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Podarcis siculus latastei (Bedriaga, 1879) of the Western Pontine Islands (Italy) raised to the species rank, and a brief taxonomic overview of *Podarcis* lizards

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Abstract. In recent years, great attention has been paid to many *Podarcis* species for which the observed intra-specific variability often revealed species complexes still characterized by an unresolved relationship. When compared to other species, *P. siculus* underwent fewer revisions and the number of species hidden within this taxon may have been, therefore, underestimated. However, recent studies based on genetic and morphological data highlighted a marked differentiation of the populations inhabiting the Western Pontine Archipelago. In the present work we used published genetic data (three mitochondrial and three nuclear gene fragments) from 25 *Podarcis* species to provide a multilocus phylogeny of the genus in order to understand the degree of differentiation of the Western Pontine populations. In addition, we analyzed new morphometric traits (scale counts) of 151 specimens from the main islands of the Pontine Archipelago. The phylogenetic analysis revealed five principal *Podarcis* groups with biogeographic consistency. The genetic distinctiveness of the *Podarcis* populations of the Western Pontine Islands is similar or even more ancient than those observed in numerous other pairs of *Podarcis* sister species. In the light of these evidences we raise the Western Pontine lizards to specific rank; thus they should be referred to as *Podarcis latastei*.

Keywords. Reptilia, *Podarcis latastei*, *Podarcis siculus*, insular lizards, Mediterranean.

INTRODUCTION

The wall lizards belonging to the genus *Podarcis* Wagler, 1830 are among the most speciose vertebrates in Europe, representing one of the most important faunistic elements of the Mediterranean insular biota. Originally, two opposite taxonomic viewpoints ("lumping" and "splitting") were – partly emotionally – discussed in the late 19th and early 20th centuries. At that time the most prominent representative of the "lumpers" was George A. Boulenger (1859-1937) who joined numerous wall lizards together under the name "*Lacerta muralis*" distinguishing, as some predecessors did, only "varieties" within this species (Boulenger, 1887, 1905, 1913, 1920). His main antagonist, representing the taxonomic "splitter" faction, was Ludwig von Méhely (1862-1953) who considered many of Boulenger's "varieties" to be distinct species (Méhely, 1907, 1909). He wrote: «Like a night-mare, the so-called *muralis* question is burdening the mind of herpetologists» (Méhely, 1907). Despite modern approaches, molecular genetics included, Méhely was closer to the current concept than his more influential contemporary colleague; however, the number of *Podarcis* species is still debated. For example, 21 taxa were recognized as

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valid species by Speybroeck et al. (2010), whereas other authors have suggested 23 (Sindaco et al., 2013; Uetz and Hošek, 2016; but see Psonis et al., 2017). The taxonomic wavering of the genus *Podarcis* is mainly due to the presence of marked intra-specific variability with multiple species complexes characterized by unresolved relationships (Harris and Arnold, 1999; Oliverio et al., 2000; Harris et al., 2005; Lymberakis et al., 2008). Table 1 summarizes this taxonomic/nomenclatural history of the currently recognized *Podarcis* species.

In contrast to the great taxonomic attention paid to numerous *Podarcis* species, *P. siculus* has undergone fewer revisions and the number of species hidden within this taxon may have been underestimated. *Podarcis siculus* (Rafinesque-Schmaltz, 1810) was originally described as *Lacerta sicula*. However, because of its distribution over a large part of Italy (Sicily, Sardinia and numerous minor islands, islets and rocks) and Dalmatia, several subspecies were described. Some of them were originally assigned to "*Lacerta*" *muralis* (more than 90 were listed together with their type localities by Henle and Klaver, 1986). This situation led some authors to hypothesize the presence of a species complex similar to those observed in other *Podarcis* assemblages (Oliverio et al., 1998, 2000; Harris and Sa-Sousa, 2002). More recent studies based on mitochondrial (Podnar et al., 2005) and nuclear (Senczuk et al., 2017) markers have supported the monophyly of *P. siculus* and revealed surprisingly high genetic divergences between the main constituent evolutionary lineages, most comparable to those observed between many recognized *Podarcis* species (Harris et al., 2005). In addition, recent studies using molecular markers (mitochondrial and nuclear DNA) and geometric morphometrics have revealed that the populations from the Western Pontine Islands represent an evolutionarily independent unit (Senczuk et al., 2018a, 2018b). The genetic distances of these populations with respect to mainland ones were extraordinary high (*p*-distances of 7-10% for the mtDNA *cytb* gene), and the head morphology was clearly distinguishable with respect to the mainland and Sicilian populations (Senczuk et al., 2018a; 2018b).

The Pontine Archipelago is located 40 km off the Tyrrhenian coast and comprises the Western Pontine islands Ponza, Palmarola, Zannone and Gavi, and the Eastern Pontine islands Ventotene and Santo Stefano. From the Pontine Archipelago, the following nominal insular intraspecific taxa have been described: *Lacerta muralis* var. *latastei* = *Podarcis siculus latastei* (Bedriaga, 1879 a, b) from Ponza; *Lacerta muralis parkeri* = *Podarcis siculus parkeri* (Mertens, 1926) from Santo Stefano; *Lacerta sicula sancti-stephani* = *Podarcis siculus sanctistephani* (Mertens, 1926) from Santo Stefano; *Lacerta sicula ventotenensis* = *Podarcis siculus ventotenensis* (Taddei, 1949) from Ventotene; *Lacerta sicula pasquinii* = *Podarcis siculus pasquinii* (Lanza, 1952) from Scoglio Cappello near Palmarola; *Lacerta sicula patrizii* = *Podarcis siculus patrizii* (Lanza, 1952) from Zannone; *Lacerta sicula lanzai* = *Podarcis siculus lanzai* (Mertens, 1967) from Gavi and *Lacerta sicula palmarolae* = *Podarcis siculus palmarolae* (Mertens, 1967) from Palmarola (cfr. Lanza and Corti, 1996; Corti et al., 2010).

Podarcis siculus parkeri was synonymized with *P. s. sanctistephani* (Mertens and Wermuth, 1960; Mertens, 1965), which is believed to have become extinct during the first decades of the last century (1914 at the latest), and replaced by *P. s. siculus* (Mertens, 1965). Henle and Klaver (1986), reviewing the intraspecific taxa, followed Mertens (1965) in considering *P. s. ventotenensis* as a synonym of the nominotypical form, and listed *P. s. latastei*, *P. s. lanzai*, *P. s. pasquinii*, *P. s. patrizii* and *P. s. palmarolae* as valid subspecies. These five taxa occur in the Western Pontine Islands, which are believed to have been connected to the mainland in the Pleistocene, whereas the Eastern Pontine Islands (Ventotene, Santo Stefano) seem never to have been, being located along the 500 m isobath (Woldstedt, 1958; Mertens, 1965, 1967).

The deep genetic distance recently found between the Eastern and the Western Pontine Islands populations (Senczuk et al., 2018a), geometric morphometrics (Senczuk et al., 2018b), classical morphometric and meristic data, as well as an updated time calibrated multilocus phylogeny of *Podarcis* (Wagler, 1830), all suggest that the Western Pontine lizards deserve their own specific status and should be referred to as *Podarcis latastei* (Bedriaga, 1879), which we characterize and redescribe here.

MATERIALS AND METHODS

Molecular phylogenetics

To obtain a robust and time calibrated phylogeny of *Podarcis* as a whole, three mitochondrial (*16s; cytb*, and *nd4*) and three nuclear (*mc1r*, *pod15b* and *pod55*) gene fragments from 25 *Podarcis* species, including several subspecies, were retrieved from GenBank (all samples are reported in Fig. 1 and Table 1, localities and accession numbers are reported in Appendix 1, Table A1). Most of the retrieved sequences for each species belong to the same individual, when not possible we selected individuals of close geographic origin. All final consensus alignments were computed for each gene separately using BioEdit 7.2 (Hall, 1999). Coding gene fragments (*cytb*, *nd4* and *mc1r*) were translated into amino acids to assess the lack of stop codons.

For each alignment we used jModelTest v.2.1.3 (Darriba et al., 2012) to assess the best model of nucleotide evolution under the corrected Akaike Information Criterion (AICc). To

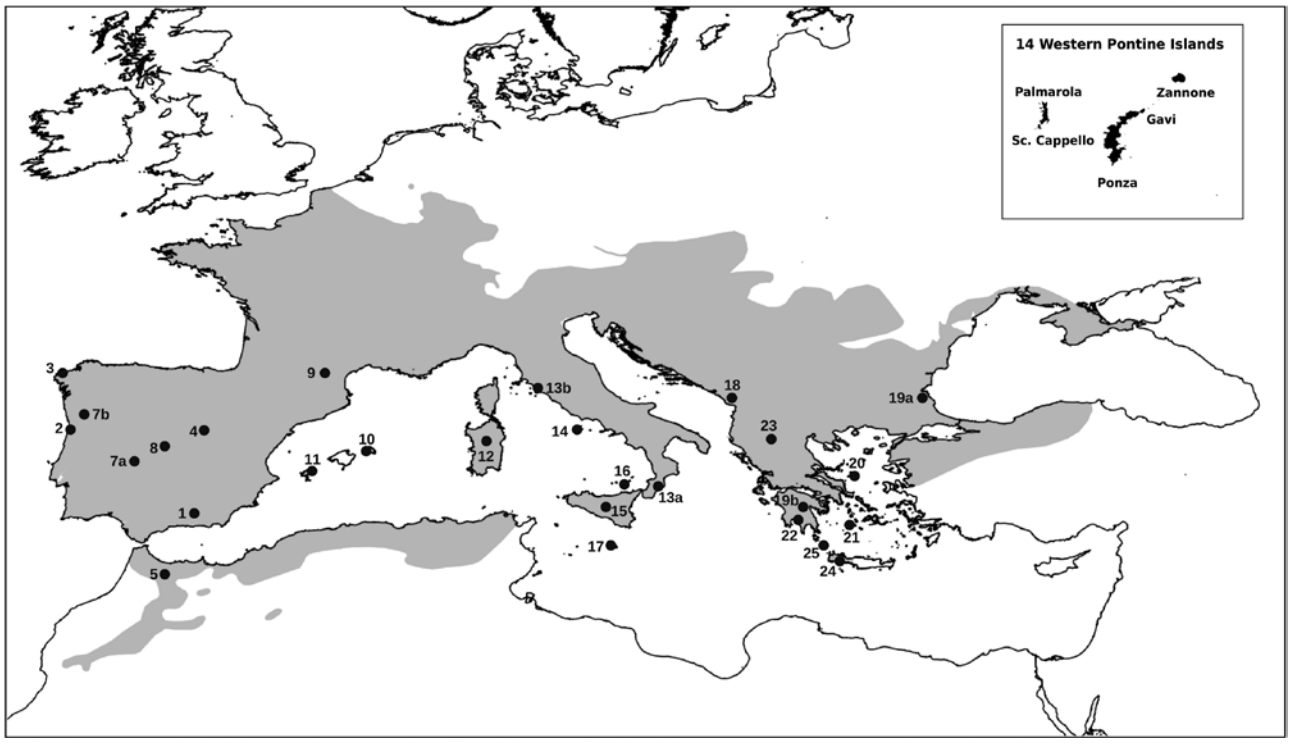

Fig. 1. Distribution of the genus *Podarcis* and location of the samples used for the phylogenetic analysis, as reported in Table 1. Geographic distribution of *Podarcis latastei* is also reported at the top right.

reconstruct phylogenetic relationships we used a Bayesian coalescent framework implemented in BEAST v.1.8 (Drummond et al., 2012). To calibrate the tree in absolute time we used two vicariant calibration points: the separation between the Peloponnesus (*P. peloponnesiacus*) and the islands of Crete and Pori (*P. cretensis* and *P. levendis*); and the separation between the islands of Menorca and Mallorca (*P. lilfordi*) and the Pytiusic Islands (*P. pytiusensis*). Both episodes occurred following the Messinian Salinity Crisis (MSC, at about 5.2 Mya) yielding the sudden separation of these landmasses (Poulakakis et al., 2003; Brown et al., 2008). A normal distribution using the mean in real space option ($\mu = 5.325$; SD = 0.2) has been incorporated for each of the aforementioned nodes. We used a Yule process in a linked tree partition and a lognormal relaxed model maintaining unlinked clock partitions. As substitution models we used GTR+I+G for *16s* and *cytb*; TVM+I+G for *nd4* and *mc1*r; HKY for *pod15b* and HKY+I for *pod55*. We performed three independent runs of 10^8 generations sampling every 10^4 steps. Convergence was checked using the software TRACER v 1.5 (Rambaut and Drummond, 2007) and after combining the trees using LogCombiner, the final consensus tree was computed in TreeAnnotator (Drummond et al., 2012).

Morphology

We used the measurements and scale counts published by Mertens (1967) for diagnosing the subspecific taxa of *P. siculus* recognized by him and compared them with our own data taken from the holdings deposited in the Florence Museum (MZUF). We measured and counted the scales of 151 specimens (60 females and 91 males) from the main islands of the Pontine Archipelago preserved at the Natural History Museum of the University of Florence (MZUF) (see Table 2). Specimens previously studied by Lanza (1952, 1967) and used for his descriptions of *P. s. patrizii* and *P. s. pasquinii* were also included. We analyzed sex, snout-vent length (SVL), and the following meristic characters: a) number of mid-body dorsal scales (DORS); b) number of ventral plates counted longitudinally along the intermediate row (VENT); c) number of gular scales counted along the throat mid-line from the collar to the confluence of maxillaries (GUL); d) number of collar scales (COLL); e), number of femoral pores on the right leg (PORF).

To test for significance of differences between sexes and islands, we used a two-way analysis of variance (ANOVA). An additional two-way ANOVA was performed to test differences between Ventotene Island and Santo Stefano Island sampled in 1954 and 1966, and the Western Pontine and Santo Stefano Island sampled in 1878.

RESULTS AND DISCUSSION

The final alignment of 3117 bp included 27 taxa (Supplementary Information). The three independent runs

Table 1. Currently accepted *Podarcis* species and their original description name and reference. The geographic localities are shown in Fig. 1.

