; Lanza et al., 2007, Andreone et al., 2009), monitoring schemes aimed at establishing demographic trends and conservation statuses are scattered and mostly limited to a few populations. In particular, the conservation status of amphibians in the southernmost part of the Italian Peninsula is severely under-investigated. It is worth noting that this part of peninsula holds the most of the intraspecific diversity for several species (Canestrelli et al., 2010; Bisconti et al., 2018a; Chiocchio et al., 2019), including most Italian amphibians investigated to date (Canestrelli et al., 2006a, 2006b, 2008, 2012, 2014). Consequently, a thorough understanding of the conservation status of amphibian populations in southern Italy is a priority for establishing effective conservation plans at both local and regional scales.

In this study, we aimed to characterize the distribution and conservation status of the endangered amphibians inhabiting the Aspromonte Massif, a mountain region in the southernmost part of the Italian Peninsula (Tab. 1). The Aspromonte Massif is an extended and heterogeneous mountain region surrounded by the sea and characterized by a high diversity of habitats spanning from dry grasslands and shrublands to oak, pine, and beech forests, and including also a wide range of humid areas that host many amphibian species. Recent studies

identified the Aspromonte region as a glacial refuge and hotspot of genetic diversity for many temperate species (Canestrelli 2010; Todisco et al., 2010), including amphibians (Canestrelli et al., 2006a; Iannella 2018). Despite the biogeographic and conservation value of this area, surveys on the distribution and conservation status of amphibians are scarce and discontinuous over space and time (Tripepi et al., 1999; Tripepi and Sperone 2007; Temi and Agristudio 2018). The only exhaustive study carried out in this area to clarify the conservation status of an endangered amphibian, the Apennine Yellow-bellied toad highlighted the strong discordance between the historical and current distribution of the investigated species (Zampiglia et al., 2019). This evidence, coupled with the scarcity of data for other endangered amphibians, suggest the need for a thorough investigation of the current distribution of amphibians in this area.

The objective of this study is to provide an updated and detailed reference framework concerning the distribution, habitat preferences, and conservation status of endangered amphibians in southern Italy. To achieve this, we apply a fine-scale field survey to map the current presence of endangered amphibians in the Aspromonte region. We will focus on four amphibian species that are endemic to the Italian Peninsula and have shown local or regional decline because of habitat reduction/alteration or pathogen outbreaks: *Bombina pachypus*, *Rana italica*, *Salamandrina terdigitata*, and *Salamandra salamandra gigliolii* (see Table 1).

3.2 Material and Methods

Study area

The Aspromonte is a mid-to-high mountain region (maximum altitude 1.957 m asl), located at the southern boundary of the Italian Peninsula (Figure 1) and characterized by a high diversity of habitats and peculiar climatic conditions. The seasonal distribution of rainfall has typical Mediterranean features, with less rainfall in the summer months than in the winter months (Colacino et al., 1997). The mountainous belts are characterized by abundant precipitation during autumn, winter, and spring, and streams and brooks are mainly perennial. In the hilly and coastal strips, most humid habitats show dependence from seasonal precipitations. In mountainous areas, the average winter temperatures are quite low, with minimum temperatures occurring in January and February and frequently dropping below 0 °C. Conversely, in the coastal area, the average summer temperatures are high, exceeding 40 °C in July and August. In general, the western and eastern sides are characterized by almost opposite microclimatic features, that is, wetter and cooler on the western side and warmer and drier on the eastern side. Brullo et al., (2021) identified two distinct macro-bioclimates in Aspromonte (then divided them into different thermotypes): a Mediterranean rain-seasonal oceanic bioclimate between 0 and 1.100 m asl and a temperate oceanic bioclimate between 1.100 and 1.957 m asl.

Climatic and orographic diversification have generated ecological gradients and highly heterogeneous vegetation typologies (Spampinato et al., 2009). In endemism, relict species, and ancient woods, the Aspromonte represents a hotspot of plant and animal biodiversity at the species, genetic, and community levels (Spampinato et al., 2009; Zampiglia et al., 2019; Piovesan et al., 2020). Biodiversity conservation within the Aspromonte region is undertaken by the Aspromonte National Park (ANP), together with 57 Natura 2000 areas, 55 of which are Special Areas of Conservation (SAC) and two are Special Protection Areas (SPA).

Field survey

The identification of potential presence sites for all the investigated species was obtained by consulting the available literature (Stoch 2000-2005; Lanza et al., 2007, and reference therein) and analyzing cartographic data. Cartographic data were retrieved from the Italian Military Geographic Institute (Military Geographic Institute) and satellite images to identify potential aquatic habitats. In line with other studies (e.g. Sindaco et al., 2006; Brandmayr et al., 2017), we considered the original standard grid defined by the European Community as a cartographic reference (reference system ETRS89-LAEA Europe - Lambert Azimuthal Equal Area

projection), a universal transverse mercator (UTM) grid with cells of 10 × 10 km mesh (N = 45, 2.804 km^2) and WGS84 projection.

Field surveys were conducted from spring 2016 to autumn 2021, during the period of activity of the investigated species, focusing on the species' reproductive season, according to the phenology of each species. We applied standard methodologies, such as the visual encounter survey (VES) and calling survey (CS), as reported by Heyer et al., (1994) and Sindaco (2016). We examined adults, subadults, larvae, and eggs. Each site was visited three times. The field survey involved both species natural habitats and artificial environments (Table S2). Investigation of *B. pachypus* covered a large part of the Aspromonte Massif (UTM grids = 19.1822 km^2) whereas investigations of the other three species were almost exclusively in the ANP and adjacent territories (UTM grids = 18, 913 km). Each site was georeferenced using a GPS device and subsequently transferred to a geographic information system (GIS) platform (ArcMap 10.7.1, © ESRI Inc., CA, USA). As *B. pachypus* and *S. terdigitata* are mentioned in Annexes II and IV of the EU Habitat Directive, for these two species, we added abundance estimates of populations using visual counts. All procedures were approved by the Italian Ministry of Ecological Transition and the Italian National Institute for Environmental Protection and Research (ISPRA; permit number: 7727, 15-04-2016).

Data analysis

For each presence site, the following environmental information was retrieved: altitude from sea level using the DTM (Digital Terrain Model), with a resolution of 10 m; habitat type according to the CORINE Biotopes (European Commission 1991) and EUNIS codes (APAT 2004); and Horton order of river courses (Horton 1945). The environmental typology surrounding the sites was defined by buffer analysis (see e.g. Plăiaşu et al., 2012; Canessa et al., 2013) with a buffer radius of 100 m (3.14 ha). Each buffer area was superimposed on the land cover map (LC, De Fioravante et al., 2022) to generate an independent set of polygons representing land cover with a resolution of 10 m. The percentage of land cover within each area was calculated. Similarly, altimetry, exposure, and slope were obtained using the DTM layer, with a resolution of 10 m. Data visualization and analysis were performed using ArcMap 10.7.1.

To produce information for conservation management purposes, we estimated the following parameters: the diffusion index (DI, Ragni 2002), the distribution of species within the study area using the minimum convex polygon method (MCP, Mohr 1947), and the pattern of distribution using the average nearest neighbor (ANN, Clark and Evans 1954). DI measures the

ratio between the number of cells with the species occurrence out of the total number of cells investigated and spans from 0 (i.e., the species is not present in any cell) to 1 (i.e., the species is present in all the cells investigated). MCP generates a polygon whose angles are the outermost locations of the presence sites. Although MCP tends to overestimate home ranges by including areas not frequently visited by the species (Borger et al., 2006), we employed this method because it allows the identification of the boundaries of the species range in the study area (Burt 1943); the minimum boundary geometry tool was used to obtain MCP. The average nearest neighbor is a method used to describe the spatial structure of occurrences (Pommerening 2020). It determines the clustering of occurrences in the study area by measuring the distance between each location and its neighboring centroid location and then calculating the average among all the nearest neighbor distances. If the average distance is less than the average for a hypothetical random distribution, the distribution is considered clustered; if the average distance is greater than the hypothetical random distribution, the distribution is considered dispersed (Clark and Evans 1954).

Finally, we estimated species habitat selection in the study area using the Manly standardized habitat selectivity index (α) (Manly et al., 1972, 2002). This index represents the proportional use of a resource (or habitat) divided by the proportional availability of each habitat. The values of α range from 0 to 1: $\alpha = 1/k$ indicates that the resource is used randomly and in proportion to the abundance in the environment; $\alpha > 1/k$ indicates positive selection of the resource; $\alpha < 1/k$ indicates resource avoidance. Habitats with the highest α were considered key habitats for the species (Desbiez et al., 2009).

3.3 Results

From 2016 to 2021, we visited 507 sites in 21 UTM cells (Figure 1), corresponding to 46.66% of the study area (mean of 24.14 sites per cell). The investigation involved most of the ANP (245 sites, 48.2%) and 20 of the 55 SAC in the study area. The investigated species were observed in 337 of the 507 visited sites (66,46%), of which 220 were within the ANP; species occurrences were detected in 16 SAC (Table S6). The distribution of species occurrences are summarized in Table 2 and Figure 2.

Fire salamander

Salamandra salamandra gigliolii was observed at 63 sites, mostly within the ANP (Figure 2A, Table S1). This species is mainly distributed along the western side of the mountain massif. The altitude and habitat distributions are summarized in Figure 3 and Table S2-S5. The altitudinal distribution spanned from 718 to 1.774 m asl, with most observations occurring between 1300 and 1.400 m asl (39%). The species was found almost exclusively in forest environments, such as beech forests (LC code 21115, 67.49%) and oro-Mediterranean mountain pine forests (LC code 21122, 19.54%). Permanent streams and brooks represented the most frequented aquatic habitats (81%), with a few interesting observations in peat bogs (5%). The most frequent river courses belonged to order 1 (38%) and order 2 (35%). This species was found in seven SAC (Table S6).

ANN analysis (Table 2) showed a random distribution pattern (NNR: 0.986; z-score: -0.197; p-value: 0.843). The Manly Index (Table S7) suggests a species positive selection for altitude range between 1.200 and 1.500 m asl, slope classes of 0–15° and 15–30°, fresh sides affected by the Tyrrhenian humid currents (i.e., N, NW, and W exposure), beech forest and mountain pine forest habitats (LC codes 21115 and 21122, respectively).

Spectacled salamander

Salamandrina terdigitata was observed at 29 sites, most of which were within the ANP (Figure 2B, Table S1). The altitude and habitat distributions are summarized in Figure 4 and Table S2-S5. The altitudinal distribution was from 122–1.573 m asl, with most observations being below 700 m asl. The species mainly occupies forest environments, such as oak forests and broad-leaved woods (LC code 21111; 25.52%), beech woods (LC code 21115; 16.78%), and olive groves (LC code 21132 - 13,96%). The species was mainly found in permanent streams and brooks (61%) and temporary streams and brooks (32%). The most frequent river course was Horton's order 4 (29%) and 3 (26%). This species was found at four SAC sites (Table S6).

ANN analysis (Table 2) showed a dispersed distribution pattern (NNR: 1.284; z-score: 2.828; p = 0.004). The Manly Index (Table S8) suggests a positive selection for altitudes of 100–200 m asl, 700–800 m, and 1500 m above sea level, as well as positive selection for slopes below 15° on cool and humid sides (i.e., W, NW, and E exposure). Interestingly, despite the large use of oak forests (LC code 21111) and beech forests (LC code 21115), the Manley index showed species positive selection for less available habitats, that is, orchards (LC code 21131) and chestnut (LC code 21114).