Loc.	Species (Author and year of description)	Described as	Reference
1	*P. hispanicus* (Steindachner, 1870)	*Lacerta oxycephala* var. *hispanica*	Geniez et al. 2007
2	*P. carbonelli* Pérez-Mellado, 1981	*Podarcis bocagei carbonelli*	Sá-Sousa and Harris, 2002
3	*P. bocagei* (Lopez-Seoane, 1885)	*Lacerta murals bocagei*	Sá-Sousa et al., 2000
5	*P. liolepis* (Boulenger, 1905)	*Lacerta muralis var. liolepis* *Lacerta muralis atrata*	Geniez et al., 2014
6	*P. vaucheri* (Boulenger, 1905)	*P. hispanicus vaucheri*	Oliverio et al., 2000
7a, 7b	*P. guadarramae* (Boscá, 1916)	*Lacerta muralis guadarramae,* *Podarcis hispanicus* "type 1A, 1B"	Geniez et al., 2014
8	*P. virescens* (Geniez et al., 2014)	*Podarcis hispanicus* "type 2"	Geniez et al., 2014
9	*P. muralis* (Laurenti, 1768)	*Lacerta muralis*	
10	*P. lilfordi* (Günther, 1874)		
11	*P. pityusensis* (Boscá, 1883)	*Lacerta muralis* var. *pityusensis*	
12	*P. tiliguerta* (Gmelin, 1789)	*Lacerta tiliguerta*	
13a, 13b	*P. siculus* (Rafinesque-Schmaltz, 1810)	*Lacerta sicula*	
14	*P. latastei* (Bedriaga, 1876)		
15	*P. waglerianus* (Gistel, 1868)	*Podarcis muralis* var. *wagleriana*	
16	*P. raffoneae* (Mertens, 1952)	*Lacerta sicula raffonei*	Capula, 1994
17	*P. filfolensis* (Bedriaga, 1876)		
18	*P. melisellensis* (Braun, 1877)		
19a, 19b	*P. tauricus* (Pallas, 1814)		
20	*P. gaigeae* (Werner, 1930)	*Lacerta taurica gaigeae*	
21	*P. milensis* (Bedriaga, 1882)	*Lacerta muralis fusca* var. *milensis*	
22	*P. peloponnesiacus* (Bibron and Bory, 1833)		
23	*P. erhardii* (Bedriaga, 1882)	*Lacerta muralisfusca* var. *erhardii*	
24	*P. cretensis* (Wettstein, 1952)	*Lacerta erhardii cretensis*	
25	*P. levendis* (Lymberakis et al., 2008)		

Table 2. Population number and relative sample size for both males and females for each island. *Individuals collected in Santo Stefano Island in 1954/1966.

N°	Island	Females	Males
1	Ponza	18	22
2	Gavi	3	9
3	Palmarola	11	21
4	Zannone	6	6
5	Santo Stefano (1878)	5	5
	Santo Stefano*	5	14
6	Ventotene	8	12
7	Scoglio Cappello	4	2
	Tot.	60	91

showed Effective Sample Size (ESS) for each parameter of more than 200. The phylogenetic tree obtained is shown in Fig. 2. The tree topology is rather well supported (most of the nodes showed posterior probabilities higher than 0.95) and the relationships among species only partly corresponds to previous phylogenetic reconstructions. Within the *Podarcis* radiation we found five principal groups with biogeographic consistency (Fig. 1-2).

1 – The *Podarcis hispanicus* complex currently includes seven species distributed from North Africa to the Iberian Peninsula and south-western France. All species from the *P. hispanicus* complex were first described as intraspecific taxa of the collective species *P. muralis* and later raised to species rank in order to resolve paraphyly (see Table 1) (Oliverio et al., 2000; Sá-Sousa and Harris, 2002; Geniez et al., 2007, 2014). Our phylogenetic analysis support a similar phylogenetic relationships among species as previously reported, and suggested, albeit with moderate support (0.91), *P. muralis* as the sister species of all the Iberian *Podarcis*.

2 – The "*erhardii*" group comprises species of the Balkan Peninsula and the Greek islands. Because of a paraphyletic relationship between *P. erhardii* (Bedriaga, 1882) and *P. peloponnesiacus* (Bibron and Bory, 1833), two new insular endemics *P. cretensis* (Wettstein, 1952) and *P. levendis* (Lymberakis et al., 2008) were raised to

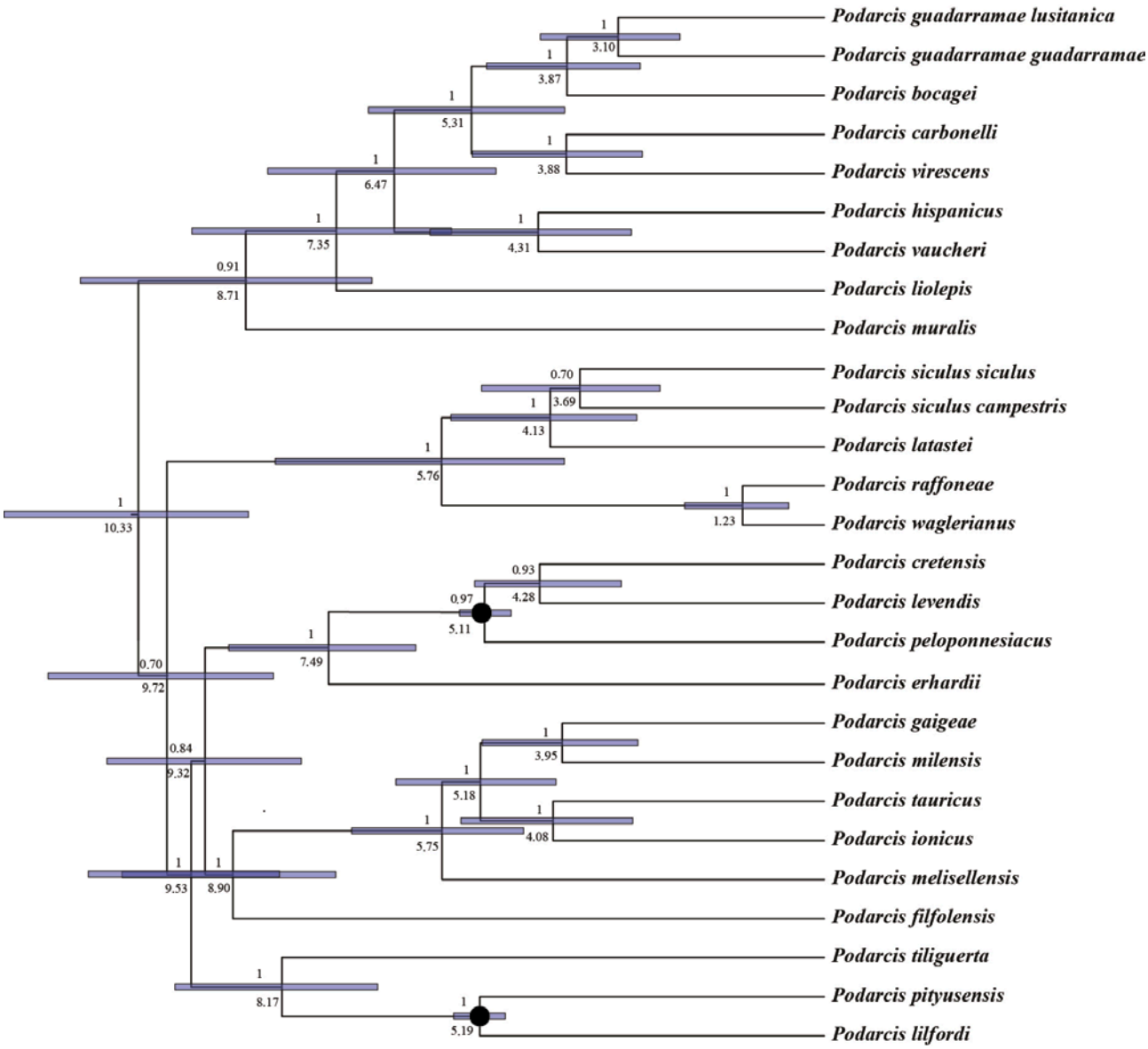

Fig. 2. Bayesian phylogenetic tree based on multilocus data (*cytb*, *16s*, *nd4*, *mc1r*, *pod15b* and *pod55*) using BEAST v. 1.8. Black filled circles indicate nodes used to calibrate phylogeny (Poulakakis et al., 2003; Brown et al., 2008). The times of the most recent common ancestor are reported for each node as well as the posterior probability.

the species rank (Poulakakis et al., 2003; 2005a; Lymberakis et al., 2008).

3 – The "*tauricus*" group includes two species *P. tauricus* (Pallas, 1814) and *P. melisellensis* (Braun, 1877) distributed over a large part of the Balkans and two endemic insular species: *P. gaigae* (Werner, 1930) from Skyros and surrounding islands, and *P. milensis* (Bedriaga, 1882) from Milos and surrounding islands (Poulakakis et al., 2005a, 2005b). However, a recent species delimitation approach (Psonis et al., 2017), suggested the presence of nine species within the *tauricus* group: *P. melisellensis, P. gaigeae, P. milensis*, and six in the *P. tauricus* complex. Based on the absence of support to the monophyly of *P. tauricus*, the authors proposed to raise the subspecies *P. t. ionicus* (Lehrs, 1902) to the species rank (Psonis et al., 2017). Our phylogenetic analysis confirms this scenario indicating an ancient divergence between *P. ionicus* and *P. tauricus* (Fig. 2).

It is interesting to note that although the geographic distribution of *P. filfolensis* (Bedriaga, 1876) would suggest a close relationship with the other two endemic species of the Siculo-Maltese area, *P. waglerianus* (Gistel,

Table 3. Scale counts after Mertens (1965) (minimum – **mean** – maximum) of the insular populations of *P. latastei* (the six left columns) and *P. siculus* (the two right columns). DORS = no. of mid-body dorsal scales; VENT = no. of ventral plates; COLL = no. of collar scales; PORF = no. of femoral pores on the right leg; m. = males; f. = females.

		Ponza	Gavi	Zannone	Palmarola	Sc. Cappello	S. Stefano 1878	S. Stefano 1963	Ventotene
DORS	m	68-**70.4**-75	71-**76.3**-81	66-**72.8**-78	69-**76.6**-86	72-**73.2**-76	72-**75.8**-79	60-**65.6**-72	61-**66.8**-78
	f	62-**67.7**-73	70-**73.2**-78	63-**68.0**-74	66-**68.7**-71	66-**68.7**-71	71-**75.3**-79	59-**61.2**-63	60-**60.3**-68
VENT	m	25-**26.1**-27	26-**26.3**-27	25-**26.7**-28	24-**25.2**-26	25-**25.7**-26	25-**26.3**-27	24-**24.8**-26	22-**24.6**-26
	f	27-**28.1**-29	27-**28.0**-30	27-**28.4**-30	27-**28.2**-29	28-**28.7**-30	27-**28.1**-30	27-**28.0**-29	25-**26.9**-29
COLL.	m	9-**10.8**-12	9-**10.0**-11	9-**10.5**-12	9-**10.4**-13	10-**10.5**-11	12-**12.1**-13	8-**9.1**-11	9-**10.6**-12
	f	10-**10.7**-11	9-**10.2**-11	9-**10.5**-11	10-**10.7**-11	11-**11.0**-11	10-**11.0**-12	7-**8.2**-09	8-**9.8**-11
PORF.	m	22-**24.8**-29	22-**24.3**-26	22-**25.1**-28	21-**24.9**-29	22-**24.7**-28	24-**25.3**-28	19-**23.8**-26	20-**23.5**-27
	f	21-**23.8**-28	22-**24.7**-26	19-**23.0**-25	22-**24.1**-26	23-**24.5**-26	22-**24.8**-27	20-**21.6**-24	20-**22.0**-23

Table 4. Snout-vent length (SVL) and scale counts (minimum – **mean** – maximum) of specimens preserved at the Natural History Museum of the University of Florence (MZUF). DORS = no. of mid-body dorsal scales; VENT = no. of ventral plates; GUL = no. of gular scales; COLL = no. of collar scales; PORF = no. of femoral pores on the right leg; m. = males; f. = females. *Individuals collected in Santo Stefano Island in 1954/1966.

		Ponza	Gavi	Zannone	Palmarola	Sc. Cappello	S. Stefano 1878	S. Stefano*	Ventotene
SVL	m.	58-**68.6**-78.8	70.5-**73.2**-78	70.4-**67.1**-76.2	58-**67**-75	62.5-**65**-67.5	69-**76.1**-81.5	67-**73.5**-81.6	60-**70.7**-77
	f.	50-**58.6**-68.6	61-**63.5**-67	52.6-**62.7**-76.1	57.8-**52**-63	56.5-**57.9**-60	55-**63.1**-69	59-**63.5**-70	53.5-**60.4**-66
DORS	m.	67-**70.1**-76	73-**74.3**-77	69-**70.1**-73	68-**72.4**-76	**72**	68-**71**-74	59-**64.1**-69	62-**66**-70
	f.	62-**68.2**-74	67-**72**-75	63-**67.3**-74	63-**68**-74	65-**67**-69	69-**71.8**-75	55-**58**-63	57-**61.4**-67
VENT	m.	18-**19.9**-21	22-**23**-24	19-**21.3**--20	18-**19.3**-21	20-**20.**5-21	20-**20.3**-21	17-**18.**9-21	17-**19.2**-20
	f.	22-**22.7**-24	18-**19.9**-23	22-**22.2**-23	21- **22.4**-24	24	19-**21**-23	20-**21.4**-23	21-**21.6**-23
COLL	m.	11-**12.7**-14	11-**12.3**-13	10-**12.1**-13	10-**12.4**-15	12-**13**-14	13-**14**-15	9-**12.3**-16	10-**12.6**-15
	f.	11-**13**-15	11-**13**-15	10-**11.5**-13	11-**12.8**-14	12-**12.8**-13	12-**13.4**-15	9-**11.2**-12	11-**12.9**-16
GUL	m.	28-**32.4**--37	31-34.**8**-38	31-**33.7**-40	27-**33**--39	31	34-**34.3**-35	23-**26.8**-32	27-**30.1**-34
	f.	27-**31.2**--35	32-33.3-35	31-**33**-36	27-**33**--36	23-**24**-25	33-**36.4**-40	23-**25.4**-28	25-**27**-29
PORF	m.	22-24.5-26	23-**24.7**-26	23-**25**-26	21-**24.8**-28	25-**25.5**-26	26-**26.5**-27	21-**22.2**-24	21-**23.7**-27
	f.	21-**23.9**-27	22-**24.4**-27	21-**23.3**-27	21-**22.8**-26	23-**24**-25	23-**27.4**-30	20-**20.8**-21	20-**22**-25

1868) and *P. raffoneae* (Mertens, 1952), previous molecular analysis has resulted in contrasting phylogenies regarding the position of these three species (Harris et al., 2005; Psonis et al., 2017; Salvi et al., 2017). Our phylogenetic reconstruction supports a tangled evolutionary history indicating *P. filfolensis* as the sister species of the *Podarcis* "*tauricus*" group (Fig. 2).

4 – *Podarcis* species from the Western Mediterranean islands include *P. tiliguerta* (Gmelin, 1789), *P. lilfordi* (Günther, 1874) and *P. pityusensis* (Boscá, 1883). *Podarcis tiliguerta* distributed in Sardinia, Corsica and surrounding islands, has also been argued to be a species complex showing very deep phylogeographic discontinuities (Harris et al., 2005; Rodriguez et al., 2017; Salvi et al., 2017; Senczuk et al., 2019). On the other hand, *P. lilfordi* and *P. pityusensis* from the Balearic and Pityusic islands showed closer phylogenetic relationship as a consequence of vicariance following the Messinian Salinity Crisis (Brown et al., 2008). The phylogenetic reconstruction reported here, confirms the close relationship of these endemic Western Mediterranean species.

5 – *Podarcis* species from the Italian Peninsula, Sicily and surrounding islands forms a monophyletic assemblage that includes *P. siculus, P. waglerianus* and *P. raffoneae*. The last of these was raised to the species rank on the basis of allozyme analysis although further studies showed relatively low genetic distances from *P. waglerianus* (3.3% at cytochrome b), far lower than those observed between many other *Podarcis* species (Capula, 1994; Harris et al., 2005). Based on our data, the lineage including *P. waglerianus* and *P. raffoneae* is sister to *Podarcis siculus* and the lizards of the Western Pontine Archipelago. The Western Pontine *Podarcis* are separated from *P. siculus* by approximately 4 Mya based on our results. The genetic distinctiveness of these insular populations is comparable or even greater than several other

Table 5. Analysis of variance (ANOVA) for SVL and meristic characters of the MZUF specimens. Significant *p*-value at 0.05 are marked in bold. Degrees of freedom (d.f.) are also reported. In the last column, ANOVA results using the endemic insular taxon (*P. latastei* + the extinct *P. s. sanctistephani*) and introduced *P. siculus* as factors, are reported. SVL = snout-to-vent length; DORS = no. of mid-body dorsal scales; VENT = no. of ventral plates; GUL = no. of gular scales; COLL = no. of collar scales; PORF = no. of femoral pores. *Individuals collected in Santo Stefano Island in 1954/1966.

		SEX	Islands	S. Stefano 1878 + W. Pontine/ S. Stefano* + Ventotene
	d.f.	1	7	2
SVL	F	152.96	8.73	4.54
	p	**<0.001**	**<0.001**	**<0.05**
DORS.	F	40.73	27.15	57.42
	p	**<0.001**	**<0.001**	**<0.001**
VENT.	F	259.4	9.88	5.4
	p	**<0.001**	**<0.001**	**<0.01**
COLL.	F	1.74	2.9	4.44
	p	0.18	**<0.01**	**<0.05**
GUL.	F	5.71	24.86	62.13
	p	**<0.05**	**<0.001**	**<0.001**
PORF.	F	15.41	10.71	57.42
	p	**<0.001**	**<0.001**	**<0.001**

pairs of *Podarcis* sister species (i.e., *P. bocagei*/*P. guadarramae*, *P. carbonelli*/*P. virescens*, *P. cretensis*/*P. levendis*, *P. gaigeae*/*P. milensis*, *P. tauricus*/*P. ionicus*).

Our morphological analysis substantially confirms what Bedriaga (1879a) and Mertens (1965) already observed. Indeed, we found significant differences comparing the specimens of the Western Pontine Islands and the Santo Stefano Island collected in 1878, with those collected in Santo Stefano in 1954/1966 and Ventotene Island (Tables 3, 4 and 5; Fig. A1). Furthermore, we also found significant differences when considering sexes and islands as factors (Tables 3, 4 and 5; Fig. A1). Smaller dorsal scales (resulting in higher dorsal scales counts) were already reported by Bedriaga (1879a) to characterize his new taxon *latastei*. Slight discrepancies between the scale counts taken by Mertens (1967) as compared with ours (Tables 3 and 4) are likely due to a different counting method, which was not precisely defined in Mertens' (1967) paper, e.g., in the number of oblique ventral rows which is dependent on whether only complete or also incomplete rows are counted.

Based on multiple sources of evidence from genetics (herein and Senczuk et al., 2018a), morphology (herein and in Senczuk et al., 2018b) we believe that this insular endemic taxon deserves specific rank and should be referred to as *Podarcis latastei* (Bedriaga, 1879). We propose to adopt as common name "Lataste's lizard" for this

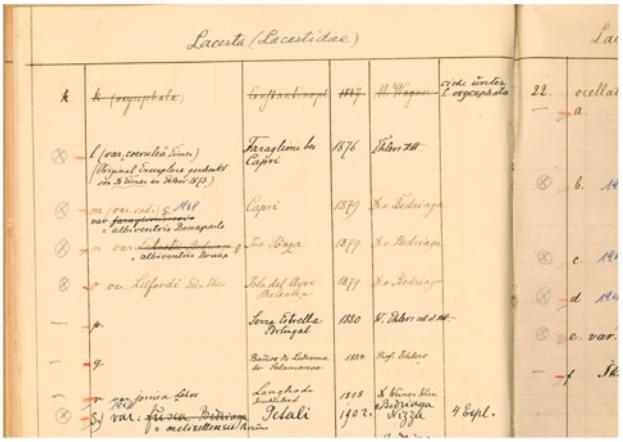

Fig. 3. Detail of the entry of a *P. latastei* specimen from Ponza collected by J. v. Bedriaga, in the catalogue of the Göttingen Zoological Museum.

species, following Bedriaga, (1979b).

In accepting the specific status for the lizards of the Western Pontine Islands, under the oldest name available *Podarcis latastei* (Bedriaga, 1879), type locality Ponza Island, we nevertheless accept the infraspecific subdivisions within the Western Pontine Islands assigned by earlier authors to *P. siculus*. This means that the former subtaxa of the latter taxon, viz. *patrizii, pasquinii, lanzai* etc. now become subspecies of *P. latastei*. The various island populations of *Podarcis latastei* in the Western Pontine Archipelago exhibit variable color patterns. The patterned color morphs often show a tendency for longitudinal stripes to dissolve into oblique bands, thus forming a reticulate pattern with light ocelli included (Fig. 4, 5 and 6). In other individuals, particularly from Gavi Island (Fig. 5a, 5b) there is a strong tendency for a reduction of black-pigmented color pattern elements, corresponding to the "*concolor*" mutation that also occurs in other *Podarcis* species. These differences in body dimensions, scalation and color pattern justify, in our opinion, the maintenance of their subspecific names, at least for conservation purposes (Senczuk et al., 2018a).

Bedriaga (1879a) based his nomen *latastei* on an unknown number of individuals – "in Anzahl" which means "in a certain quantity" – collected by himself on Ponza Island in Summer 1878, plus one individual from a rock west off Ponza which he called Faraglioni of Ponza. Obviously, he kept all specimens in a cage alive during his travel and brought them via Nice (Nizza), France, from where he sent a part of them to F. Lataste to Paris, to his residential town of Heidelberg, Germany, where he continued to observe them in life, mainly in respect to colour change phenomena (Bedriaga, 1879a). In 1879 and 1902 he sent preserved specimens to some German museums,

Fig. 4. Detail of plate IX. (Bedriaga, 1879b) with a specimen (right) of his "*Lacerta muralis* var. *latastei* (= *Podarcis latastei*).

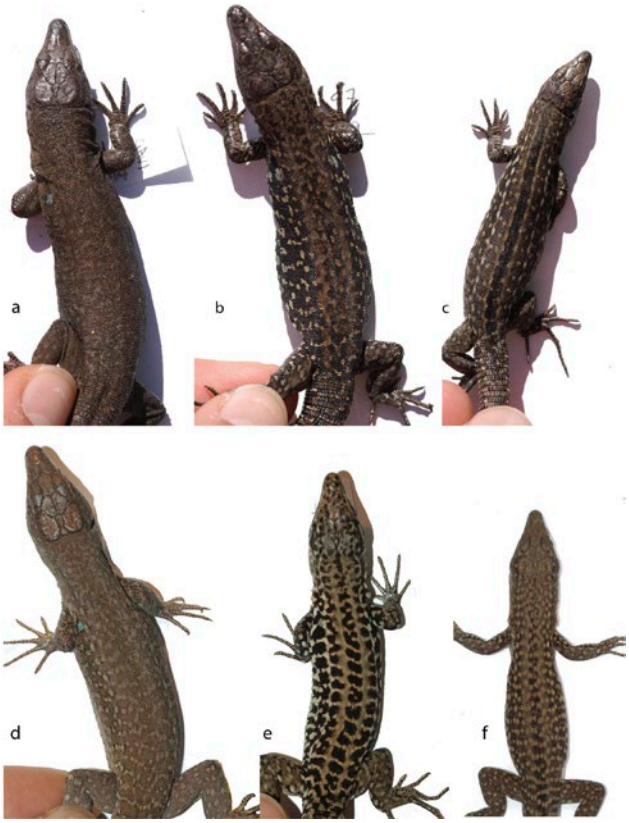

Fig. 5. Examples of living representatives of the Pontine Islands populations. Gavi: a, b, c; Ponza: d, e; Palmarola: f.

including Frankfurt and Munich as well as the Zoological Museum of the University of Göttingen (whose herpetological holdings have been in Bonn since 1977), and one specimen of his Ponza lizard is still documented in the old Göttingen catalogue, although unfortunately it was lost some time before 1968 (Böhme, 2014, Fig. 3). We failed to retrieve any of these old syntypes in any of the mentioned collections. So, there seems to be no extant type material of this taxon, and the single colour image provided by Bedriaga (1879b), can be regarded as the figure of the individual that could have been chosen as a lectotype if it would be still extant (Fig. 4). A neotype selection, however, seems presently to be unnecessary in this case.

According to the genetic and geometric morphometric data published by Senczuk et al. (2018a, b) and to our data, the wall lizards of the Western Pontine Islands, so far classified as belonging to *Podarcis siculus*, clearly merit their own specific status und should be treated under the oldest

Fig. 6. Two different color morphs from Zannone Island: a "quasi" concolor individual above and a dark reticulated individual below.

available name for these Western Pontine populations, i.e., *Podarcis latastei* (Bedriaga, 1879). Because of the marked morphological differences between these populations, their former insular subspecific names (Ponza: *latastei*, Gavi: *lanzai*, Zannone: *patrizii*, Palmarola: *palmarolae*, and Scoglio Cappello: *pasquinii*) which were ranked as subspecies of *P. siculus* before, should be maintained but now attached as subspecific names to *Podarcis latastei*. Each of these island populations has its own characteristics and may well turn out to be a distinct conservation unit.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 25159.

REFERENCES

Bedriaga, J. von (1879a): Herpetologische Studien (Fortsetzung). Arch. f. Naturges. **45**: 234-339.

Bedriaga, J. von (1879b): Mémoire sur les variétés européennes du Lézard des murailles. Bull. Soc. zool. France **4**: 194-228.

Böhme, W. (2014): Herpetology in Bonn. Mertensiella **21**: 1-256.

Boulenger, G.A. (1887): Catalogue of the lizards in the British Museum (Natural History). III. (Lacertidae, Gerrhosauridae, Scincidae, Anelytropsidae, Dibamidae, Chamaeleontidae). Trustees of the British Museum, London.

Boulenger, G.A. (1905): A contribution to our knowledge of the varieties of the wall lizard (*Lacerta muralis*). Trans. Zool. Soc. London **17**: 351-436.

Boulenger, G.A. (1913): Second contribution to our knowledge of the varieties of the wall lizard (*Lacerta muralis*). Trans. Zool. Soc. London **20**: 135-231.

Boulenger, G.A. (1920): Monograph of the Lacertidae. Vol. 1. Trustees of the British Museum, London.

Brown, R.P., Terrasa, B., Pérez-Mellado, V., Castro, J.A., Hoskisson, P.A., Picornell, A., Ramon, M.M. (2008). Bayesian estimation of post-Messinian divergence times in Balearic Island lizards. Mol. Phylogenet. Evol. **48**: 350-358.

Capula, M. (1994): Genetic variation and differentiation in the lizard, *Podarcis wagleriana* (Reptilia: Lacertidae). Biol. J. Linn. Soc. **52**: 177-196.

Corti C., Biaggini M., Capula M. (2010): *Podarcis siculus* (Rafinesque-Schmaltz, 1810). In: Fauna d'Italia. Reptilia, pp. 407-417. Corti C., Capula M., Luiselli L., Razzetti E., Sindaco R., Eds, Edizioni Calderini, Bologna, Italy.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D. (2012): jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods **9:** 772-772.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A. (2012): Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution **29**: 1969-1973.

Geniez, P., Cluchier, A., Sa-Sousa, P., Guillaume, C.P., Crochet, P.A. (2007): Systematics of the *Podarcis hispanicus* complex (Sauria,Lacertidae) I: Redefinition, morphology and distribution of the nominotypical taxon. Herpetol. J. **17**: 69-80.

Geniez, P., Sa-Sousa, P., Guillaume, C.P., Cluchier, A., Crochet, P.A. (2014): Systematics of the *Podarcis hispanicus* complex (Sauria, Lacertidae) III: valid nomina of the western and central Iberian forms. Zootaxa **3794**: 1-51.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41**: 95-98.

Harris, D.J., Arnold, E.N. (1999): Relationships of Wall Lizards, *Podarcis* (Reptilia: Lacertidae) based on mithocondrial DNA sequences. Copeia **1999**: 749-754.

Harris, D.J., Pinho, C., Carretero, M.A., Corti, C., Böhme, W. (2005): Determination of genetic diversity within the insular lizard *Podarcis tiliguerta*, using mtDNA sequence data, with a reassessment of the phylogeny of *Podarcis*. Amphibia-Reptilia **26**: 401-407.

Harris, D.J., Sà-Sousa (2002): Molecular phylogenetics of Iberian wall lizards (*Podarcis*): is *Podarcis hispanica* a species complex? Mol. Phylogenet. Evol. **23**: 75-81.

Henle, K., Klaver, C.J.J. (1986): *Podarcissicula* Rafinesque-Schmaltz, 1810 – Ruineneidechse. In: Handbuch der Reptilien und Amphibien Europas, vol. 2/II (Echsen III, *Podarcis*), pp. 254-342. Böhme, W., Ed., AULA Verlag, Wiesbaden, Germany.

Lanza, B. (1952): Note critiche su alcune lucertole italiane e diagnosi preliminare di una nuova razza insulare. Riv. Sci. Nat., Natura **43**: 69-82.

Lanza, B. (1967): Su due nuove razze insulari di *Lacerta sicula* e di *Lacerta tiliguerta*. Arch. Zool. Ital. **51**: 511-522.

Lanza, B., Corti, C. (1996). Evolution of the knowledge on the Italian herpetofauna during the 20th century. Boll. Mus. civ. St. Nat. Verona **20**: 373-436.

Lymberakis, P., Poulakakis, N., Kaliontzopoulou, A., Valakos, E., Mylonas, M. (2008): Two new species of *Podarcis* (Squamata; Lacertidae) from Greece. Syst. Biodiv. **6**: 307-318.

Méhely, L. v. (1907): Zur Lösung der „*Muralis*-Frage". Vorläufige Mitteilung. Ann. Mus. Natl. Hungar. **5**: 84-88.