The number of observed individuals at each site during each visit spanned from 1 to approximately 1000: 1 to 7 adult individuals were observed at 10 sites; up to 50 individuals (larvae and/or adults) were observed at 15 sites; more than 50 individuals were observed at four sites, of which, at one we counted approximately 100 individuals (mainly larvae) and at another approximately 1000 larvae.

Appennine yellow-bellied toad

Bombina pachypus was observed at 83 sites, most of which were outside the ANP (Figure 2C, Table S1). The altitude and habitat distributions are summarized in Figure 5. The altitudinal distribution spanned from 51–1.613 m asl, with most observations occurring between 400–600 m asl (49%). The species mainly occupy open environments, such as pastures (LC code 22110–27.66%) and shrublands (LC code 21220–14.87%), but also oak woods and evergreen broad-leaved trees (LC code 2111–15.21%). Artificial environments were the most frequent aquatic habitat (64%). The species was found at seven SAC (Table S6).

ANN analysis (Table 2) showed a strongly aggregated distribution pattern (NNR: 0.753; z-score: -4.328; p-value: 0.000). The Manly Index (Table S9) showed a general positive selection for altitudes between 400–1.100 m asl, slopes from 0° to 45°, for the sunniest sides (i.e., SE, W, SW, S, and NW exposures), as well as for pastures (LC code 22110), artificial abiotic surfaces (LC code 11000), and grassy soils (LC code 22200).

The number of observed individuals spanned from 1 to 120 adult individuals: up to 50 individuals were counted at 71 sites and more than 50 were observed at 12 sites, of which 5 showed more than 100 individuals. It is worth noting that *B. pachypus* was not found at 14 sites of historical presence.

Italian stream frog

Rana italica was observed at 161 sites, most of which were within the ANP (Figure 2D, Table S1). The altitude and habitat distributions are summarized in Figure 6. The altitudinal

distribution spans from 118 to 1.822 m asl, with a peak of observations around 1.300 m asl (12%). The species has been frequently observed in beech forests (code LC 21115, 32.23%) and oak and evergreen broad-leaved tree forests (code LC 21111, 22.91%). The species has been mostly observed in permanent and temporary streams and river courses of 1–4 Horton's code. These species were observed in 14 SAC (Table S6).

ANN analysis (Table 2) showed a strongly aggregated distribution pattern (NNR: 0.74; z-score: -6.092; p-value: 0.000). Inspection of the Manly Index (Table S10) suggests a positive selection at an altitude of 100 m asl and from 1.200–1.500 m asl. Positive selection was also observed for slopes between 0–30° for wetter and cooler sides (i.e., N, NE, E, and NW exposure), deciduous oak forests (LC code 21112), and oro-Mediterranean and mountain pine forests (LC code 21122) (S7).

3.4 Discussion

Results from this survey updates the information on distribution and conservation status of four endangered amphibian species inhabiting the Italian Peninsula. We focused on the southernmost portion of the Italian Peninsula, as it is an acknowledged hotspot of biodiversity within the Mediterranean region and one of the most unexplored regions of Europe in terms of amphibian ecology and conservation. We collected data on a large number of new and previously unknown breeding sites for the four investigated species, which depicts an encouraging frame on the conservation status of the populations in this area.

Overall, we found that these four species were widespread in the study area. The inspection of the distribution maps (Figure 2) outlined *R. italica* as the most abundant species and *S. terdigitata* as the least abundant species. *S. terdigitata* also showed the most fragmented distribution, as supported by ANN analysis with a highly dispersed distribution pattern. These distribution patterns are in line with those shown by these species on the Italian Peninsula in previous studies (Lanza et al., 2007). By contrast, *S. s. gigliolii* and *B. pachypus* were more widespread in the Aspromonte Massif than in the northern regions of the Italian Peninsula (Lanza et al., 2007), despite their non-uniform distributions in the study area. Indeed, *S. salamandra* was mainly distributed on the western side of the mountain massif, with only a few observations on the eastern side. This distribution reflects the needs of the species for cooler microclimate conditions, which are more common on the western and northwestern mountain sides in this region. Interestingly, the distribution map of *S. s. gigliolii* occurrences (Figure 2A) highlighted strong contiguity of populations, as supported by ANN analysis, which excluded either clustered or dispersed patterns of distribution. Conversely, *B. pachypus* was more abundant in the southwestern part of the Aspromonte region, despite many observations were localized to the eastern side of the ANP. Furthermore, inspection of the *B. pachypus* distribution (Figure 2C) showed a strongly clustered distribution, as confirmed by ANN analysis. This pattern of distribution could reflect the patchy availability of the artificial environments colonized by *B. pachypus*, that is, irrigation tanks, drinking troughs, and artificial ponds, which currently constitute the species-preferred habitats (Figure 5).

The habitat use of the four species in the Aspromonte Massif (Figure 3-6) agrees with available information on species ecology, although there were some relevant differences. *S. s. gigliolii* showed a marked preference for a higher altitudinal range in Aspromonte (1.200–1.300 m asl) compared to the rest of the peninsula (where the species shows a preference for altitudes around 800–1.000 m) (Lanza et al., 2007). This difference is even more marked in *R. italica*, which is commonly more frequent below 800 m asl (Lanza et al., 2007) but showed a peak

frequency distribution around 1.300 m asl in Aspromonte. In contrast, *B. pachypus* showed a wider distribution at lower altitudes than the rest of the peninsula. We also found a population at 1.613 m asl, which, along with observations reported in the Pollino Massif (around 1.600 m asl: Barbieri et al., 2004), is the highest record for *B. pachypus*. We also reported a wider range of habitats for *S. terdigitata*, suggesting a wider ecological tolerance of populations in Aspromonte than in the northernmost populations. Finally, our results emphasize the importance of preserving forests, which comprised the most frequented habitats of *S. terdigitata*, *S. s. gigliolii*, and *R. italica*.

The comparison of our data with those of previously published studies (Tripepi and Sperone 2007) highlights a general increase in species presence, especially for *B. pachypus* and *S. terdigitata*. This increase can be attributed to the wider geographic coverage of this survey, compared to that of previous surveys, rather than an increase in species distribution. It is worth noting that the unavailability of exact locations from previous surveys does not allow for direct comparison and inferences on population trends. However, we visited 26 known historical sites for *B. pachypus* (see Zampiglia et al., 2019) and in 14 of them (54%), the presence of the species was not confirmed. A negative trend was also reported by local people (shepherds, farmers, woodcutters, and walkers), who confirmed that *B. pachypus* is less widespread now compared to the early 1990s when it was ubiquitous and abundant in hilly and mountain streams and ponds, as well as in artificial aquatic environments used for agriculture and local pastoralism. Our data suggest a change in species habitat suitability, as we only observed *B. pachypus* in five watercourses (6% of the observations), with the majority of observations (53) occurring in artificial habitats: irrigated tanks (35), drinking troughs (15), and artificial ponds (3). Thus, despite our data outline a good conservation status of *B. pachypus* populations in Aspromonte compared to that in central and northern Italy, we report a negative trend in population that has to be accounted by conservation stakeholders. This evidence, coupled with the expected reduction in habitat availability in the next 50 years due to climate change (Zampiglia et al., 2019) and the scarcity of populations enclosed in protected areas (Figure 2C), makes clear the need for rapid and effective conservation actions for *B. pachypus* in the Aspromonte region.

3.5 Conclusions

This study provides further evidence on the vital role of the Aspromonte Massif in biodiversity conservation. In this area, all investigated species were found to be more widespread and relatively more abundant than in the central and northern parts of the Italian Peninsula (see e.g. Vanni and Nistri 2006). The better conservation status of the populations in this area can be

attributed to two main reasons. First, during the Anthropocene, human activity has been less intensive in this region than in the rest of Italy. Because of this, Aspromonte contains several patches of ancient forests which act as "biodiversity tanks" in the face of anthropogenic habitat degradation. Second, Aspromonte harbors a hotspot of genetic diversity for all four investigated species (Canestrelli et al., 2006a, 2006b, 2008; Mattoccia et al., 2011; Bisconti et al., 2018b; Iannella 2018; Zampiglia et al., 2019). Because genetic diversity provides populations with the potential to adapt to environmental changes, hotspots of genetic diversity represent invaluable resources for species to adapt to global changes (Hampe and Petit, 2005; Zachos and Habel, 2011). Therefore, preserving populations located within the Aspromonte region preserves populations with the highest potential to survive extinction threats that can be used for effective, genetically informed, translocation, and repopulation programs. Nevertheless, even though most of the populations were within the ANP boundaries, our data showed only a few occurrences within the Natura 2000 network. In particular, almost all observed populations of *B. pachypus*, one of the most endangered vertebrates in Italy, were outside protected areas. These results highlight the need for rapid implementation of new distribution data for these species in future conservation policies concerning the Aspromonte region. In this respect, it is worth mentioning that Aspromonte underwent several destructive fires during the last 10 years, many of which were considered human-induced. In particular, during the summer of 2021, after our field survey was complete, a large fire swept through 17.733 ha of the Aspromonte Massif, destroying 3.200 ha of forest, of which approximately 25 ha were ancient forests, with severe impacts on biodiversity. This fire event involved 34 of 337 sites in this study (10%), of which 18 were *B. pachypus* breeding sites (20% of *B. pachypus* breeding sites in this study). This event stresses the need to implement management methods that reduce the spread of fires and, thus, their impact on biodiversity, avoiding to set the hotspot on fire.

Funding

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Competing interests

The Authors have declared no competing interests

Acknowledgment

This paper is dedicated to the memory of Sergio Tralongo, who supported this research but suddenly passed away before seeing the results published. We thank Mauro Zampiglia, Paolo Nucera, Chiara Martino, Eugenio Potitò, Manuela Policastrese, and Emanuele Rosace for their help in the field survey activity. We also thanks Antonio Barca, Francesco Manti, Giuseppe Bruzzaniti, Francesco d'Aleo and Valentina Laface for their advices and suggestions on potential breeding sites in the study area.