Méhely, L. v. (1909): Materialien zu einer Systematik und Phylogenie der *muralis*-ähnlichen Lacerten. Ann. hist.-nat. Mus. Natl. Hungar. **7**: 409-621.

Mertens, R. (1926): Zwei neue Inselrassen der Gattung *Lacerta*. Zool. Anz. **68**: 319-322.

Mertens, R. (1965): Das Rätsel der Eidechsen von Santo Stefano. Zool. Jahrb., Abt, Syst. **92**: 91-102.

Mertens, R. (1967): Unterlagen zu einer „Herpetologia tyrrhenica" VIII. Die Reptilien der Pontinischen Inseln. Senckenberg. biol. **44**: 125-144.

Mertens, R., Wermuth, H. (1960): Die Amphibien und Reptilien Europas (Dritte Liste nach dem Stand von vom 1. Januar 1960). Kramer, Frankfurt am Main.

Oliverio, M., Bologna, M.A., Mariottini, P. (2000): Molecular biogeography of the Mediterranean lizards *Podarcis* Wagler, 1830 and *Teira* Gray, 1838 (Reptilia, Lacertidae). J. Biogeogr. **27**: 1403-1420.

Podnar, M., Mayer, W., Tvrtković, N. (2005): Phylogeography of the Italian wall lizard, *Podarcis sicula*, as revealed by mitochondrial DNA sequences. Mol. Ecol. **14**: 575-588.

Poulakakis, N., Lymberakis, P., Antoniou, A., Chalkia, D., Zouros, E., Mylonas, M., Valakos E. (2003): Molecular phylogeny and biogeography of the wall-lizard *Podarcis erhardii* (Squamata: Lacertidae). Mol. Phylogenet. Evol. **28**: 38-46.

Poulakakis, N., Lymberakis, P., Valakos, E., Pafilis, P., Zouros, E., Mylonas, M. (2005): Phylogeography of Balkan wall lizard (*Podarcis taurica*) and its relatives inferred from mitochondrial DNA sequences. Mol. Ecol. **14**: 2433-2443.

Poulakakis, N., Lymberakis, P., Valakos, E., Zouros, E., Mylonas, M. (2005b): Phylogenetic relationships and biogeography of *Podarcis* species from the Balkan Peninsula, by Bayesian and maximum likelihood analyses of mitochondrial DNA sequences. Mol. Phylogenet. Evol. **37**: 845-857.

Psonis, N., Antoniou, A., Kukushkin, O., Jablonski, D., Petrov, B., Crnobrnja-Isailović, J., Poulakakis, N. (2017): Hidden diversity in the *Podarcis tauricus* (Sauria, Lacertidae) species subgroup in the light of multilocus phylogeny and species delimitation. Mol. Phylogenet. Evol. **106**: 6-17.

Rambaut, A., Suchard, M., Xie, D., Drummond, A. (2014): Tracer v1. 6. http://beast.bio.ed.ac.uk/Tracer/ [*accessed on 29 May 2015*]

Rodríguez, V., Buades, J.M., Brown, R.P., Terrasa, B., Pérez-Mellado, V., Corti, C., Delaugerre, M., Castro, J. A., Picornell, A., Ramon, C. (2017): Evolutionary history of *Podarcis tiliguerta* on Corsica and Sardinia. BMC Evol. Biol., **17**: 27.

Salvi, D., Pinho, C., Harris, D.J. (2017): Hotspot of genetic diversity: decoupled mito-nuclear histories in the evolution of the Corsican-Sardinian endemic lizard *Podarcis tiliguerta.* BMC Evol. Biol. **17**: 63.

Sà-Sousa, P., Harris, D.J. (2002): *Podarcis carbonelli* Pérez-Mellado, 1981 is a distinct species. Amphibia-Reptilia **23**: 459-468.

Senczuk, G., Colangelo, P., De Simone, E., Aloise, G., Castiglia, R. (2017): A combination of long term fragmentation and glacial persistence drove the evolutionary history of the Italian wall lizard *Podarcis siculus*. BMC Evol. Biol. **17**: 6.

Senczuk, G., Havenstein, K., Milana, V., Ripa, C., De Simone, E., Tiedemann, R., Castiglia, R. (2018a): Spotlight on islands: on the origin and diversification of an ancient lineage of the Italian wall lizard *Podarcis siculus* in the western Pontine Islands. Sci. Rept. **8**: 15111.

Senczuk, G., Colangelo, P., Avramo, V., Castiglia, R. Böhme, W., Corti, C. (2018b): A study in scarlet: incipient speciation, phenotypic differentiation and conservation implications of the *Podarcis* lizards of the western Pontine Islands, Italy. Biol. J. Linn. Soc. **125**: 50-60.

Senczuk, G., Castiglia, R., Colangelo, P., Delaugerre, M., Corti, C. (2019): The role of island physiography in maintaining genetic diversity in the endemic Tyrrhenian wall lizard (*Podarcis tiliguerta*). J. Zool. **309**: 140-151.

Sindaco, R, Venchi, A, Grieco, C. (2013): The reptiles of the western Palearctic, Volume 2 Annotated checklist and distributional atlas of the snakes of Europe, North Africa, Middle East and Central Asia, with an update to Volume 1. Edizioni Belvedere, Latina, Italy.

Speybrock, J., Beukema, W., Crochet, P.-A. (2010): A tentative species list of the European herpetofauna (Amphibia and Reptilia) – an update. Zootaxa **2492**: 1-27.

Taddei, A. (1949): Le Lacerte (*Archaeolacerta* e *Podarcis*) dell'Italia peninsulare e delle isole. Coment. Pont. Acad. Sci. Vaticano **13**: 197-174.

Woldstedt, P. (1958): Das Eiszeitalter. Grundlinien einer Geologie des Quartärs, II. Europa, Vorderasien und Nordafrika im Eiszeitalter, 2nd Edition. Enke, Stuttgart, Germany.

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Substrate type has a limited impact on the sprint performance of a Mediterranean lizard

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Abstract. Environmental factors may affect animal performance in diverse ways, even among different populations of a single species. Here, we assess the impact of substrate type on the sprint performance (maximum speed and acceleration) of Schreiber's fringe-fingered lizard (*Acanthodactylus schreiberi*). This species is a skillful runner that also bears micro spike-like protruding scales on its toepads (toe fringes), an adaptation for locomotion on sand. We worked with three populations living in habitats that differ in substrate type (sand, soil and rock). We measured sprint performance using a race-track with custom substrate platforms replicating the different substrate types. We formulated two hypotheses: first, we anticipated that the three populations would differ in their sprint performance due to the differences in substrate type; second, we expected that each population would perform better on its home substrate. Our results generally refuted the hypothesis that sprint performance would differ on different substrate types. Our results suggest that there is a restricted effect of substrate type on locomotion and indicate a multifactor interplay among alternative underlying parameters.

Keywords. Ecophysiology, morphology, locomotion, Lacertidae, Cyprus.

INTRODUCTION

Sprint performance is very important for all animals, as it affects most of their daily activities. Sprinting is quite common among lizards during foraging, antipredator defense and inter- and intraspecific competitive behavior (Losos and Irschick, 1996; Husak et al., 2006; McElroy et al., 2008). Speed and acceleration, the main components of sprint performance, may be crucial for the overall fitness of individuals (Jayne and Bennett, 1990; Robson and Miles, 2000; Miles, 2004). Interactions between the ecology and morphology of species act as driving factors that exert strong selective pressures leading to optimal locomotor performance (Van Damme et al., 2003; Husak et al., 2006). For instance, gekkonid and lacertid lizards were shown to adopt different locomotion patterns because of their distinct ecology (Aerts et al., 2000). Also, Losos (1990) reported that locomotion parameters and morphological features evolved concordantly in 15 *Anolis* species.

The results of previous studies on the effects of substrate type on locomotion are puzzling (Korff and McHenry, 2011; Tulli et al., 2012; Vanhooydonck et al., 2015). The texture complexity (e.g., particle size, shape and roughness) of the substrate type is also known to affect locomotor performance (Brandt et al., 2015; Bergmann et al., 2017). The propulsive forces applied by the toes on non-solid substrates (e.g., sand) may be reduced because of insufficient grip and friction with the substrate, therefore leading to suboptimal sprint performance (Redfern et al., 2001; Korff and McHenry, 2011; Brandt et al., 2015). Stiff, rough substrates provide more

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friction that enhances grip, thus allowing higher performance (Kerdok et al., 2002; Van der Tol et al., 2005; Brandt et al., 2015; Bergmann et al., 2017).

Morphological features that are often beneficial on certain substrate types include adaptations of the epidermis that covers the digits. For instance, the adhesive ability of many Gekkonidae species depends on toe pad microarchitecture (setae) that allows them to run easily on smooth and vertical surfaces such as walls or even glass (Autumn et al., 2002; Autumn et al., 2005). Also, some other taxa bear fringes on their toes (e.g. genera *Acanthodactylus*, *Basiliscus* and *Uma*) enabling them to run fast on non-solid substrates (e.g., sand or water) without 'sinking', as fringes increase the amount of toe surface that comes into contact with the substrate while running (Salvador, 1982; Luke, 1986; Carothers, 1986).

In this study, we aimed to evaluate the impact of substrate type on sprint performance (maximum speed and maximum instant acceleration) in Schreiber's fringe-fingered lizard (*Acanthodactylus schreiberi* Boulenger, 1878). We worked with three Cypriot populations that reside in habitats with different substrate types (soil free from rocks, rock and sand). We formulated two hypotheses. First, we anticipated that the sprint performance of the focal populations would differ due to the presence of different substrate types in the habitats and due to the different running styles required on each one (Van Damme et al., 1998). Second, we predicted that individuals would perform better on their home substrates than those coming from other habitats (Goodman et al., 2008).

MATERIALS AND METHODS

Study system

Acanthodactylus schreiberi is a medium-sized lacertid lizard (snout-vent length 73-93 mm for males and 55-76 mm for females), inhabiting various habitats all over Cyprus (Baier et al., 2009). Even though it is considered to be mostly a sand-dwelling lizard, it can be found from coastal areas to mountain pine forests (over 1,300 m a.s.l) (Baier et al., 2009). The species is a skillful and swift runner that can use bipedalism while running (Savvides et al., 2017).

The habitats of the three focal populations vary considerably in substrate and vegetation type (Fig. 1). Geri (35º05'50"N, 33º26'21"E, elevation 183 m a.s.l.) is a sub-urban shrubland characterized by the presence of boxthorn (*Lycium ferocissimum*), thorny burnet (*Sarcopoterium spinosum*) and conehead thyme (*Thymbra capitata*). The substrate consists mostly of solid soil without any rocks. Agros (34º56'27"N, 33º00'14"E, elevation 1,348 m a.s.l.) is in a pine forest (*Pinus brutia*) with dense shrubs and has a rocky soil as its substrate. Akrotiri (34º56'27"N, 33º00'14"E, elevation 1 m a.s.l.) is a coastal dune habitat with quite sparse phrygana, where the substrate is fine-grained sand.

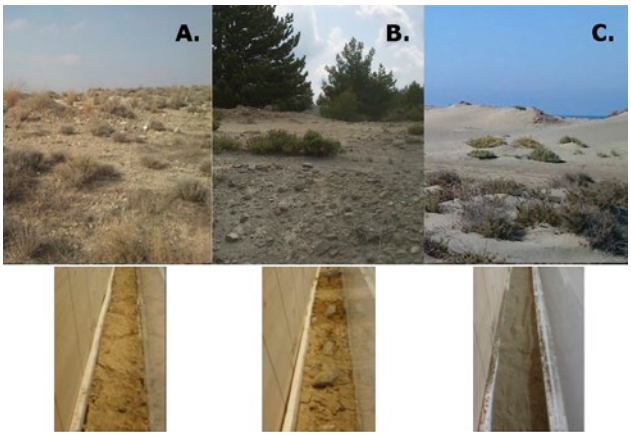

Fig. 1. The three habitats studied. A. Geri, B. Agros, C. Akrotiri and the respective platforms that were used in the laboratory to simulate the substrate of each habitat.

We captured a total of 67 adult individuals of both sexes (excluding gravid females) (Geri, N = 22; Agros, N = 22; Akrotiri, N = 23). Lizards were housed in individual terraria (30 x 30 x 30 cm) in the laboratory under a constant temperature (30 ºC) and controlled photoperiod (16-h light and 8-h dark), and were provided with mealworms (*Tenebrio molitor* larvae) and fresh water *ad libitum*. All lizards were released at their sampling sites after the completion of the experiments (experimental lizards remained in the laboratory for two weeks).

Based on the substrate type of each habitat, we constructed three removable substrate platforms that were used on a custom-made wooden racetrack (240 x 12 cm^2), bearing 10 cm increments on its back and clear acrylic glass on its front in order to allow video recording of each trial (Fig. 1).

Morphological measurements

Snout-vent length (SVL), hind limb length (HLL) and hind toe length (HT) were recorded with a digital caliper (Silverline 380244, accurate to 0.01 mm), before running trials. Also, the length of the largest right hind toe, the total number of fringes on it and the length of the three largest fringes from base to tip were measured using stereoscopic images of their toes (N = 60, 10 males and 10 females from each population) (Fig. 2), in order to test for correlation between toe length and fringe microarchitecture (length and number of fringes).

Sprint performance

All individuals were allowed to thermoregulate for an hour in a specifically designed terrarium (Van Damme et al., 1986) before each trial, so as to perform at the highest level possible (Irschick and Losos, 1998). After this period, lizards were placed in the racetrack. We triggered motion with a brush touching the lizard's tail base. Each individual performed five trials on each substrate type in a single day. Between trials on

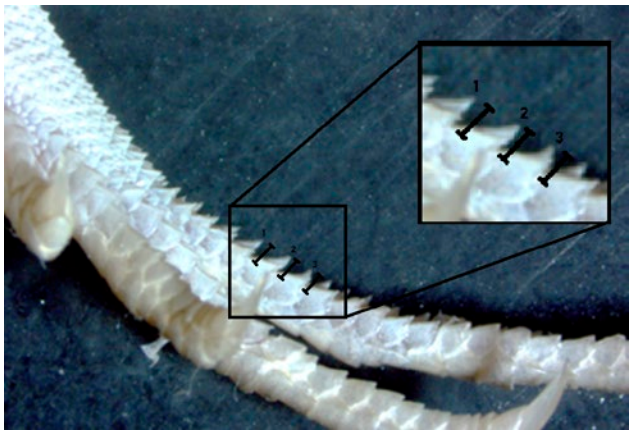

Fig. 2. Stereoscopic image of the toes and an example of how we measured the three largest toe fringes.

different substrate types, lizards were left to rest for a day and were then tested on the new type (in total, 15 trials per individual within five days). Trials were recorded with a video camera (Olympus SH-60) viewing the racetrack from the side (covering all the distance from start to finish), at a rate of 240 frames per second. Sprint performance was estimated from the video recordings (Martin and Avery, 1998; Kaliontzopoulou et al., 2012; Vanhooydonck et al., 2015; Savvides et al., 2017).

Maximum speed was calculated for all trials, based on the number of frames needed to cover a distance of 20 cm within a known time interval (Savvides et al., 2017). We chose the highest values for each individual to represent its best trial. Maximum instant acceleration was calculated by digitizing the position of the lizard's snout in every frame, for all trials, on x- (movement) and y- (gravity) axes (MATLAB DLTdataviewer3; Hedrick, 2008). In each frame, we estimated the displacement of the snout and converted it from pixels to meters. We filtered the curve of the instantaneous displacement of the snout over time (i.e. instantaneous velocity) using a fourth-order zero phase shift Butterworth low-pass data noise filter (40Hz; VBA application in Office Excel). The time differential of the instantaneous velocity yields the instantaneous acceleration and we chose the highest value (i.e. peak) for each individual's best trial (Van Wassenbergh, 2007). We only used trials during which lizards ran continuously for at least 50 cm.

Using the Pearson product moment correlation coefficient, we found that the length of the largest hind toe correlated strongly with the length of the three longest fringes in all populations (all r values > 0.6 and P values < 0.05), so we used this measurement as a proxy of fringe size to detect possible fringe effects on sprint performance.

Statistical analyses

We used the Shapiro – Wilks and Levene's tests to check for data normality and homogeneity of variance, respectively. All data were log-transformed based on the results of these tests. Log-transformed data for the morphological characters were compared between sexes for each population using one-way MANCOVA and taking SVL as covariate. One-way MANCOVA was also used to search for differences among populations in relation to the transformed data for the morphological characters (HLL and HT), using again SVL as a covariate. One-way MANOVA was used to compare their sprint performance among different substrate types and populations. A post-hoc Tukey test was used in order to determine the differences among populations. The Friedmann test and the Bonferroni correction were used to compare the performance of each individual on the three types of substrate. The effects of the log-transformed values of morphological characters (HLL and HT) on sprint performance were identified independently, using linear regression.

RESULTS

Morphological characters showed significant differences between the sexes in each population, with female individuals having relatively smaller hind limbs and hind toe length than male individuals (one-way MANCOVA: Geri, $F_{2,18}$ = 9.965 Roy's largest root = 1.107; Agros, $F_{2,18}$ = 29.373 Roy's largest root = 3.264; Akrotiri, $F_{2,18}$ = 5.517 Roy's largest root = 0.613; all P values < 0.05). Thus, hind limbs and hind toes length were therefore compared among populations separately for males and females (Table 1), but no significant differences were found (all P values > 0.05).

When comparing sprint performance among populations, we observed that the Agros males (home habitat with rocky substrate) were significantly faster than the other populations (one-way MANOVA: $F_{6,26}$ = 4.072, Roy's largest root = 0.940, p = 0.005). They achieved the highest maximum speed (Post hoc Tukey HSD: Agros vs. Geri, P = 0.012; Agros vs. Akrotiri, P = 0.002) and maximum instant acceleration (Post hoc Tukey HSD: Agros vs. Geri, P = 0.001; Agros vs. Akrotiri, P = 0.002) on soil

Table 1. Mean values for morphological characters between males and females from the three populations. SVL: snout-vent length, HLL: hind limb length, HT: longest hind toe length. Values are in cm.

Character		Geri		Agros		Akrotiri	
		Mean	SD	Mean	SD	Mean	SD
SVL	♂♂	7.10	0.43	6.90	0.64	6.35	0.40
	♀♀	6.45	0.41	6.50	0.34	6.00	0.44
HLL	♂♂	4.60	0.23	4.65	0.14	4.49	0.43
	♀♀	4.00	0.28	4.00	0.16	3.96	0.22
HT	♂♂	1.29	0.14	1.24	0.12	1.22	0.10
	♀♀	1.13	0.22	1.10	0.09	1.00	0.10

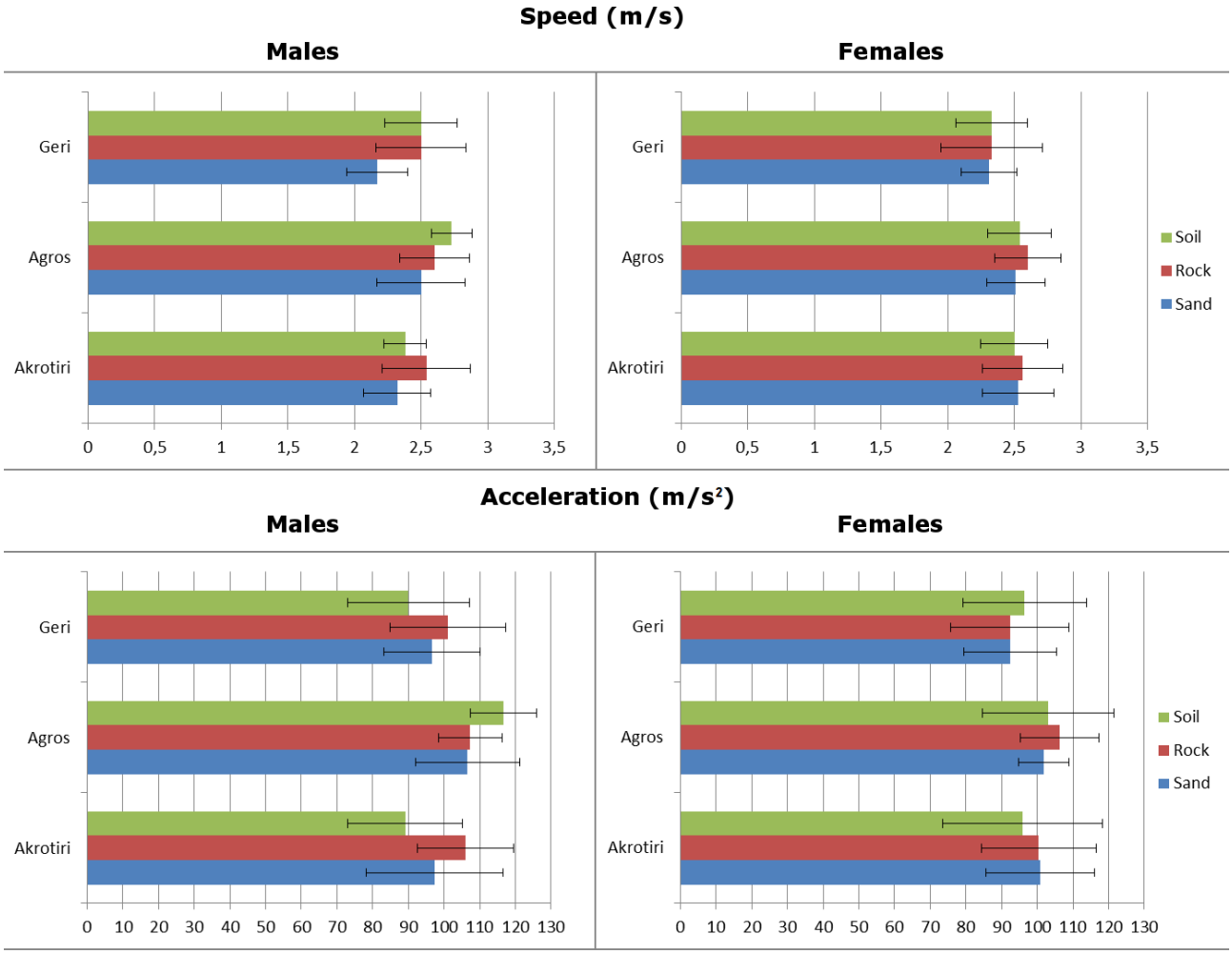

Fig. 3. Mean values for speed and acceleration on each substrate within populations and for males and females.

(Fig. 3). We did not observe further differences in the sprint performance among other populations on the other substrates.

The sprint performance of all lizard populations (for both sexes) did not differ among the three types of substrate (all P values > 0.05) (Fig. 3).

The length of hind limbs and toes showed no significant effects on performance among populations and substrates (all regression P values > 0.05).

DISCUSSION

Locomotion patterns may change in response to endogenous or extraneous factors (Vanhooydonck and Van Damme, 2003; Sathe and Husak, 2018). Among the latter, the type of substrate is known to affect locomotion in lizards. In some cases, specific substrate types favor higher performance (e.g., solid substrates like rock), while others restrict locomotion (e.g., substrates not providing sufficient grasp, e.g., sand or mud) (Vanhooydonck et al., 2005; Tulli et al., 2012). In our study, we did indeed see certain differences in sprint performance among conspecific populations of Schreiber's fringe-fingered lizard. However, these differences followed a rather unclear pattern and indicated a limited effect of substrate type on locomotion.

Interestingly, when we analyzed sprint performance taking into account the effect of hind limb length, we did not find any significant effects for any type of substrate. Also, the multiple regression analyses did not show a beneficial effect of toe fringes (using the hind toe as a proxy). Hind limbs have been repeatedly reported to affect lizard locomotion (Vanhooydonck et al., 2001; Herrel et al., 2002; Savvides et al., 2017). The absence of such effects in our study might indicate an interplay among

other morphological features (e.g., fore limbs, tail length, etc.) involved in locomotion in response to substrate type requirements (Herrel et al., 2002). On the other hand, we cannot rule out the possibility that despite our best efforts, lizards might have underperformed and have failed to achieve their maximum performance levels.

Contrary to our first hypothesis regarding sprint performance on different substrates, no differences emerged from the comparison among the three populations, save the single case of the Agros males (rock substrate) that were the fastest sprinters. However, subsequent analysis based on morphological features, failed to provide an underlying reason for this finding. In our initial hypothesis, we considered the three types of substrate to be of different quality. We presumed that sand would be the more challenging substrate because of its grainy texture that "sinks" under the weight of a running lizard (Clemente, 2014; Sathe and Husak, 2015). As such, we expected that sprint performance therein would be the lowest. On the other hand, solid substrates are known to provide high traction and thus facilitate locomotion (Lejeune et al., 1998; Claussen et al., 2002; Brandt et al., 2015; Bergmann et al., 2017). Apparently, these predictions were not valid in our study system, as we failed to find any significant deviations. It seems that there is no ideal substrate type for *A. schreiberi* locomotion and all populations are well adapted in their home habitat.

According to our second hypothesis, lizards should have performed at higher levels on their home substrate. However, we did not find such a pattern in our study system. All populations performed at similar levels on all substrate types, indicating that the species conserves generalized running capabilities that allow a high level of performance on various types of substrate. The rejection of the specialized populations hypothesis, might be due to overlapping genetic pools or mixed habitat characteristics, leading the lizards in similar directions regarding their kinematics and their interactions between morphology, biomechanics and environmental factors. We also have to highlight the fact that long periods of draught in Cyprus can change abruptly to rainy periods (especially during late spring and early summer). This would cause the substrate to change its properties, such as its roughness and its potential to provide grip and would thus favor the generalized pattern we observed in this study.

This study will enhance the growing body of literature on saurian locomotion, as it examines some of the widely accepted principles in the field. According to our findings, the type of substrate has a limited impact on sprint performance, and at least for Schreiber's fringe-fingered lizard, there were no strict patterns observed. Further research including more species will shed light on the fascinating interplays taking place in lizard locomotion.

ACKNOWLEDGMENTS

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REFERENCES

Aerts, P., Van Damme, R., Vanhooydonck, B., Zaaf, A., Herrel, A. (2000): Lizards locomotion: how morphology meets ecology. Netherl. J. Zool. **50**: 261-277.

Autumn, K., Buehler, M., Cutkosky, M., Fearing, R.S., Full, R.J., Goldman, D.I., Groff, R., Provancher, W., Rizzi, A.A., Saranli, U., Saunders, A., Koditschek, D.E. (2005): Robotics in scansorial environments. In: Unmanned ground vehicle technology VII, pp. 291-303. Gerhart, G.R., Shoemaker, C.M., Gage, D.W., Eds, SPIE Proceedings.

Autumn, K., Sitti, M., Liang, Y.A., Peattie, A.M., Hansen, W.R., Sponberg, S., Kenny, T.W., Fearing, R., Israelachvili, J.N., Full, R.J. (2002): Evidence for van der Waals adhesion in gecko setae. Proc. Natl. Acad. Sci. **99**: 12252-12256.

Baier, F., Sparrow, D.J., Wield, H.J. (2009): The amphibians and reptiles of Cyprus. Edition Chimaira, Frankfurt am Main.

Brandt, R., Galvani, F., Kohlsdorf, T. (2015): Sprint performance of a generalist lizard running on different substrates: grip matters. J. Zool. **297**: 15-21.

Bergmann, P.J., Pettinelli, K.J., Crockett, M.E., Schaper, E.G. (2017): It's just sand between the toes: how particle size and shape variation affect running performance and kinematics in a generalist lizard. J. Exp. Biol. **220**: 3706-3716.

Carothers, J.H. (1986): An experimental confirmation of morphological adaptation: toe fringes in the sand-dwelling lizard *Uma scoparia*. Evolution **40**: 871-874.

Claussen, D.L., Lim, R., Kurz, M., Wren, K. (2002): Effects of slope, substrate, and temperature on the locomotion of the ornate box turtle, *Terrapene ornata*. Copeia **2002**: 411-418.