3.6 Figures and tables

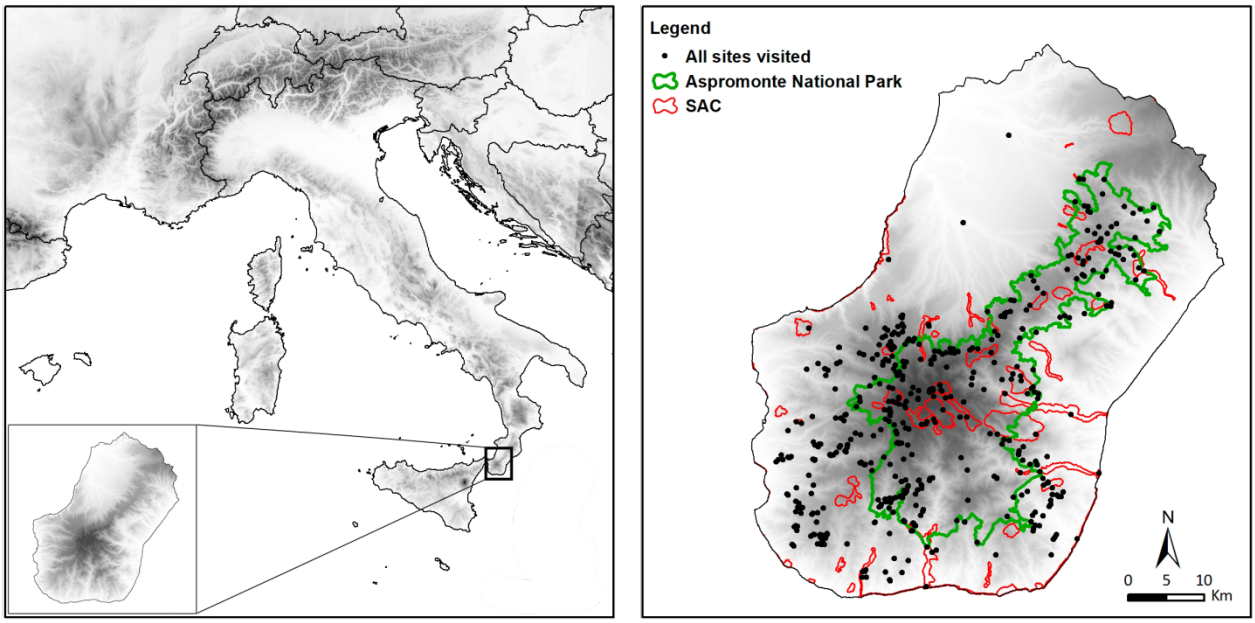

Figure 1 - Geographic location of the study area. Location of the Aspromonte massif within the Italian peninsula (**left**), and the distribution of the sites investigated during the field survey (**right**); black dots indicate occurrences, green line indicates the ANP boundary, red lines indicate SAC boundaries.

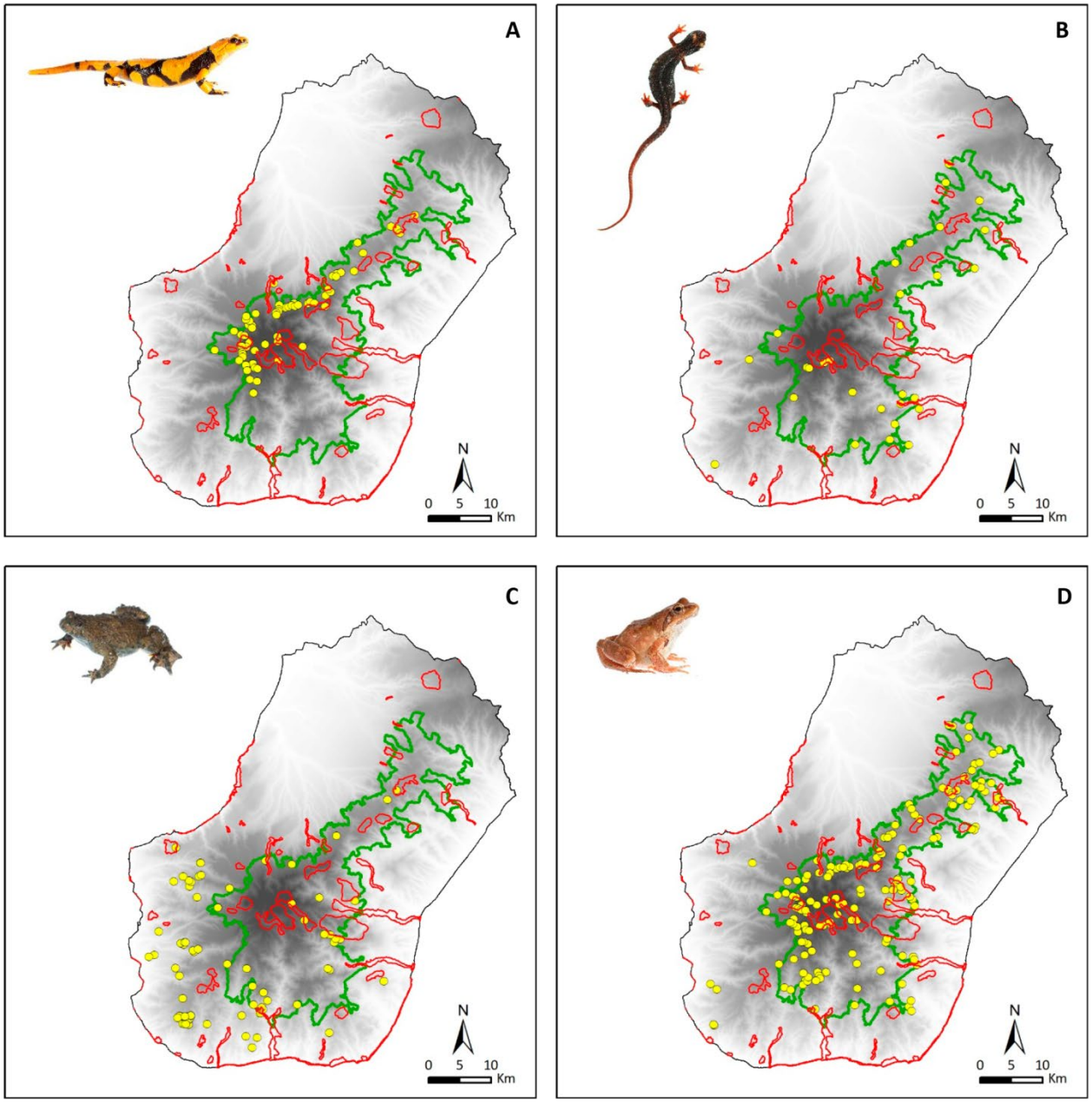

Figure 2 - Location of occurrences for each species investigated in this study. Yellow dots indicate species occurrences, green line indicates the ANP boundary, red lines indicate SAC boundaries. A: *Salamandra salamandra gigliolii*; B: *Salamandrina terdigitata*; C: *Bombina pachypus*; D: *Rana italica*.

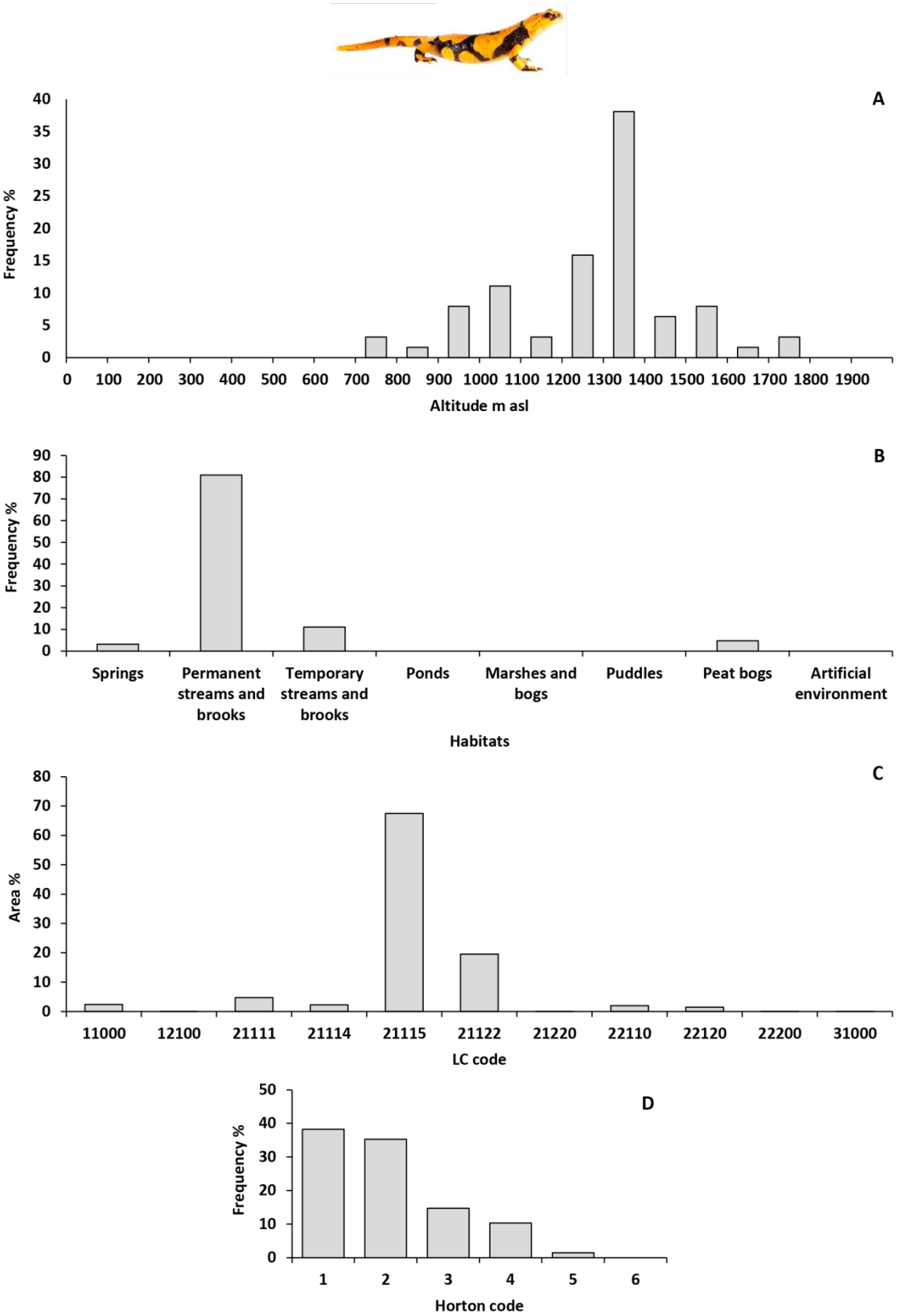

Figure 3. Frequency distribution of the *Salamandra salamandra gigliolii* occurrences for (A) altitude, (B) habitats, (C) land cover, and (D) Horton categories.