Clemente, C.J. (2014): The evolution of bipedal running in lizards suggests a consequential origin may be

exploited in later lineages. Evolution **68**: 2171-2183.
Goodman, B.A., Miles, D.B., Schwarzkopf, L. (2008): Life on the rocks: habitat use drives morphological and performance evolution in lizards. Ecology. **89**: 3462-3471.
Herrel, A., Meyers, J.J., Vanhooydonck, B. (2002): Relations between microhabitat use and limb shape in phrynosomatid lizards. Biol. J. Linn. Soc. **77**: 149-163.
Husak, J.F., Fox, S.F., Lovern, M.B., Van Den Bussche, R.A. (2006): Faster lizards sire more offspring: sexual selection on whole-animal performance. Evolution **60**: 2122-2130.
Irschick, D.J., Losos, J.B. (1998): A comparative analysis of the ecological significance of maximal locomotor performance in Caribbean *Anolis* lizards. Evolution **52**: 219-226.
Jayne, B.C., Bennett, A.F. (1990): Selection on locomotor performance capacity in a natural population of garter snakes. Evolution **44**: 1204-12.
Kaliontzopoulou, A., Adams, D.C., van der Meijden, A., Perera, A., Carretero, M.A. (2012): Relationships between head morphology, bite performance and ecology in two species of *Podarcis* wall lizards. Evol. Ecol. **26**: 825-845.
Kerdok, A.E., Biewener, A.A., McMahon, T.A., Weyand, P.G., Herr, H.M. (2002): Energetics and mechanics of human running on surfaces of different stiffnesses. J. Appl. Physiol. **92**: 469-478.
Korff, W.L., McHenry, M.J. (2011): Environmental differences in substrate mechanics do not affect sprinting performance in sand lizards (*Uma scoparia* and *Callisaurus draconoides*). J. Exp. Biol. **214**: 122-130.
Lejeune, T.M., Willems, P.A., Heglund, N.C. (1998): Mechanics and energetics of human locomotion on sand. J. Exp. Biol. **201**: 2071-2080.
Lleonart, J., Salat, J., Torres, G.J. (2000): Removing allometric effects of body size in morphological analysis. J. Theor. Biol. **205**: 85-93.
Losos, J.B. (1990): Concordant evolution of locomotor behaviour, display rate and morphology in *Anolis* lizards. Anim. Behav. **39**: 879-890.
Losos, J.B., Irschick, D.J. (1996): The effect of perch diameter on escape behaviour of *Anolis* lizards: laboratory predictions and field tests. Anim. Behav. **51**: 593-602.
Luke, C. (1986): Convergent evolution of lizard toe fringes. Biol. J. Linn. Soc. **27**: 1-16.
Martin, J., Avery, R.A. (1998): Effects of tail loss on the movement patterns of the lizard, *Psammodromus algirus*. Funct. Ecol. **12**: 794-802.
McElroy, E.J., Hickey, K.L., Reilly, S.M. (2008): The correlated evolution of biomechanics, gait and foraging mode in lizards. J. Exp. Biol. **211**: 1029-1040.
Miles, D.B. (2004): The race goes to the swift: fitness consequences of variation in sprint performance in juvenile lizards. Evol. Ecol. Res. **6**: 63-75.
Redfern, M.S., Cham, R., Gielo-Perczak, K., Grönqvist, R., Hirvonen, M., Lanshammar, H., Marpet, M., Pai, C.Y.C., Powers, C. (2001): Biomechanics of slips. Ergonomics **44**: 1138-1166.
Robson, M.A., Miles, D.B. (2000): Locomotor performance and dominance in male tree lizards, *Urosaurus ornatus*. Funct. Ecol. **14**: 338-344.29.
Salvador, A. (1982): A revision of the lizards of the genus *Acanthodactylus* (Sauria: Lacertidae). Bonn. Zool. Monogr. **16**: 1-167
Sathe, E.A., Husak, J.F. (2015): Sprint sensitivity and locomotor trade-offs in green anole (*Anolis carolinensis*) lizards. J. Exp. Biol. **218**: 2174-2179.
Sathe, E.A., Husak, J.F. (2018): Substrate-specific locomotor performance is associated with habitat use in six-lined racerunners (*Aspidoscelis sexlineata*). Biol. J. Linn. Soc. **124**: 165-173.
Savvides, P., Stavrou, M., Pafilis, P., Sfenthourakis, S. (2017): Tail autotomy affects bipedalism but not sprint performance in a cursorial Mediterranean lizard. Sci. Nat. **104**: 3.
Tulli, M.J., Abdala, V., Cruz, F.B. (2012): Effects of different substrates on the sprint performance of lizards. J. Exp. Biol. **215**: 774-784.
Van Damme, R., Aerts, P., Vanhooydonck, B. (1998). Variation in morphology, gait characteristics and speed of locomotion in two populations of lizards. Biol. J. Linn. Soc. **63**: 409-427.
Van Damme, R., Bauwens, D., Verheyen, R.F. (1986): Selected body temperatures in the lizard *Lacerta vivipara*: variation within and between populations. J. Therm. Biol. **11**: 219-222.
Van Damme, R., Vanhooydonck, B., Aerts, P., De Vree, F. (2003): Evolution of lizard locomotion: context and constraint. In: Vertebrate biomechanics and evolution, pp. 267-282. Bels, V., Gasc, J.P., Casinos, A., Eds, BIOS Scientific, Oxford.
Van der Tol, P.P.J., Metz, J.H.M., Noordhuizen-Stassen, E.N., Back, W., Braam, C.R., Weijs, W.A. (2005): Frictional forces required for unrestrained locomotion in dairy cattle. J. Dairy Sci. **88**: 615-624.
Vanhooydonck, B., Andronescu, A., Herrel, A., Irschick, D.J. (2005): Effects of substrate structure on speed and acceleration capacity in climbing geckos. Biol. J. Linn. Soc. **85**: 385-393.
Vanhooydonck, B., Measey, J., Edwards, S., Makhubo, B., Tolley, K.A., Herrel, A. (2015): The effects of substratum on locomotor performance in lacertid lizards. Biol. J. Linn. Soc. **115**: 869-881.

Vanhooydonck, B., Van Damme, R. (2003): Relationships between locomotor performance, microhabitat use and antipredator behaviour in lacertid lizards. Funct. Ecol. **17**: 160-169.

Vanhooydonck, B., Van Damme, R., Aerts, P. (2001): Speed and stamina trade-off in lacertid lizards. Evolution **55**: 1040-1048.

Van Wassenbergh, S. (2007): https://www.uantwerpen.be/en/staff/sam-vanwassenbergh/my-website/excel-vba-tool.

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Coping with aliens: how a native gecko manages to persist on Mediterranean islands despite the Black rat?

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Abstract. How a native gecko manages to coexist with an alien rodent in the Mediterranean since thousands of years? What kind of eco-ethological adaptations or evolutionary adjustments enables this gecko to persist? The present study explores the interaction between the endemic European Leaf-toed gecko (*Euleptes europaea*) and the alien Black rat (*Rattus rattus*). In the last 30 years, we compared 26 populations inhabiting "rat" and "rat-free" islands and islets in Tunisia, Sardinia, Corsica and Southern France. Geckos' populations can persist despite the occurrence of rats. In the presence of rats: 1) geckos' average body size tends to decrease towards medium-sized individuals; 2) geckos shift their spatial behaviour avoiding to forage "in the open"; 3) geckos' body condition is not affected by the presence of rats. Moreover, shortly after rats' eradication, geckos' population structure seems to change and larger sized geckos prevail while the spatial behaviour is much more conservative. The mechanisms driving the interactions between the two species still need to be explained. Rats could represent a stressor for geckos, compete for space, be pest vectors and even predators. Coexistence of natives and aliens requires adaptive plasticity and evolutionary adjustments. In contexts where the risk of reinvasion is high, eradication programs need to be carefully evaluated, since the arrival of "new rats" on an island could have much more damaging effects on the insular biota than those caused by the eradicated population.

Keywords. Behavioural shift, disturbance, ecological plasticity, evolutionary processes, predation, rat eradication, Euleptes europea, Rattus rattus.

In memory of Michel Pascal (1947-2013)

INTRODUCTION

The long term dynamics of insular biota reflect the interplay between recurrent immigration and extinction events (Brown and Lomolino, 2000). Mainly because of human expansion on global scale, the natural phenomenon of sea dispersal has been greatly amplified favouring introductions and even biological invasions (Simberloff et al., 2013). In the last centuries, with a speeding up in the last decades human-induced dispersal of species has implied: a) huge acceleration of the introductions' rate; b) great number of species involved; c) remote origins of some propagules; d) possible genetic modifications originated from captive breeding and farming activities. Insularity-associated features, such as high sensitivity to climate and sea level changes, high rate of speciation, immigration and extinction processes, naiveté of native species, make insular biota particularly fragile (Moser et al., 2018).

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Over the last decades, conservation biology has emphasized the detrimental effects of introduced species (mostly mammals) on insular ecosystems, but little attention has been paid to the understanding of the eco-ethological adaptations that enable a native species to persist despite the presence of aliens (Martin et al., 2000; Hoare et al., 2007; Ruffino et al., 2009). In systems with novel combination of species (native and introduced ones) that do not show co-adaptation, rapid evolutionary changes may occur (Berthon, 2015; Mooney and Cleland, 2001; Strauss et al., 2006; Sax et al., 2007; Stuart et al., 2014). On islands, in particular, morphological, ecological and behavioural shift are more easily detectable than in more complex systems (Simberloff, 1974).

Within the Mediterranean, reptiles, notably lizards, are good tools to investigate insular evolution. These herpetological communities, which are currently found on continental islands and on many land-bridge islets, origin from the neighbouring continents or large islands before their isolation, dating back to the sea level raise of the last interglacials. Trans-marine dispersal is less frequent in the Mediterranean arid islands (Foufopoulos and Ives, 1999).

Most of the endemic herpetofauna still remains on some big islands (e.g., Corsica, Sardinia) and relative satellite islands, while on other major Mediterranean islands the endemic herpetofauna has been completely replaced by human-introduced species, and endemic species persist only on some of their satellite islands (Corti et al., 1999; Silva-Rocha et al., 2018, 2019).

In the present work we investigated the interaction between the native European Leaf-toed gecko *Euleptes europaea* (Gené, 1839) and the alien Black rat, *Rattus rattus* (Linnaeus, 1758) on some small islands of the Western Mediterranean. The European Leaf-toed gecko, a Western Mediterranean endemic species listed in Appendix II of the European Habitat Directive, is a very old inhabitant of this region, presumably since several million years ago (Müller, 2001). This species underwent a process of historic extinction or steep demographic decline along the North-Western (Provence, France) and Southern (Tunisia) edges of its range (Delaugerre et al., 2011). The Black rat colonized the Western Mediterranean approximately 2000-2400 years ago (Thibault et al., 1987; Vigne and Valladas, 1996; Ruffino and Vidal, 2010). Thus, the coexistence between the two species might last since hundreds of geckos' generations and thousands of rats' generations, since *Euleptes* lives longer than rats (Salvidio et al., 2010 and ref. therein). According to Ruffino et al. (2009), in the Western Mediterranean, 74% of the islands between 1 and 5 ha (and 99% of islands larger than 30 ha) are colonized by Black rats. Among the human introduced species, rats can dramatically impact on island biodiversity (Recher and Clark, 1974; Atkinson, 1985; Courchamp et al., 2003; Bennett et al., 2005) and Black rats in particular, have caused rapid extinctions on islands, as reported for New Zealand and the Hawaiian Archipelago (Towns et al., 2006; Drake and Hunt, 2009). Alien rats can predate and compete with native species and modify islands' food chains (Traveset and Richardson, 2006). The worldwide success of the Black rat as colonizer is due to its ability to exploit a large range of habitats and resources (Jones et al., 2008), as well as to shift to seasonal resources (Caut et al., 2008). This plasticity is crucial to survive in poor insular ecosystems, characterized by strong resource variation.

On small islets *E. europaea* and *R. rattus* are often the only sedentary vertebrates. They are both nocturnal, good climbers on rocky substrates, and they both forage on rocky outcrops and on low vegetation.

Rat eradication programs are often focused on one or few emblematic species (Capizzi et al., 2010), without a comprehensive understanding of insular assemblages. Investigating the possible interactions between the European Leaf-toed gecko and the Black rat the present work could provide useful data for long-term insular conservation plans.

In particular, we explored the effects of the Black rat on the Leaf-toad gecko population structure (analysed on the basis of the relative proportion of size classes), body condition and habitat use, comparing populations living on islands colonized by rats and islands free of rats. Because we expect rats to interfere with geckos' habitat use, feeding habits and thermoregulation, geckos living on islands on which also rats live should show: 1) population structure with smaller-sized individuals resulting from a shorter lifespan; 2) poor body conditions; 3) modification of spatial behaviour by minimizing or even avoiding foraging in open spaces.

MATERIAL AND METHODS

Ecological or evolutionary responses of native species to aliens cannot be easily distinguished from the pre-existing ecological differences (Strauss et al., 2006). However, even if each micro insular population differs from the others (e.g., due to age of isolation, presence of competitors and/or predators, etc.), any detectable trend can provide strong correlative evidences.

Study species

Euleptes europaea is a small gecko (average snout-vent length, hereafter SVL, 38 mm; average adult weight 1.2 g; hatchling weight 0.25 g) endemic to the Western Mediterranean, mostly found on islands in rocky habitats. It is strictly noctur-

nal, spending daytime in narrow rocky crevices (opening 2-4 mm wide) where dorsal and ventral parts of the body are in contact with the rock. These crevices are also used for egg laying (Salvidio et al., 2010). Achieving its sexual maturity at the age of 3 years (Salvidio and Delaugerre, 2003), it might live 6 to 8 years in the wild and its maximum longevity in captivity attains 21 years (F. Molle in Mertens, 1970).

It feeds on flying and ground dwelling invertebrates. On islets, densities are higher than on the mainland and population size ranges from several hundreds to only few dozen adults. This gecko is able to live on tiny islets (hundreds of square meters) characterized by the presence of few vascular plants, where it represents the only sedentary vertebrate (Delaugerre and Cheylan, 1992). Body size greatly varies on islets with trends towards gigantism, dwarfism and variation of the sexual size difference (Delaugerre and Cheylan, 1992; Salvidio et al., 2010).

Rattus rattus (average weight 170 g), is a good climber, both on rocks and trees, but not a long lasting swimmer and therefore considered with poor marine dispersal capacities (≤ 500 m) (Cheylan, 1988; Ruffino et al., 2009). In the Mediterranean, rats feed mainly on plants, avoiding or rarely eating halophilous and nitrophilous ones (Cheylan, 1988; Cassaing et al., 2007). Black rats and *Euleptes europaea* are both nocturnal and share the same habitat.

Study sites and data collection

We focused on islands characterized by simple ecosystems, where strong interactions between the target species are more likely to occur, namely small islands characterised by seasonal shortage of food availability for rats, and higher probability of encounters between the two species. We selected 26 islands and islets (Table 1) throughout most of the Leaf-toed gecko range (Fig. 1). Islands were classified as "rat" vs "rat-free", depending on the presence or absence of rats; islands where rats were eradicated were considered as "rat-free". The presence of rats was detected thanks to at least one of the following evidences: direct sightings, fresh and/or old faeces, remains of chewed olive seeds or plants, rats' nests, rats' urine scent. Past presence/absence of rats on islands was investigated through literature and observations made by locals. Nine islands (the largest ones) were inhabited by rats probably since their early colonization (Abdelkrim et al, 2009), whereas 13 islands (the smallest and most remote ones, without edible plants) were presumably never inhabited by rats. On Lavezzu Island (Corsica) we collected data before and after rat eradication (performed in 2000): the island was considered as "rat" before the eradication and as "rat-free" after eradication (Table 1). Five islands with "transient" rat populations (i.e., small islets close to a colonization source but lacking resources to support a permanent rat population) were treated as "rat", because rat frequency on these islands is unknown.

Sampling methods

Observations were carried out in spring, summer and autumn from July 1983 until August 2016. Sampling sessions (n = 47) consisted of 1 up to 6 nights per island (Table 1). Active geckos were searched using battery-powered lamps starting one or two hours after dusk and lasted until dawn. Total sampling effort achieved more than 380 hours (Table 1). Geckos behaviour was classified as follow: "in the open" when found on bare rocks, on the ground or on plants; "under cover" when found hidden by the vegetation at the base of rocks (Fig. 2). Distance from the ground (height of the first sight) was also measured. Geckos were carefully caught by hand and temporarily stored in bags; sex and adulthood was determined according to Delaugerre and Dubois (1985). Snout-vent length (SVL) of 1795 individuals was measured to the nearest 0.01 mm using a digital calliper, weight (W) of 424 adults was recorded to the nearest 0.01g using a digital scale. Geckos were released in the area of original sighting. Body condition index (BCI) was calculated as from Bonnet and Naulleau (1994). Spatial behaviour of 1012 geckos (i.e., "in the open" or "under cover"), recorded for 24 sampling sessions carried out on 17 islets, was assessed for one hour "catch per-unit-effort" (CPUE).

Statistical analyses

*Population structure***.** In order to assess whether the populations structure of *E. europaea* was affected by the presence of rats, we compared gecko's size between islands with and without rats. Since Leaf-toed geckos' size varies among islands independently of rats, we firstly normalized body sizes by dividing each measure by the greatest observed value in order to have all values in the 0-1 range (Legendre and Legendre, 2012). Each individual was classified depending on its inclusion in the quartile of the distribution of the normalized body size as "small" (1st and 2nd quartiles), "medium" (3rd quartile), and "large" (4th quartile). Geckos' population structure was assessed by computing the proportion of individuals per size class. Different population structures were computed for spring and autumn. Sample size included 1871 individuals from 24 islets (Mean values ± SE: 78 ± 16); islands with less than 10 individuals measured (San Bainzu, Piana and Porro) were excluded. Data were analysed using permutational multivariate analysis of variance, PERMANOVA (Anderson, 2001; McArdle and Anderson, 2001) on the basis of Euclidean distances among islands' population structures. The PERMANOVA allows the multivariate information to be partitioned according to the full experimental design without any *a priori* assumption regarding the distributions of the original variables. The predictors were rat occurrence (yes/no), season (spring/autumn), number of reptile species, number of sampling sessions (to account for repeated measures within an island), and island size. P-values were obtained by permutation, and the number of permutations was set to 9999.

Body condition. The effect of rat occurrence on the body condition of geckos was assessed using a linear mixed model in which the BCI was the dependent variable; rat occurrence, sex, and season were the fixed effects, whereas the island was the random factor accounting for repeated measuring.

Spatial behaviour. Linear mixed models were also used to investigate the effect of rat occurrence on the habitat use by geckos. In a first analysis we checked if Leaf-toed geckos avoided

Table 1. Map identification numbers (see Figure 1) of the study islands, geographical region, surface, presence ("rat") or absence ("rat-free") of the Black rat, and number of reptile species. For each island we also indicate sampling dates (and number of sampling nights), sampling effort (in minutes), number of geckos measured (n Biometry), number of geckos for which activity data were recorded (n Activity).

n°	Islet/island	Region	Surface (ha)	Rat status	N sp reptiles	Year (month): n nights	Sampling effort (min)	n Biometry	n Activity
1	Gallo	N_Tunisia	8.9	rat	2	2008 (05):3	1320	49	/
				rat		2010 (07):1	270	/	30
2	Toro	Sardinia	13.22	rat free	3	2015 (06):1	195	34	45
3	Carpa	Sardinia	0.4	rat	2	2011 (09):1	430	50	67
4	Porco	Sardinia	4.8	rat	4	2012 (05):1	285	44	55
5	Spargiotto	Sardinia	11.13	rat free	3	2014 (05):1	360	66	79
6*	Lavezzu	Corsica	62.73	rat	3	1986 (08):1	390	50	/
				rat free		2010 (06):3	550	40	41
				rat free		2011 (06):3	427	67	41
				rat free		2012 (06):2	720	66	/
7	Porraggia Grande	Corsica	1.25	rat free	2	1985 (08):1	600	50	/
8	Porraggia piccola	Corsica	0.61	rat free	2	1986 (08):1	410	59	/
9	Sperduto grande	Corsica	0.92	rat free	1	1984 (10):2	785	40	/
				rat free		1986 (08):1	330	81	/
				rat free		2011 (06):1	435	43	43
10	Toro grande	Corsica	1.62	rat free	2	1986 (08):1	360	59	/
				rat free		2005 (04):1	200	50	/
				rat free		2012 (07):1	140	/	52
				rat free		2014 (07):1	250	48	59
11	1st islet NE of Toro piccolo	Corsica	0.11	rat free	1	1986 (08):1	240	15	/
12*	Vacca	Corsica	0.65	rat free	2	1985 (08):3	1395	95	/
				rat free		2012 (07):1	165	8	15
13	Roscana	Corsica	0.2	rat free	1	1986 (08):1	720	94	/
				rat free		2008 (10):3	2258	125	/
				rat free		2012 (09):2	824	122	/
14	Locca	Corsica	0.79	rat	2	2010 (04):1	90	/	10
15	Mezzumare	Corsica	35.66	rat	3	2012 (08):1	213	15	18
16	A Botte	Corsica	0.52	rat free	1	2010 (09):3	630	35	43
				rat free		2011 (06):6	990	/	62
17	Gargalu	Corsica	20.06	rat	4	1985 (04):2	1060	50	/
				rat		1990 (07):2	505	19	/
18	Palazzu	Corsica	0.47	transient	1	1986 (08):1	225	26	/
19	Palazzinu	Corsica	0.1	transient	1	1985 (07):2	490	34	/
20	Porri	Corsica	0.27	rat free	1	1983 (07):2	590	32	/
				rat free		1986 (07):1	335	69	/
21	Brocciu	Corsica	1.11	transient	1	2012 (06):1	132	30	60
22	Giraglia	Corsica	10.35	rat free	4	2000 (09):1	360	38	/
				rat free		2012 (08):2	406	33	33
				rat free		2012 (10):1	230	/	50
				rat free		2014 (07):1	230	/	19
				rat free		2014 (10):1	190	36	38
				rat free		2015 (08):1	220	/	32
23	Saint Ferreol	Provence	0.45	rat	2	2016 (05):1	700	23	40
24	La Tradelière	Provence	1.05	rat	2	2016 (05):1	460	23	37
25	Rascas	Provence	0.76	transient	2	1985 (09):1	290	50	/
26*	Gabinière	Provence	3.42	transient	2	2003 (10):1	345	69	/
				transient		2016 (05):1	187	37	43

* On Lavezzu Island (n. 6) rats were eradicated in 2000; on the Toro islets (n. 10 and 11) in 1992, after 2-4 years of presence on the islets; on Vacca (n. 12) in January 2011, after having been detected in July 2010; on Gabinière (n. 26) rats were present in 1937, eradicated in 1966, rats re-invaded the island in 2010 and were eradicated again in 2014. The islets San Bainzu, Piana and Porro, all inhabited by rats, were escluded from the analysis because less than 10 geckoes were measured.

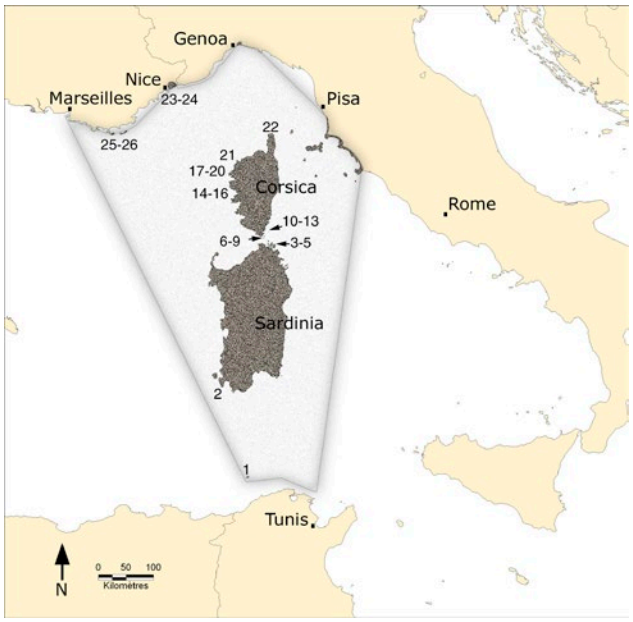

Fig. 1. Geographic range of *Euleptes europaea* and studied populations. Numbers refers to Table 1.

Fig. 2. Vegetation (mostly *Lotus cytisoides*) covering the base of the granite boulders inhabited by *Euleptes europaea* on Spargiotto Islet (Maddalena Archipelago). May 2012.

"in the open" microhabitats on islands with rats: the catch per unit effort (CPUE) was the dependent variable, while rat occurrence, habitat type ("in the open" *vs* "under cover"), island size and the number of reptile species were the fixed predictors. We also added the rat occurrence × habitat type interaction to account for differential habitat use between "rat" and "rat-free" islands. We could not include in the model the season as all but three islands were sampled in spring, and the island entered the model as random effect to control for double measuring within island (i.e., CPUE in open and close microhabitat). Sample size included 16 islands for which we collected more than 10 individuals (n = 1001, Mean values ± SE: 62 ± 10) for computing CPUE indexes for both "in the open" and "under cover" microhabitats. In a second analysis we checked if the height above the ground of the first sighting differed in islands with or without rats, using rat occurrence, season, number of reptile species, island size, and the rat occurrence × season as fixed predictors. As in the previous analysis, the islands entered the model as random effect, and geckos observed "under cover" were excluded. The sample for this last analysis included 469 geckos from 14 islands.

Analyses were performed using the package lme4 (Bates et al., 2014) in R ver. 3.2.4 (R Core Team, 2018), and otherwise stated, data reported are means ± standard error.

RESULTS

Population structure

The PERMANOVA showed that the population structure significantly varied in response to rat occurrence, season, number of reptile species living on the island, but was invariant in respect to the sampling effort (Table 2). In particular, a) the structure of geckos' populations living on the islands with rats showed a larger proportion of medium-sized individuals and a lower proportion of large-sized geckos compared to populations occurring on "rat-free" islands (Fig. 3a); b) irrespective of rat occurrence, smaller geckos were frequent in spring, while larger ones prevailed in autumn, and the relative abundance of medium-sized individuals did not vary between seasons (Fig. 3b); c) the greater was the number of reptile species on the island, the smaller was the frequency of larger geckos and the higher the number of medium-sized individuals (Fig. 3c). Moreover, large geckos were more frequent on islets rather than on islands (Fig. 3d).

Body condition

Body condition of the Leaf-toed geckos did not differ between "rat" and "rat-free" islands (0.0292 ± 0.0005

Table 2. Results of permutational ANOVA for the variability of the population structure of the Leaf-toed geckos in response to rat occurrence, season and number of reptile species after having been controlled for island size and number of sampling years. P-values are computed with 9999 permutations.

Variable	df	F	R^2	P
Rats	1,24	3.916	0.08	0.0374
Islet size	1,24	3.775	0.08	0.0374
Season	1,24	10.69	0.23	0.0010
N. reptiles	1,24	3.975	0.08	0.0336
N. replicates	1,24	0.0033	<0.01	0.9958

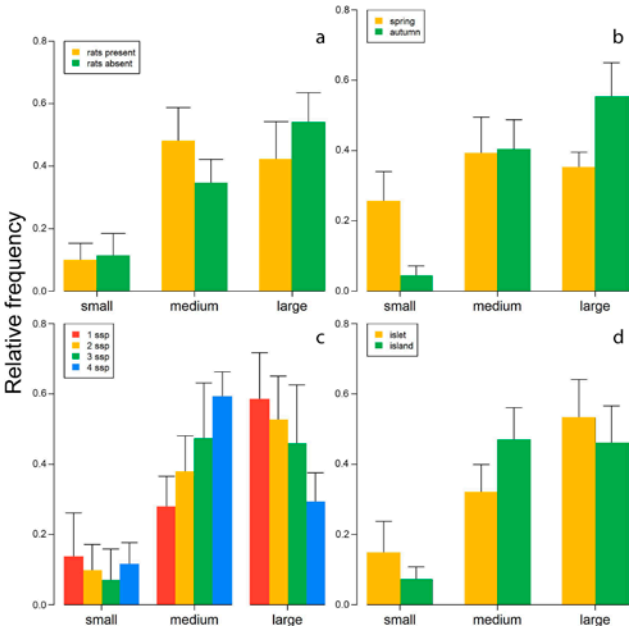

Fig. 3. Variation of the population structure of the European Leaf-toed gecko on 22 Mediterranean islands in response to (a) rat occurrence (present in yellow, absent in green), (b) season (Spring in yellow, Autumn in green), (c) number of reptile species living on the islands (1 sp. in red, 2 spp. in yellow, 3 spp. in green, 4 spp. in blue), and (d) island size (islet: < 10,000 m^2 in yellow, island: > 10,000 m^2 in green). Bars = 95% confidence interval.

and 0.0285 ± 0.0002 respectively, $F_{1,5}$ = 0.0053, P = 0.94), either between males and females (0.0282 ± 0.0003 and 0.0292 ± 0.0004 respectively, $F_{1,413}$ = 2.545, P = 0.11), or season (autumn: 0.0269 ± 0.0004; spring: 0.0295 ± 0.0003, $F_{1,5}$ = 0.608, P = 0.46) (Table 3). By contrast, the random effect (island) was highly significant (L-ratio χ^2 = 24.0, d.f. = 1, P < 0.001), and the effect (σ = 0.0021) accounted for 32% of the total variance, suggesting that body condition is highly dependent on the island features.

Spatial behaviour

Leaf-toed geckos were more active on "rat-free" islands than on "rat" islands (values of CPUE being 7.85 ± 2.35 and 6.33 ± 2.30, respectively), but this difference was significantly depending on the habitat (rat occurrence × habitat type interaction: $F_{1,24}$ = 11.99, P = 0.0019, Table 4). Geckos on "rat-free" islands were active "in the open" rather than "under cover" (P = 0.057), while the opposite occurred on "rat" islands (P = 0.0091, Fig. 4), suggesting that the Leaf-toed geckos actually avoid insecure micro-habitats in presence of rats. All other predictors were not significant (see Table 4 for details). The random effect of islands (L-ratio χ^2 = 2.92, d.f. = 1, P = 0.09) was not significant, suggesting that CPUE was not affected by island features other than rat occurrence and habitat.

Table 3. Results of the linear mixed model for the variability of the body condition index of male and female Leaf-toed geckos in response to rat occurrence. Only fixed effects are reported.

Predictor	df	F	P
Rat occurrence	1,6	0.0053	0.94
Season	1,6	0.608	0.46
Sex	1,5	2.545	0.11

Table 4. Results of the linear mixed model for the variability of the activity of Leaf-toed geckos in response to rat occurrence (df have been calculated using the Satterthwaite approximation). Only fixed effects are reported.