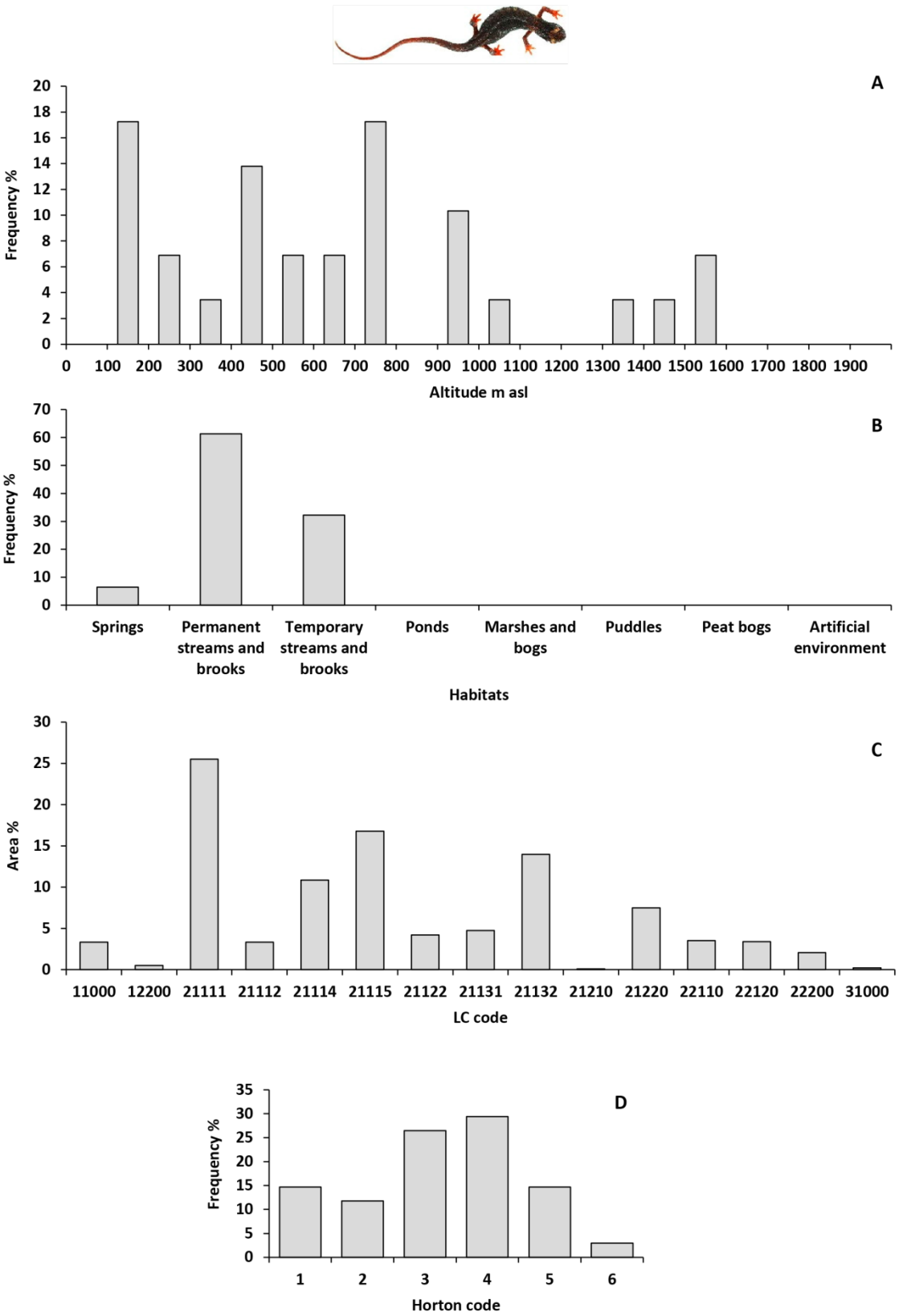

Figure 4 - Frequency distribution of the *Salamandrina terdigitata* occurrences for (A) altitude, (B) habitats, (C) land cover, and (D) Horton categories.

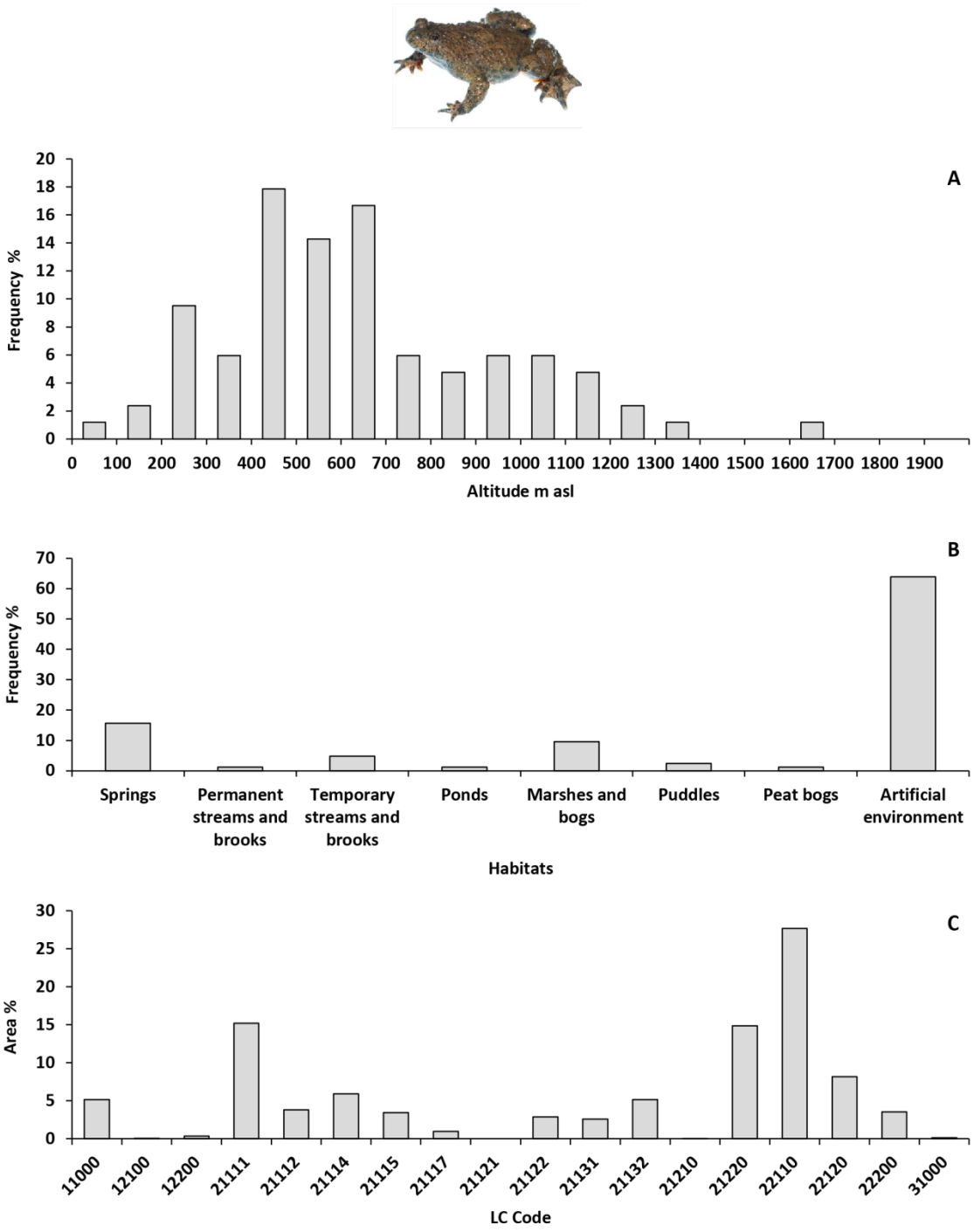

Figure 5 - Frequency distribution of the *Bombina pachypus* occurrences for (A) altitude, (B) habitats, (C) land cover.

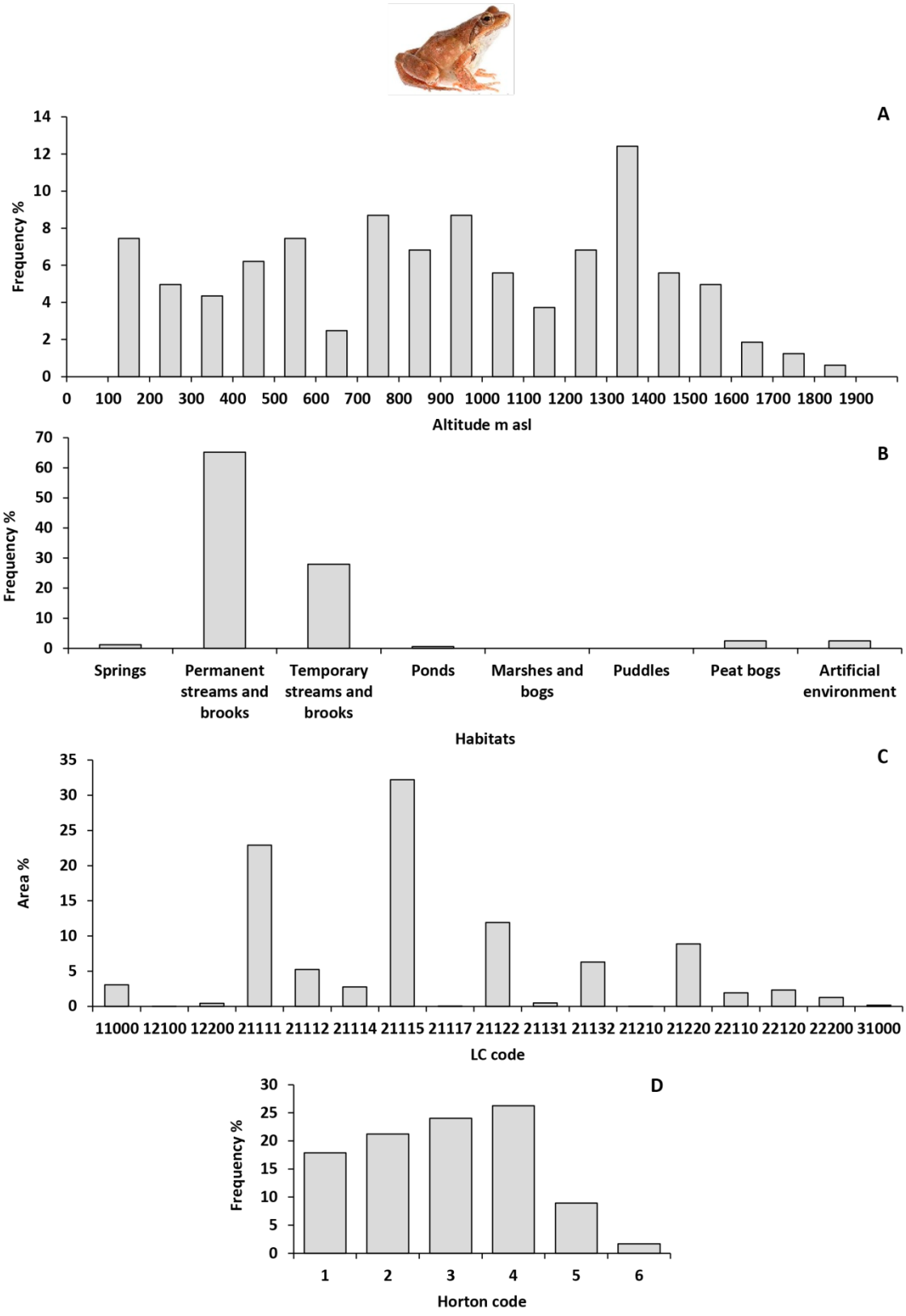

Figure 6 - Frequency distribution of the *Rana italica* occurrences for (A) altitude, (B) habitats, (C) land cover, and (D) Horton categories.

Taxon	Endemic	IUCN Italy	IUCN Europe	Habitat Directive Annexes II or IV	Pathogen threats
Salamandra salamandra gigliolii	Yes	LC	LC		Yes[1,2,3]
Salamandrina terdigitata	Yes	LC	LC	II, IV	Yes[1]
Bombina pachypus	Yes	EN	EN	II, IV	Yes[4,5]
Rana italica	Yes			IV	Yes[3,5]

Table 1 - Conservation status of the taxa investigated in this study. [1]Costa et al., 2021; [2]Grasselli et al., 2019; [3]Zampiglia et al., 2013; [4]Canestrelli et al., 2013; [5]Zampiglia et al., 2019; [5]Fagotti et al., 2019.

Taxon	Occurrencies	Presence cell	Altitude range	DI	MCP (Km^2)	ANN			
						OBN	NNR	z-score	p-value
Salamandra salamandra gigliolii	63	7	718 - 1774	0.33	239	968.43 m	0.986	-0.197	0.843
Salamandrina terdigitata	29	13	122 - 1573	0.62	821	3541.27 m	1.284	2.828	0.004
Bombina pachypus	84	17	51 - 1613	0.81	1191	1418 m	0.753	-4.328	0.000
Rana italica	161	16	118 - 1822	0.76	1002	944.40 m	0.74	-6.092	0.000

Table 2 - Summary of the species occurrences within the Aspromonte massif. DI: Diffusion Index; MCP: Minimum Convex Polygon; ANN: Average Nearest Neighbor; OBN; Observed Mean Distance; NNR: Nearest Neighbor Ratio; details in the main text.

4. You startle me, you don't

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Yellow-and fire-bellied toads (genus *Bombina*) exhibit textbook examples of a peculiar behavior known as the "unken-reflex", a defense mechanism used to startle predators. When a predator's attack is perceived as imminent and unavoidable, these dorsally camouflaged toads contract their dorsal muscles, suddenly arch their body, expose their vividly colored ventral side, and release toxins from their skin glands. Documented in many species of amphibians, this behavior is considered a stereotyped, rather invariant, response that serves as a tactic to evade predation. While surveying populations of the endangered Apennine yellow-bellied toad (*Bombina pachypus*) in Aspromonte National Park (southern Italy), we found that, even under repeated stimulation (we did our best to mimic a persistent predatory bird) only about half of the local population of toads exhibited this behavior, and that individual toads were highly consistent in their response over multiple trials. In other words, the "unken-reflex" seems to be a polymorphic and repeatable trait, rather than a fixed impulse. This unexpected intraspecific variation raises several intriguing questions for future research. What is the origin and adaptive value of this behavioral variation? Does frequency-dependent selection affect the efficacy of startling displays? Could the observed variation in these displays among toads reflect individual personalities?

Figure. An individual exhibits unken-reflex and one does not exhibit it (**left image**); a detail of the posture during unken-reflex (**right image**).

5. Shock or jump: deimatic behaviour is repeatable and polymorphic in a yellow-bellied toad

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Published: under review

Abstract

Inter-individual variation in antipredatory strategies has long attracted curiosity among scientists. Deimatism combine camouflage and aposematism in a complex and time-structured antipredatory strategy consisting in prey suddenly unleashing unexpected defences to frighten predators and stop their attack. Being deimatism traditionally considered as a stereotyped antipredatory response, the inter-individual variation in phenotypic traits related to deimatic displays is almost unexplored. In this study, we employed common garden experiments on the Apennine yellow-bellied toad *Bombina pachypus* to investigate the extent and pattern of inter-individual variation in the *unken-reflex* behaviour, a deimatic display performed by this species and some other amphibians. Results show that deimatic displays consistently differ among *B. pachypus* individuals. We found that only about half of the individuals reacted to the predation stimuli by exhibiting the display, which varied in responsiveness, duration and intensity. All the investigated behavior descriptors showed significant repeatability ($R > 0.50$, $p < 0.01$). Finally, we found significant correlations between all the measured parameters, defining two alternative behavioral profiles: individuals quickly doing *unken-reflex*, with high intensity and long duration of the display, and individuals avoiding the *unken-reflex* but rather escaping. Such dichotomy fit with the alternative behavioral profiles described by copying style theory. Such an unexpected level of within-population variation in deimatic behavior raises intriguing questions on the evolutionary processes shaping multiple adaptive responses to predation within populations.

Keywords: deimatism, antipredatory behaviour, phenotypic variation, *unken-reflex*, amphibians.

5.1 Introduction

The boundless variety of antipredatory strategies spread in the animal kingdom has long attracted curiosity among scientists and inspired evolutionary ecology research through the last two centuries (reviewed in Ruxton et al., 2018). Antipredatory strategies typically involve the concerted evolution of suites of visual, chemical, acoustic, and behavioral traits, which allow the prey to survive the encounter with the predator (Swaffer & O'Brien 1996; Croft et al 2009; Stevens and Merilaita, 2011; Bateman & Fleming 2014; Barry et al., 2015; Briolat et al 2019). Several studies reported large within-species variation in phenotypic traits related to the antipredatory strategies. Interestingly, a large part of this variation regards the individual ability to actively modulate the antipredatory responses. Still, inter-individual variation has been reported also in more complex anti-predator strategies where variation was traditionally considered as non-adaptive, such as camouflage and aposematism (Coppinger 1970; Skelhorn & Rowe, 2006). Such variation has been explained by differential selection exerted by predators on prey populations (Kettlewell, 1973; McPeek 1997; Gunnarsson, 1998; Reale and Festa-Bianchet 2003; Edgell & Rochette, 2008; Strobbe et al. 2009, 2010), and by the interaction between contrasting evolutionary forces and ecological trade-offs acting on the many phenotypic traits involved (e.g. balancing the costs of foraging and mating with the costs of sequestering/synthesizing compounds for colorful signals and/or chemical defenses; see Mappes et al., 2005, Nokelainen et al., 2012, Stevens and Ruxton, 2012). However, the data available do not allow a full understanding of the processes maintaining this variation within populations.

Deimatism is an appealing antipredatory strategy which consists in displaying striking and transitory behaviors aimed at inhibiting predator attacks (i.e. startling behaviours), and it is spread among several animal taxa, especially arthropods, cephalopods, and amphibians (Cott 1940, Edmunds, 1974, Skelhorn et al., 2016, Umbers et al., 2015, Umbers et al., 2017; Ruxton et al., 2018). Deimatic displays are multicomponent as they combine multiple defensive strategies, such as camouflage and aposematism, and typically involves either chromatic and behavioral components (Rowe & Halpin, 2013; Umbers et al., 2017). Furthermore, deimatic displays are dynamic behaviors as individuals can actively modulate duration, frequency, and intensity of the exhibition depending e.g. on the type and/or behavior of predators and the prey risk perception. Thus, deimatic species provide an appealing system to investigate the trade-offs between alternative behavioral phenotypes when facing with a threat. Yet, the extent of inter-individual variation in deimatic behavior within and among populations is almost unexplored for the most of deimatic species.

In this study, we investigate the extent of inter-individual variation in deimatic behavior in the Apennine yellow-bellied toad *Bombina pachypus*. Toads of the genus *Bombina* are textbook examples of the *unken-reflex,* a deimatic behavior exhibited by many other toads and salamanders. These species usually bear cryptic colorations on their dorsal side (Bordignon 2018). However, when the attack by a predator is perceived as imminent and unavoidable, these toads contract dorsal muscles, suddenly arch their body, expose their vividly colored ventral side, and release toxins from their skin glands as a reinforcement to the warning signal (Hinsche 1926; Toledo 2011). Preliminary evidence showed some level of variation in the *unken-reflex* behavior among distinct populations in either *B. variegata* and *B. bombina* (Löhner, 1919; Bajer 1980). Such variation has been linked to the presence of different predator sets among the tested populations (Bajer, 1980). However, there are no data on the extent of this variation among individuals within populations, which cannot be imputed to differential predator selective pressures.

Here, we collected Apennine yellow-bellied toads from southern Italy and scored their *unken-reflex* behavior in a series of common garden experiments, with the aim to address the following questions: (i) Is the *unken reflex* polymorphic and repeatable within populations? (ii) What are the main components of its variation?

5.2 Material and Methods

Ethical note

Sampling procedures were performed under the approval of the Italian Ministry of Ecological Transition and the Italian National Institute for Environmental Protection and Research (ISPRA; permit number: 20824, 18-03-2020). Permission to temporarily house amphibians was granted by Local Health and Veterinary Centre, with license code 050VT427. All handling procedures outlined in the present study followed the Ethical Committee of the University of Tuscia for the use of live animals.

Study species

The Apennine yellow-bellied toad *B. pachypus* is an amphibian endemic to the Italian peninsula. It is a diurnal, thermophilic toad, active from April to October, and breeding in small seasonal ponds, from 100 up to 1600 masl (Lanza et al., 2007). According to the altitude and local climate conditions, it starts reproducing from May to July; females produce up to 60 eggs and tadpoles usually metamorphose after one month; juveniles reach sexual maturity after two years. Adults' diet is composed by small invertebrates. This species is characterized by a dorsal cryptic habitus, and a ventral yellow-black aposematic coloration, which is exposed to the potential predator through arching the body, suddenly releasing toxins from skin glands (i.e. the *unken-reflex* deimatic behavior). The main predators of *B. pachypus* are birds and larger aquatic animals like grass snakes (*Natrix natrix*). Once abundant through the Italian peninsula, populations have been declining because of a combination of habitat alteration, climate change and disease (Zampiglia et al., 2019).

Sampling and housing

A total of 71 *B. pachypus* male individuals were collected during the spring 2020 from a number of close ponds in the surroundings of Reggio Calabria (Aspromonte massif, southern Italy: 38°00'N, 15°51'E). We collected adults males from close but distinct ponds, in order to ensure same environmental conditions and predator set, and to avoid collecting related individuals. Also, we selected this region because it has not been affected by either recent and historical population decline, and all the individuals can be considered belonging to a unique and panmittic population (Zampiglia et al., 2019). Males were identified by the presence of forelimb nuptial pads. All the collected individuals were transported within three days to the experimental facilities, and then housed individually in cages (25 cm × 25 cm × 25 cm). Toads were provided with a water tank, an oak wood as shelter. The cages were placed in a temperature-controlled

room set to 24/25°C (monitored using a Hobo MX2201 temperature data-logger), 60%-80% of humidity, and natural photoperiod (Staniszewski, 1995). Individuals were fed ad libitum with mealworm beetle larvae (*Tenebrio molitor*) and crickets (*Acheta domestica*).

Before any experiment started, we ascertained that the day-night activity rhythms were normalized (higher activity at night-time) by hourly visual check for two weeks; observations included reactivity to food and calling activity at night. No adverse effects on the overall health toad conditions were observed during the procedures. Finally, we tested for the occurrence of Chytrid fungus (*Batrachochytrium dendrobatidis*) infection in all the individuals: skin swabs were collected from all the individuals, and the molecular diagnostic essay was performed following the procedures described in Zampiglia et al. (2013). All the individual tested negative and could attend the behavioral essays.

Behavioural essays

To obtain behavioural descriptors of the *unken-reflex* response under predator attack, *B. pachypus* individual were submitted to the Tonic Immobility (TI) test, a standardized method that induces thanatosis-like antipredatory reactions in many species of different taxa, including amphibians (Suzuky et al., 2013; Toledo et al., 2010). The TI test usually consists in simulating the attack of a predator by hitting or caughting the prey head for few seconds, and then scoring qualitative and quantitative parameters describing the prey immobility reaction. In the case of *unken-reflex*, toads under threat are expected to arch their body and expose their colored ventral side for the time necessary to the predator to recognize the aposematic signal and give up the attack. Thus, we (i) simulated the attack of a predator in artificial condition, (ii) recorded the toad reaction by means of a camera, and (iii) scored the parameters describing the *unken-reflex* reaction, i.e. latency to react, duration and intensity of the reaction.

Before the simulation, individuals were individually placed in the centre of a container (50 x 30 x 10 cm) and covered with a plastic cup for 2 minutes. Then, the plastic cup was raised up and the stimulation began. The stimulation consisted in gentle hitting the head of the toad with a finger for a maximum of 60 seconds. If a toad moved away, the stimulation continued for a maximum of 60 seconds, then it was considered that *unken-reflex* had not been induced and a score of 0 s was given for all the parameters. Conversely, if toads arched the body, not showing any movement, TI immobility parameters were measured. If the *unken-reflex* lasted for more than 5 minutes, the test was terminated and a score of 300 s was given for duration. Animals were always handled by the same researcher (GM). The tests were recorded with a camera (Panasonic FZ300) fixed at 50 cm from the container, and the recordings were evaluated by the

same operator, who scored all the response variables. The following variables were decoded to characterize the *unken-reflex* reaction: (1) *unken-reflex* occurrence, expressed as *yes* or *not*; (2) latency, expressed as the time delay in showing the *unken-reflex* after the test began (from 0 to 60); (3) the duration time of the immobility, in seconds (from 0 to 300); (4) the intensity of the response, expressed as a scale from 0 to 3 where 0 is no response, 1 is light response (only the feet are lift up), 2 is moderate response (all the limbs are lift up), and 3 is strongest reaction (the limbs are lift up and the whole body is arched). All the variables were measured using the Boris 7.9.6 software package for video analysis (Friard & Gamba 2016). The test was repeated for each individual under the same conditions, after a seven days interval.

Statistical analyses

Individual behavioral consistency was assessed by calculating repeatability estimates on repeated measures of each measured parameter (i.e. latency, duration and intensity of the reaction) using mixed models (Nakagawa and Schielzeth 2010). Repeatability coefficients R, that is, intraclass correlation coefficients, for each parameter were calculated using the "rpt." function of the "rptR" package (Stoffel et al., 2017, Schielzeth and Nakagawa 2011) in R software (R software version 4.0.0; R Core Team 2020). Repeatability was estimated as the ratio of between-individual variance to total variance with generalized linear mixed effects models (GLMM) for Poisson distributed data, using individual identity as a random factor and the link-scale approximation of R (Stoffel et al., 2019); for assessing repeatability for *unken-reflex* occurrence, we applied the "rpt." function for binary data. The 95% confidence intervals (CI) around repeatability estimates was generated by performing 1,000 parametric bootstrap iterations. Variables were considered 'highly' repeatable if $R > 0.5$ or 'marginally' repeatable if $R > 0.2$, and the lower bound of the CI was >0.0 (Aplin et al., 2015; Baker et al., 2018; Dzieweczynski & Crovo, 2011).

Correlation among the measured behavioral descriptors was tested using the Spearman's rank correlation method implemented in the "spearman.test" function of the "pspearman" R package (Savicky 2009).

5.3 Results

Results from the TI tests showed inter-individual variation in the *unken-reflex* deimatic behavior within the investigated *B. pachypus* population, as well as consistence in the individual response across multiple trials. During the first trial, 38 out of 71 individuals (53.5%) reacted to the predation stimuli performing *unken-reflex*; all the other individuals tried to escape without showing any deimatic display. During the second trial, 41 out of 71 individuals (57.7%) performed the *unken-reflex* display. The repeatability test confirmed that the *unken-reflex* response is a highly repeatable behaviour (R = 0.56, CI= 0.16 - 0.70, P = 7.34E-05).

We also found marked inter-individual variation in the way individual performed *unken-reflex*. Latency spanned from 0.24s to 42.59s in the first trial (mean 8.83s, median 3.88, sd.± 11.73) and from 0.40s to 25s in the second trial (mean 5.77s, median 4.18, sd.± 6.22); duration spanned from 0.31s to 236.50s in the first trial (mean 56.11s, median 22.03s, sd.± 68.03s) and from 0.01 to 157.99s in the second trial (mean 38.88s, median 12.47s, sd.± 49.69s); intensity spanned from 1 to 3 in the first trial (mean 2.37, median 3, sd.± 0.79s) and from 1 to 3 in the second trial (mean 2.22, median 3, sd.± 0.88). We found consistency in the individual behavior between the two trials as all the measured behavioral descriptors resulted significantly and highly repeatable (see Table 1). Finally, the behavioral descriptors resulted significantly correlated: quickly responding individuals showed high intensity and long duration of the reaction (Table 2).

Results of the first trial are shown in Figure 1 and overall show two alternative behavioral strategies: about half of the individuals did not perform *unken-reflex* (latency = 60s, intensity and duration = 0); on the other hand, most of the individuals performing *unken-reflex* showed low latency, high intensity and long duration of the reaction.

5.4 Discussion

Anti-predatory behavior can differ among individuals, although the extent of this variation has been poorly characterized in the context of deimatic behavior. In simulated attacks on the Apennine yellow-bellied toads, we found that deimatic behavior varies among individuals, and that this variation is repeatable across multiple trials. We observed about half of the toads reacting to a simulated attack by arching the body and exposing the aposematically-colored ventral side. The other half did not show any display, but rather moved away. These results are among the first evidences of inter-individual variation in deimatic behavior, and raise questions on the evolutionary processes shaping multiple defensive strategies within populations.

The inspection of the pattern of variation in the unken-reflex behavior revealed inter-individual variation in all the scored behavioral descriptors, i.e. latency, duration and intensity of the deimatic display (Figure 1). The latency and the intensity of the display showed a bimodal pattern, with the extreme phenotypes way more frequent than intermediate phenotypes. This pattern suggests that no display (flee) or alternatively rapid and intense deimatic displays perform better than intermediate behaviors. The duration of the display has a uniform distribution, despite the display lasted more than one minute for the most of the individuals performing the unken-reflex. This pattern suggests best performance of long displays respect to short display in halting predator attacks. The best performance of extreme behaviours is confirmed by the significant correlation shown by these traits (Table 2), which allow to define two alternative antipredatory strategies in *B. pachypus*: individuals quickly doing unken-reflex, with high intensity and long duration of the display, versus individuals avoiding the unken-reflex but rather escaping. These results supports the occurrence of a trade-off between speed and accuracy (Sih and Del Giudice, 2012) and are in line with the hypothesis that intermediate deimatic phenotypes could produce ambiguous stimuli, failing in startling or halting the predator attack (Ruxton et al., 2018).

We found all the behavioral descriptors highly repeatable across multiple trials (Table 1). This evidence has two main implications. First, the inter-individual variation in deimatic behavior of *B. pachypus* is consistent through time. Evidences of consistent behavioral differences among individuals within species have been accumulating in the last years and had major implications on the study of animal personalities (reviewed in Bell et al., 2009). Recent studies on the katydid crickets *Acripeza reticulata* revealed within-species differences in deimatic display (linked to the sexual dimorphism), but these differences were not consistent across multiple trials (De Bona et al., 2020). Our results show that deimatic behavior can consistently vary among individuals within species, and could therefore be linked to other personality traits. The second implication of this result is that the tonic immobility test applied here is repeatable, and it can be employed to

quantify consistent individual differences in deimatic behaviour across multiple contexts, which is a promising subject for future investigations on *Bombina* toads.

The two antipredatory strategies of *B. pachypus* remind to the dichotomy in the way individual cope with environmental challenges, as described by the coping style theory (Koolhaas et al., 1999, 2010). Indeed, employing deimatic display or rather escaping can be linked to the reactive-proactive copying styles. Accordingly, the reactive pattern involves parasympathetic reactivity, resulting in a conservation-withdrawal response, conditioned immobility, and low levels of aggression (Koolhaas et al., 1999, 2010, Oster et al., 2015). This pattern fits with the rapid employing of deimatic display observed in *B. pachypus*. Conversely, animals with a proactive coping pattern have high sympathetic activity and are characterized by active avoidance behaviour (commonly fight-or-flee response) and high levels of aggression. This pattern fits with the escaping behaviour observed in the toads not employing deimatic display. However, further behavioral, physiological and genetic investigations (i.e. gene expression profiles) are mandatory to define deimatic phenotypes according to the copying style theory.

Overall, results from this study show that unken-reflex is not a stereotyped deimatic behavior in *Bombina* toads. This evidence provides interesting insights on the evolutionary pathways to deimatism in *Bombina*, as well as on the mechanisms maintaining alternative antipredatory strategies within populations. The effectiveness of deimatic display, when chemical defences are also involved – as in the case of *Bombina* toads – does not require the predators learn the signal from the focal prey. Yet, predators can learn to ignore the deimatic component, but not the chemical defence (Umbers et al., 1997). In this frame, possessing a defensive 'repertoire' could provide populations with the opportunity to get away with a wider range of predators, including predators who learned to ignore the deimatic component. Therefore, behavioral polymorphism would be adaptive for populations. However, more data are needed on extent of defensive repertories within populations of other deimatic species, either with and without further defenses. Moreover, further investigations should be addressed to evaluate the link between the observed variation and other variables, like personality traits, predator-exogenous stimuli, and the association between phenotypic and genetic variation.

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5.5 Figures and tables

Table 1 – Repeatability estimates of the behavioral descriptors of the *unken-reflex* reaction among different trials, obtained by fitting the generalized linear mixed effects models (GLMM) implemented in the "rptR" R package. R: repeteability (i.e. intraclass correlation coefficient); SE, standard error; CI, 95% confidence intervals obtained from 1,000 parametric bootstrap iterations; P: significance, obtained by likelihood ratio tests. N = 71.

Repeatability				
	Occurrence	**Intensity**	**Latency**	**Duration**
R	0.56	0.65	0.70	0.80
SE	0.15	0.08	0.07	0.07
CI	[0.16, 0.70]	[0.47, 0.77]	[0.55, 0.82]	[0.64, 0.90]
P [LRT]	7.34E-05	1.04E-08	2.22E-08	8.68E-11

Table 2 - Matrix of the Spearman's rank correlation coefficients (below) and significance (above) among the 3 descriptors of *unken-reflex* reaction (latency, intensity and duration). *, significant; N = 71.

Spearman's Rho			
	Latency	**Intensity**	**Duration**
Latency	-	0.033	0.046
Intensity	-0.35*	-	0.004
Duration	-0.32*	0.46**	-

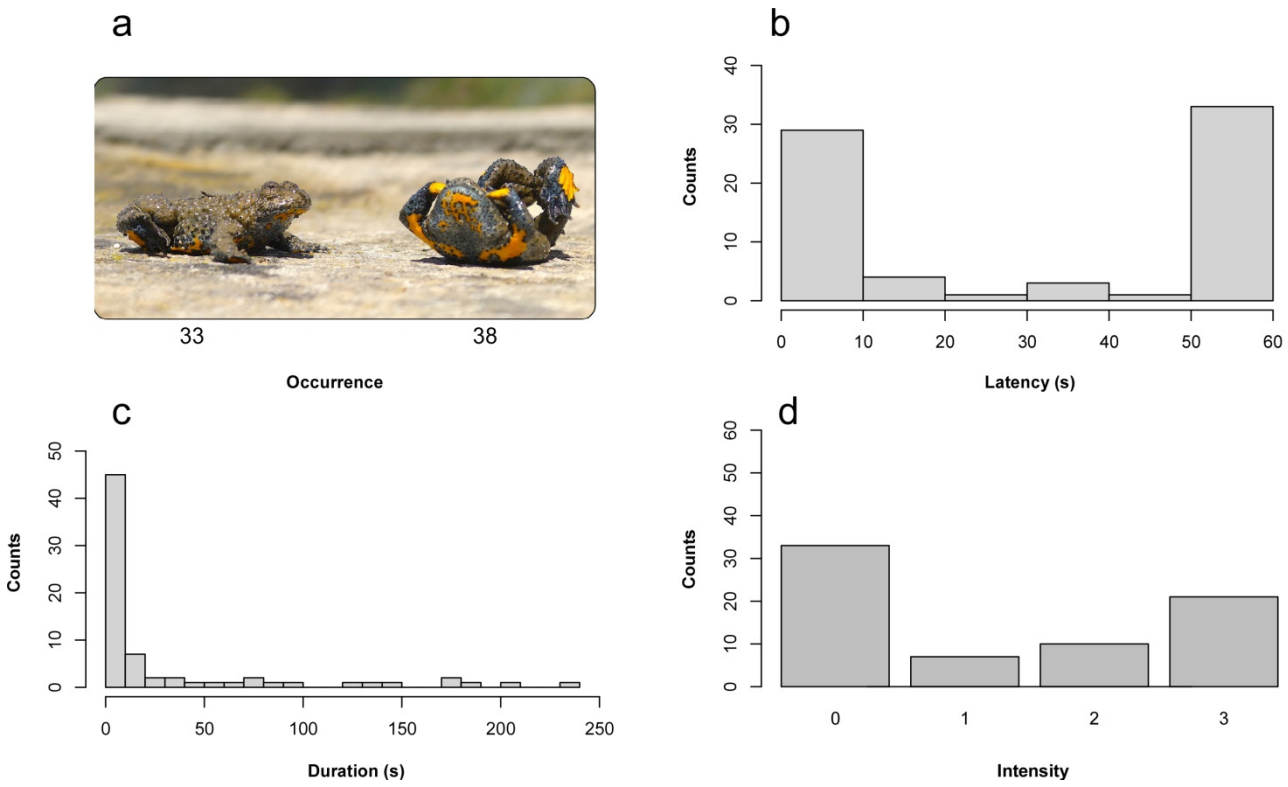

Figure 1 – Representation of the inter-individual variation in the *unken-reflex* behavior in the 71 tested *B. pachypus* individuals (details are given in the main text): a) image representing a non-responding individual (left) and a high-responding individual (right), with relative occurrence (below); b) latency of the response; c) duration of the *unken-reflex* display; d) intensity of the response, expressed as a scale from 0 to 3, where 0 is no response and 3 is the most intense reaction. Data refer to the first trial.

6. Conclusion

This study provided fundamental novel insights into the conservation of the Appenine yellow bellied-toad *B. pachypus*, emphasizing the fundamental importance of considering the individual population as the unit for conservation programs. Overall, results from this study defines the *B. pachypus* population form the Aspromonte region as an independent evolutionary unity with high level of genetic and phenotypic variation and apparently stronger resitance to the decline. These charachteristics elict this population as a priority for both regional anc large-scale conservation programs. In fact, bringing together genetic, demographic, ecological, epidemilogic and behavioural data support for the Aspromonte population holding the highest potential for species survival to the extinction threaths in the entire species range. Thus, population from the aspromonte should be considered as source for breedeers in both in-situ and ex-situ restocking programs.

It is worth noting that despite *B. pachypus* in the Aspromonte region showed the best conservation status in the entire species range, traces of decline has also occurred in this area, as consequence of human-induced habitat alteration. This evidence claim for urgent conservation actions addressed to preserve *B. pachypus* natural habitats. Also, as we reported species local abundance also in several artificial environments which are not included in any protected areas, our results stress the need for a reassesment of Natura 2000 Network sites in this region.

Besides from applied conservation implications, our results provide interesting insights also in evolutionary ecology research. Indeed, our finding of repeteable intra-population polimorphisms in deimatic behaviour of *B. pachypus* open for further on the link between the observed variation and other variables, like personality traits, predator-exogenous stimuli, and the association between phenotypic and genetic variation. Furthermore, investigation on the geographic structure of the phenotypic variation should ascertain the link among species evolutionary history, species genetic structure, and the structure of phenotypic variation among population. Finally, genome scale studies should be addressed to investigate the genetic underpinnings of behaviourhal polimorphisms and, eventually, of phenotypic plasticity of populations.

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Supplementary material

Drilling down hotspots of intraspecific diversity to bring them into on-ground conservation of threatened species

Materials and methods

Microsatellite PCR

F22: forward primer was labelled with TAMRA; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 2 min, 35 cycles of 30 sec at 95° C, 30 sec at 47° C, 30 sec at 72° C and a final extension at 72° C for 15 min.

9H: forward primer was labelled with 6-FAM; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 2 min, 35 cycles of 30 sec at 95° C, 30 sec at 52° C, 30 sec at 72° C and a final extension at 72° C for 15 min.

5F: forward primer was labelled with 6-FAM; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 5 min, 35 cycles of 30 sec at 95° C, 30 sec at 50° C, 45 sec at 65° C and a final extension at 60° C for 15 min.

Bv11: forward primer was labelled with 6-FAM; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 5 min, 35 cycles of 30 sec at 95° C, 30 sec at 53° C, 45 sec at 65° C and a final extension at 60° C for 15 min.

1A: forward primer was labelled with TAMRA; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP,GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 2 min, 35 cycles of 30 sec at 95° C, 30 sec at 56° C, 30 sec at 72° C and a final extension at 72° C for 15 min.

8A: forward primer was labelled with HEX; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP,GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 2 min, 35 cycles of 30 sec at 95° C, 30 sec at 55° C, 30 sec at 72° C and a final extension at 72° C for 15 min.

10F: forward primer was labelled with 6-FAM; reaction mix (10 µl) contained 10-20 ng/µl of DNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP,GoTaq Flexi Buffer 1X and 0.5 U of GoTaq DNA Polymerase (Promega); PCR cycling included an initial denaturation at 95° C for 2 min, 35 cycles of 30 sec at 95° C, 30 sec at 58° C, 30 sec at 72° C and a final extension at 72° C for 15 min.

PCR products were assembled in multiplex post-PCR: one including 10F, 1A and Bv11 and another including 9H, 8A and F22; 5F was genotyped alone.

Spatial thinning of the occurrence database

To limit the effect of spatial clustering of the occurrences used to calibrate the different algorithms, we applied a spatial thinning in the geographical space to the original 361 occurrence points (Aiello-Lammens et al. 2015). We used the R package SPTHIN which requires the definition of a minimum distance used to randomly remove occurrence records that are closer than the same distance. To empirically determine the thinning distance, we generated 10.000 sets of 361 random points (same number as the available species' occurrences) covering the calibration area (i.e., the area sampled by the occurrence points); for each of the 10.000 sets we calculated the mean distance between nearest neighbor points, and then we calculated the mean over all 10,000 sets of random points, corresponding to 11.277 meters. Using the same distance, we obtained five alternative datasets with 182 occurrences (each distant at least 11.277 meters from the closest one) used for model calibration and 179 occurrences used for model evaluation.

				Salamandra s. gigliolii		*Salamandrina terdigitata*		*Bombina pachypus*		*Rana italica*	
Habitat	**Codes**	**Corine codes**	**Eunis codes**	**RF**	**%**	**RF**	**%**	**RF**	**%**	**RF**	**%**
Springs - Brooks and ponds supplied by springs and resurgences.	1	54.1	C2.1	2	3	2	6	13	16	2	1
Permanent streams and brooks - Permanent watercourses with a nival rain regime.	2	24.11	C2.2	51	81	19	61	1	1	105	65
Temporary streams and brooks - Temporary watercourses with a nival rain regime. Absence of water in summer.	3	24.16 (HD)	C2.5	7	11	10	32	4	5	45	28
Ponds - Small and medium-sized collections of water of natural origin	4	22.1 (HD)	C1	0	0	0	0	1	1	1	1
Marshes and bogs - Small and medium-sized water collections with vegetation and mud	5	-	D5.3	0	0	0	0	8	10	0	0
Puddles - Small and medium periodic and temporary water collections	6	22.1 (HD)	C1, C3.8	0	0	0	0	2	2	0	0
Peat bogs - Small wetlands with accumulation of considerable amount of decomposed moss (mostly *Sphagnum*) and vegetation matter.	7	54.5 (HD)	D2.23	3	5	0	0	1	1	4	2
Artificial environments - Irrigation tanks, drinking troughs, troughs and artificial ponds	8	89	J5.31, J5.5	0	0	0	0	53	64	4	2

Supplementary table S2. Habitat distribution for each studied species (RF - Relative Frequency). The correspondence to the habitats of Annex 1 of the Habitats Directive is specified (HD).

	Study area		*Salamandra s. gigliolii*		*Salamandrina terdigitata*		*Bombina pachypus*		*Rana italica*	
Land cover	**ha**	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**
11000	6708.16	3.68	4.64	2.38	2.77	3.33	12.19	5.16	14.60	3.05
12100	260.22	0.14	0.02	0.01	0.00	0.00	0.14	0.06	0.05	0.01
12200	1868.03	1.03	0.00	0.00	0.42	0.51	0.88	0.37	2.10	0.44
21111	31642.73	17.38	9.15	4.70	21.21	25.51	35.93	15.21	109.73	22.91
21112	5672.58	3.11	0.00	0.00	2.77	3.33	8.97	3.80	25.11	5.24
21114	13841.75	7.60	4.42	2.27	9.01	10.84	14.00	5.93	13.25	2.77
21115	20563.47	11.29	131.39	67.49	13.95	16.78	8.15	3.45	154.36	32.23
21117	2279.49	1.25	0.00	0.00	0.00	0.00	2.30	0.97	0.20	0.04
21121	1123.28	0.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
21122	8887.18	4.88	38.04	19.54	3.51	4.22	6.85	2.90	57.06	11.91
21131	3982.41	2.19	0.00	0.00	3.94	4.74	6.12	2.59	2.42	0.51
21132	16081.69	8.83	0.00	0.00	11.60	13.95	12.20	5.17	30.16	6.30
21210	2612.28	1.43	0.00	0.00	0.08	0.10	0.06	0.03	0.08	0.02
21220	21987.34	12.07	0.07	0.04	6.21	7.47	35.11	14.87	42.63	8.90
22110	21104.61	11.59	3.84	1.97	2.93	3.52	65.32	27.66	9.30	1.94
22120	18085.15	9.93	2.91	1.49	2.81	3.38	19.32	8.18	11.10	2.32
22200	5027.92	2.76	0.13	0.07	1.72	2.07	8.32	3.52	6.04	1.26
31000	378.31	0.21	0.08	0.04	0.20	0.24	0.30	0.13	0.80	0.17
40000	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplementary table S3. Land cover distribution in study area and for each studied species.

	Salamandra s. gigliolii		*Salamandrina terdigitata*		*Bombina pachypus*		*Rana italica*	
Altitude	**RF**	**%**	**RF**	**%**	**RF**	**%**	**RF**	**%**
0	0	0	0	0	1	1	0	0
100	0	0	5	17	2	2	12	7
200	0	0	2	7	8	10	8	5
300	0	0	1	3	5	6	7	4
400	0	0	4	14	15	18	10	6
500	0	0	2	7	12	14	12	7
600	0	0	2	7	14	17	4	3
700	2	3	5	17	5	6	14	9
800	1	2	0	0	4	5	11	7
900	5	8	3	10	5	6	14	9
1000	7	11	1	3	5	6	9	6
1100	2	3	0	0	4	5	6	4
1200	10	16	0	0	2	2	11	7
1300	24	38	1	3	1	1	20	12
1400	4	6	1	3	0	0	9	6
1500	5	8	2	7	0	0	8	5
1600	1	2	0	0	1	1	3	2
1700	2	3	0	0	0	0	2	1
1800	0	0	0	0	0	0	1	1
1900	0	0	0	0	0	0	0	0

Supplementary table S4. Altitude distribution for each studied species (RF - Relative Frequency).

	Salamandra s. gigliolii		*Salamandrina terdigitata*		*Bombina pachypus*		*Rana italica*	
Horton order	**RF**	**%**	**RF**	**%**	**RF**	**%**	**RF**	**%**
1	26	38	5	15	2	40	32	18
2	24	35	4	12	0	0	38	21
3	10	15	9	26	0	0	43	24
4	7	10	10	29	1	20	47	26
5	1	1	5	15	2	40	16	9
6	0	0	1	3	0	0	3	2

Supplementary table S5. Horton order distribution for each studied species (RF - Relative Frequency).

SAC	*Salamandra s. gigliolii*	*Salamandrina terdigitata*	*Bombina pachypus*	*Rana italica*
IT9350133	x		x	x
IT9350134	x			x
IT9350145			x	x
IT9350146				x
IT9350147		x		x
IT9350152	x			x
IT9350153	x			x
IT9350154	x	x		x
IT9350155	x			x
IT9350157			x	x
IT9350163		x		x
IT9350166		x		x
IT9350167	x			
IT9350175				x
IT9350177			x	
IT9350180				x

Supplementary table S6. SAC (Special area of Conservation - Habitat Directive) with presence of species studied.

Salamandra s. gigliolii

Land Cover	ui	mi	α	1/K
11000	0.02	0.02	0.16	
12100	0.00	0.00	0.01	
12200	0.01	0.00	0.00	
21111	0.26	0.05	0.02	
21112	0.04	0.00	0.00	
21114	0.04	0.02	0.06	
21115	0.20	0.67	0.26	
21117	0.00	0.00	0.00	
21121	0.00	0.00	0.00	
21122	0.09	0.20	0.24	0.05
21131	0.01	0.00	0.00	
21132	0.06	0.00	0.00	
21210	0.00	0.00	0.00	
21220	0.13	0.00	0.00	
22110	0.04	0.02	0.05	
22120	0.05	0.01	0.03	
22200	0.03	0.00	0.00	
31000	0.00	0.00	0.02	
40000	0.00	0.00	0.00	

Altitude	ui	mi	α	1/K
0	0.00	0.00	0.00	
100	0.03	0.00	0.00	
200	0.05	0.00	0.00	
300	0.06	0.00	0.00	
400	0.07	0.00	0.00	
500	0.09	0.00	0.00	
600	0.09	0.00	0.00	
700	0.09	0.01	0.01	
800	0.09	0.01	0.01	
900	0.09	0.07	0.03	
1000	0.06	0.12	0.08	0.05
1100	0.06	0.04	0.03	
1200	0.05	0.16	0.14	
1300	0.05	0.36	0.32	
1400	0.04	0.09	0.10	
1500	0.03	0.08	0.12	
1600	0.02	0.02	0.04	
1700	0.01	0.03	0.11	
1800	0.00	0.00	0.01	
1900	0.00	0.00	0.00	

Slope	ui	mi	α	1/K
0° - 15°	0.28	0.53	0.59	
15° - 30°	0.41	0.40	0.30	
30° - 45°	0.27	0.07	0.07	0.16
45° - 60°	0.04	0.00	0.03	
60° - 75°	0.00	0.00	0.00	
75° - 81°	0.00	0.00	0.00	

Exposure	ui	mi	α	1/K
N	0.13	0.18	0.18	
NE	0.14	0.11	0.10	
E	0.12	0.07	0.07	
SE	0.13	0.06	0.05	0.12
S	0.14	0.10	0.09	
SO	0.13	0.13	0.12	
O	0.11	0.18	0.19	
NO	0.11	0.17	0.19	

Supplementary table S7. Manly index of *Salamandra salamandra gigliolii* for environmental variables considered.

Salamandrina terdigitata

Land Cover	ui	mi	α	1/K
11000	0.02	0.03	0.10	
12100	0.00	0.00	0.00	
12200	0.01	0.01	0.02	
21111	0.26	0.26	0.05	
21112	0.04	0.03	0.04	
21114	0.04	0.11	0.13	
21115	0.20	0.17	0.02	
21117	0.00	0.00	0.00	
21121	0.00	0.00	0.00	
21122	0.09	0.04	0.02	0.05
21131	0.01	0.05	0.26	
21132	0.06	0.14	0.11	
21210	0.00	0.00	0.02	
21220	0.13	0.07	0.03	
22110	0.04	0.04	0.04	
22120	0.05	0.03	0.03	
22200	0.03	0.02	0.04	
31000	0.00	0.00	0.05	
40000	0.00	0.00	0.00	

Altitude	ui	mi	α	1/K
0	0.03	0.00	0.00	
100	0.05	0.11	0.12	
200	0.06	0.11	0.10	
300	0.07	0.02	0.02	
400	0.09	0.15	0.10	
500	0.09	0.09	0.06	
600	0.09	0.04	0.03	
700	0.09	0.17	0.11	
800	0.09	0.01	0.01	
900	0.06	0.11	0.10	0.05
1000	0.06	0.03	0.03	
1100	0.05	0.00	0.01	
1200	0.05	0.00	0.00	
1300	0.04	0.04	0.06	
1400	0.03	0.03	0.06	
1500	0.02	0.07	0.18	
1600	0.01	0.01	0.03	
1700	0.01	0.00	0.00	
1800	0.00	0.00	0.00	
1900	0.00	0.00	0.00	

Slope	ui	mi	α	1/K
0° - 15°	0.28	0.36	0.35	
15° - 30°	0.41	0.41	0.27	
30° - 45°	0.27	0.19	0.19	0.16
45° - 60°	0.04	0.03	0.18	
60° - 75°	0.00	0.00	0.00	
75° - 81°	0.00	0.00	0.00	

Exposure	ui	mi	α	1/K
N	0.13	0.11	0.10	
NE	0.14	0.15	0.13	
E	0.12	0.14	0.14	
SE	0.13	0.12	0.12	0.12
S	0.14	0.14	0.12	
SO	0.13	0.11	0.11	
O	0.11	0.11	0.12	
NO	0.11	0.13	0.15	

Supplementary table S8. Manly index of *Salamandrina terdigitata* for environmental variables considered.

Bombina pachypus

Land Cover	ui	mi	α	1/K
11000	0.04	0.05	0.09	
12100	0.00	0.00	0.03	
12200	0.01	0.00	0.02	
21111	0.17	0.15	0.06	
21112	0.03	0.04	0.08	
21114	0.08	0.06	0.05	
21115	0.11	0.03	0.01	
21117	0.01	0.01	0.05	
21121	0.01	0.00	0.00	
21122	0.05	0.03	0.04	0.05
21131	0.02	0.03	0.08	
21132	0.09	0.05	0.04	
21210	0.01	0.00	0.00	
21220	0.12	0.15	0.08	
22110	0.12	0.28	0.16	
22120	0.10	0.08	0.06	
22200	0.03	0.04	0.09	
31000	0.00	0.00	0.04	
40000	0.00	0.00	0.00	

Altitude	ui	mi	α	1/K
0	0.06	0.02	0.02	
100	0.08	0.02	0.02	
200	0.09	0.09	0.07	
300	0.10	0.06	0.04	
400	0.09	0.16	0.13	
500	0.09	0.16	0.12	
600	0.08	0.13	0.10	
700	0.07	0.08	0.07	
800	0.07	0.06	0.06	
900	0.07	0.07	0.08	0.05
1000	0.05	0.06	0.07	
1100	0.04	0.05	0.09	
1200	0.03	0.02	0.05	
1300	0.02	0.01	0.04	
1400	0.02	0.00	0.00	
1500	0.02	0.01	0.06	
1600	0.01	0.00	0.00	
1700	0.01	0.00	0.00	
1800	0.00	0.00	0.00	
1900	0.00	0.00	0.00	

Slope	ui	mi	α	1/K
0° - 15°	0.36	0.28	0.19	
15° - 30°	0.38	0.45	0.29	
30° - 45°	0.22	0.25	0.27	0.16
45° - 60°	0.03	0.02	0.19	
60° - 75°	0.00	0.00	0.06	
75° - 81°	0.00	0.00	0.00	

Exposure	ui	mi	α	1/K
N	0.12	0.09	0.09	
NE	0.12	0.09	0.10	
E	0.11	0.09	0.10	
SE	0.12	0.16	0.17	0.12
S	0.13	0.14	0.13	
SO	0.13	0.14	0.14	
O	0.13	0.15	0.14	
NO	0.13	0.14	0.14	

Supplementary table S9. Manly index of *Bombina pachypus* for environmental variables considered.

Rana italica

Land Cover	ui	mi	α	1/K
11000	0.02	0.03	0.14	
12100	0.00	0.00	0.00	
12200	0.01	0.00	0.03	
21111	0.26	0.23	0.07	
21112	0.04	0.05	0.10	
21114	0.04	0.03	0.05	
21115	0.20	0.32	0.05	
21117	0.00	0.00	0.01	
21121	0.00	0.00	0.00	
21122	0.09	0.12	0.10	0.05
21131	0.01	0.01	0.04	
21132	0.06	0.06	0.08	
21210	0.00	0.00	0.01	
21220	0.13	0.09	0.05	
22110	0.04	0.02	0.04	
22120	0.05	0.02	0.04	
22200	0.03	0.01	0.04	
31000	0.00	0.00	0.06	
40000	0.00	0.00	0.00	

Altitude	ui	mi	α	1/K
0	0.00	0.00	0.00	
100	0.03	0.05	0.07	
200	0.05	0.05	0.05	
300	0.06	0.05	0.04	
400	0.07	0.05	0.03	
500	0.09	0.08	0.04	
600	0.09	0.04	0.02	
700	0.09	0.08	0.04	
800	0.09	0.07	0.04	
900	0.09	0.09	0.05	0.05
1000	0.06	0.06	0.04	
1100	0.06	0.04	0.03	
1200	0.05	0.07	0.06	
1300	0.05	0.13	0.13	
1400	0.04	0.06	0.08	
1500	0.03	0.05	0.07	
1600	0.02	0.02	0.05	
1700	0.01	0.01	0.05	
1800	0.00	0.01	0.11	
1900	0.00	0.00	0.00	

Slope	ui	mi	α	1/K
0° - 15°	0.28	0.42	0.37	0.16
15° - 30°	0.41	0.43	0.26	
30° - 45°	0.27	0.13	0.12	
45° - 60°	0.04	0.02	0.10	
60° - 75°	0.00	0.00	0.14	
75° - 81°	0.00	0.00	0.00	

Exposure	ui	mi	α	1/K
N	0.13	0.16	0.16	0.12
NE	0.14	0.17	0.16	
E	0.12	0.14	0.14	
SE	0.13	0.12	0.11	
S	0.14	0.11	0.10	
SO	0.13	0.10	0.10	
O	0.11	0.09	0.10	
NO	0.11	0.11	0.13	

Supplementary table S10. Manly index of *Rana italica* for environmental variables considered.

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