Predictor	df	F	P
Open vs close habitats			
Rat occurrence	1,12.7	0.781	0.39
Habitat type	1,24.9	1.344	0.26
N. reptile species	1,8.5	0.629	0.45
Island size	1,8.3	0.802	0.39
Rat occurrence × Habitat type	1,24.9	11.99	0.0019
Height above ground			
Rat occurrence	1,14.9	1.068	0.32
Season	1,22.3	0.659	0.42
N. reptile species	1,2	0.142	0.74
Island size	1,4.4	2.168	0.21
Rat occurrence × Season	1,19.2	2.446	0.13

By contrast, the height from the ground where the geckoes were observed "in the open" was not affected by the rat occurrence ("rat-free" islands: 47.6 ± 2.6; "rat" islands: 32.6 ± 6.5), either by season (autumn: 51.7 ± 5.0; spring: 44.3 ± 2.7) or by their interaction (Table 4). Similarly, the effects of both island size and the number of reptile species were also negligible (see Table 4 for statistics).

We report some additional observation on the spatial behaviour. Before rat eradication, on Lavezzu Island in 1982, on 112 sightings ≈85% of geckos were active under plant cover; in 1986 (n = 50) the proportion was ≈80-85% (MD pers. obs.). Ten years after rat eradication, the percentage of geckos active under plant cover was lower but still high: 67% in 2010 (n = 41), 68% in 2011 (n = 41). On the remote Vacca Islet, rats were detected for the first time in 2010 and eradicated by the Bouches de Bonifacio Natural Reserve in less than one year (O. Bonnenfant pers. com.). We do not know the activity pattern before eradication, but in July 2012, 87% of geckos were observed in the open. Analogously, few miles away,

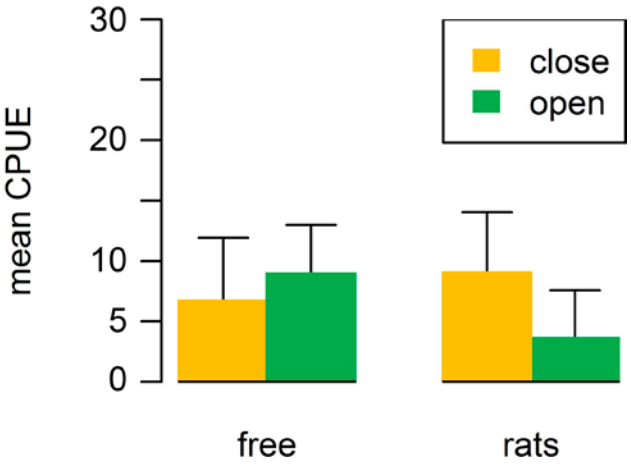

Fig. 4. Variation of the foraging mode of the European Leaf-toed geckos, "in the open" (open) and "under cover" (close) in response to the presence of rats. The relative activity of geckoes was obtained from sightings of one hour of "catch per-unit-effort" (CPUE) on 15 islets. Bars = 95% confidence interval.

rats invaded the Toro Islands where they have stayed for about 4 years, until 1992, when eradication occurred (Thibault, 1992). In April 2005 (n = 50) ≈90%, in July 2012 (n = 52) 96% and in July 2014 (n = 59) 93% geckos were observed in the open.

DISCUSSION

Two out of the three predictions on how the Black rat may influence the activity and demography of *Euleptes europaea* were confirmed by our results: a) in the presence of rats smaller sized geckos prevail, and b) geckos are less active "in the open"; c) by contrast, no significant effect on body condition was found.

Transition to populations characterized by smaller sized individuals could be caused by selective predation on larger geckos. On Ohınau Islet in New Zealand, juveniles of the giant gecko *Hoplodactylus duvaucelii* are more vulnerable to predation by *Rattus exulans* (Hoare et al., 2007), likely because adult geckos are similar in size to the Pacific rat. Conversely, a 170 g Black rat could easily predate European Leaf-toed geckos of any size (from 0.3 to 2 g). If this would be the case, a random predation of any size class could ultimately also result in a shortage of the larger sized geckos. In the Mediterranean, despite some detailed studies on this topic (Cassaing et al., 2005; Pérez-Mellado et al., 2008; Petralia et al., 2010; Ruffino et al., 2011), direct predation of *Rattus rattus* on Sauria was never observed, although predation on lizards has been supposed to occur in other regions (Caut et al., 2008; Gasc et al., 2010); ascertained (Harper and Bunbury, 2015; Thibault et al., 2016; Clapperton et al. 2019) or observed for other rat species (Towns et al., 2006). Abundance of lizards (and Tuataras) has been related to the presence of rats but the mechanisms still deserve to be explained and likely could involve competitive processes less obvious than predation (Towns et al., 2006).

Other Vertebrates or invertebrates living on the studied islands could interact with the Leaf-toed gecko as possible competitors and/or predators such as: the ant *Crematogaster scutellaris,* competing for rock crevices and predating hatching geckos (Delaugerre, 1981); introduced cows, grazing on vegetation used by geckos foraging "under cover", e.g., on Lavezzu (Delaugerre and Brunstein, 1987); the bird *Monticola solitarius* and the ant *Tapinoma erraticum* predating *E. europaea* respectively on Lavezzu (Delaugerre and Cheylan, 1992) and Porraggia Grande islands (Delaugerre and Brunstein, 1987) and perhaps the Western Whip snake *Hierophis viridiflavus,* that on Giraglia Island has been observed to have nocturnal habits (Delaugerre, 2013). However 73% of the studied islands, host just one or two reptile species (Table 1), the second species being typically a *Podarcis* (diurnal) lizard, that is unlikely a predator of the Leaf-toed gecko. On the remaining 27% islands, the presence of three or four (just on three islands) reptile species is equally distributed between rat and rat-free islands.

Alternatively, rats may affect gecko's population structure by interfering with their growth process. Survival of geckos can be reduced by increased physiological stress, which ultimately may cause physiological shifts in life history strategy and demographic fitness components (Rödl et al., 2007; Trompeter and Langkilde, 2011; Narayan et al., 2013). Rats could act as stressors for geckos just roaming around. Moreover, geckos could be subjected to infections carried by black rats (Prenter et al., 2004), often infested by the adult nematode *Mastophorus muris* (Cassaing et al., 2005). According to Lafferty et al. (2010), native geckos of the central Pacific islands might serve as paratenic hosts of *M. muris;* this stage being the most incline in affecting host fitness (Kuris, 2003).

Rats might also interact with geckos in a more indirect way, for instance inducing vegetation changes. *Lotus cytisoides*, for example, is a plant that provides high quality shelters for geckos and for a lot of invertebrates (geckos' preys) and it is consumed by rats (Cassaing et al., 2005; Ruffino et al., 2011). Behavioural shift in microhabitat use might be costly for geckos, since they would be forced to forage under plant cover with consequently limited access to bare rock surfaces more favourable for nocturnal thermoregulation (thigmothermy).

Information gathered on Lavezzu before and after rat eradication seems to confirm an influence of the presence of rats on geckos' population structure. Before rat removal, as observed for other islands with rats, geckos' size class structure was skewed towards medium-sized individuals. A decade after eradication (three geckos generations) we observed an upward trend towards larger sized individuals, even if the sample size of some size classes is too small to perform a statistical test.

Rats seem to change geckos' population structure without affecting body condition of individuals. Indeed, geckos' body condition was not influenced by the presence of rats, sex or season. However, we found a strong significant random effect of islands, suggesting that body condition in both males and females strongly depends on some island features rather than on the presence of rats.

Activity, assessed by the catch per unit effort, did not vary just according to rat occurrence, but also depending on the microhabitat. In the presence of rats, geckos avoided to forage "in the open" and were more active "under cover", probably to avoid disturbance and/or predation risk induced by the ground-dwelling rodents (Whitaker, 1973; Hoare et al., 2007). On Lavezzu Island, the "under cover" was the most common mode both before and after rat eradication, even if the percentages of geckos active "under cover" decreased ten years after eradication. This pattern (even if related to one island) may suggest a certain persistence of an avoidance behaviour likely resulted from a prolonged coexistence with rodents. Though in geckos as well as in most reptiles, young individuals cannot learn through parental care or imitation, the acquisition of a novel behaviour might take long time. Unlike how observed for the New Zealand *Hoplodactylus duvaucelii* who recovered its arboreal habitat six months after rat removal (Hoare et al., 2007), Lavezzu *Euleptes* gecko did not show a similar behavioural plasticity. Dealing with antipredator behaviours on islands Blumstein (2002) reported that «experience dependant behaviours change rapidly following isolation» (and the loss of predator), «whereas more hard wired behaviours may persist for many generations.» Does *Euleptes* geckos have acquired an avoidance behaviour over the last 2000 years while coexisting with Black rats? Or, does this behaviour date back to the Pleistocene when geckos coexisted with mammals nowadays extinct (e.g., the Tyrrhenian field rat, *Rhagamys orthodon*, M. Masseti and G. Cheylan (pers. comm.)? However, geckos' behavioural changes due to the presence of rats seem to be very variable (Hoare et al., 2007; Krebs et al., 2015).

As suggested by our results, geckos may benefit from rat eradication in most contexts but a lot of caution should be paid when undertaking rat eradications on islands that likely can be reinvaded (Harris et al., 2011; Savidge et al., 2012). Once settled on an island, a rat population may repel new invading rats through aggressive interactions, as observed by Granjon and Cheylan (1989) and Abdelkrim et al. (2009) who found that the Lavezzu population was founded by a single colonization event without further gene flow. Hence, eradication might entice new invaders to settle in a competition-free ecosystem.

Moreover, island "old-resident" rat populations are adapted to exploit seasonal resources adjusting home range, demography and intraspecific interactions (Cheylan, 1988; Clark, 1980; Cassaing et al., 2007; Ruffino et al., 2011; Pisanu et al., 2011). New invading rats (e.g., mainland rats), not familiar with the island conditions, might severely affect the local biota. Observations carried out on the Corsican Toro islands 2-4 years after rat invasion, besides the well-known detrimental effects on the Cory's shearwater and on the Pallid swift, revealed that rats preyed almost to extinction *Silene vellutina* (Caryophyllaceae) (Thibault, 1992), a rare endemic plant that elsewhere was not (or only marginally) consumed by "old-resident" rats.

Since several eradication actions have 'failed', and reinvasions occurred (Cheylan and Granjon, 1987; Howald et al., 2007; Russell et al., 2010; Savidge et al., 2012; Sposimo et al., 2012; Ragionieri et al., 2013), the urge to learn from failures led to the proposal to adopt the metapopulational (Russell et al., 2008) or the eradication units approach (Robertson and Gemmell, 2004) by considering first islands' assemblages and relative reinvasion sources in order to properly guide eradication programs. Native island species must cope with an increasing number of biological invasions; understanding the mechanisms of invasions, as well as the interaction between native species and long coexisting aliens, may be crucial to adopt proper conservation actions.

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REFERENCES

Abdelkrim, J., Pascal, M., Samadi, S. (2009): Genetic structure and functioning of alien ship rat populations from a Corsican micro-insular complex. Biol. Invasions **11**: 473-482.

Anderson, M.J. (2001): A new method for non-parametric multivariate analysis of variance. Austral Ecol. **26**: 32-46.

Atkinson, I.A. (1985): The spread of commensal species of *Rattus* to oceanic islands and their effects on island avifaunas. Conservation of Island Birds **3**: 35-81.

Bates, D., Mächler, M., Bolker, B., Walker, S. (2014): Fitting linear mixed-effects models using lme4. **ArXiv**:*1406.5823*.

Bennett, P.M., Owens, I.P.F., Nussey, D., Garnett, S.T., Crowley, G.M. (2005): Mechanisms of extinction in birds: phylogeny, ecology and threats. In: Phylogeny and Conservation (Conservation Biology), pp. 317-336. Purvis, A., Gittleman, J., Brooks, T., Eds, Cambridge University Press, Cambridge.

Berthon, K. (2015): How do native species respond to invaders? Mechanistic and trait-based perspectives. Biol. Invasions **17**: 2199-2211.

Blumstein, D.T. (2002): Moving to suburbia: ontogenetic and evolutionary consequences of life on predator-free islands. J. Biogeogr. **29**: 685-692.

Bonnet, X., Naulleau, G. (1994): Utilisation d'un indice de condition corporelle (BCI) pour l'étude de la reproduction chez les serpents. Comptes Rendus de l'Académie Des Sciences. Série 3, Sciences de La Vie **317**: 34-41.

Brown, J.H., Lomolino, M.V. (2000): Concluding remarks: historical perspective and the future of island biogeography theory. Global Ecol. Biogeogr. **9**: 87-92.

Capizzi, D., Baccetti, N., Sposimo, P. (2010): Prioritizing rat eradication on islands by cost and effectiveness to protect nesting seabirds. Biol. Conserv. **143**: 1716-1727.

Cassaing, J., Derré, C., Moussa, I., Parghentanian, T., Bocherens, H., Cheylan, G. (2005): Le régime alimentaire du rat noir *Rattus rattus* dans les îles d'Hyères analysé par la biochimie isotopique et les contenus stomacaux. Sci. Rep. Port-Cros Natl. Park, Fr. **21**: 85-115.

Cassaing, J., Derré, C., Moussa, I., Cheylan, G. (2007): Diet variability of Mediterranean insular populations of *Rattus rattus* studied by stable isotope analysis. Isotopes in Environmental and Health Studies **43**: 197-213.

Caut, S., Angulo, E., Courchamp, F. (2008): Dietary shift of an invasive predator: rats, seabirds and sea turtles. J. Appl. Ecol. **45**: 428-437.

Cheylan, G. (1988). Les adaptations écologiques de *Rattus rattus* à la survie dans les îlots méditerranéens (Provence et Corse). Bull. Ecol. **19**: 417-426.

Cheylan, G., Granjon, L. (1987). Ecologie du rat noir à Lavezzi (Corse du Sud): abondances, déplacements et reproduction. Travaux Scientifiques—Parc Naturel Régional et Réserves Naturelles de Corse **12**: 71-91.

Clapperton, K., Maddigan, F., Chinn, W., Murpy, E. (2019): Diet, Population Structure and Breeding of *Rattus rattus* L. in South Island Beech Forest. New Zeal. J. Ecol. **43**: 1-8.

Clark, D.B. (1980): Population ecology of *Rattus rattus* across a desert-montane forest gradient in the Galápagos Islands. Ecology **61**: 1422-1433.

Corti, C., Böhme, W., Delfino, M., Masseti, M. (1999): Man and lacertids on the Mediterranean islands: conservation perspectives. Natura Croatica **8**: 287-300.

Courchamp, F., Chapuis, J.-L., Pascal, M. (2003): Mammal invaders on islands: impact, control and control impact. Biol. Rev., **78**: 347-383.

Delaugerre, M. (1981). Sur l'histoire naturelle de *Phyllodactylus europaeus* Gené, (Gekkonidae, Sauria, Reptiles): Port-Cros : étude d'une population naturelle. Trav. Sci. Parc. Nation. Port-Cros **6**: 147-175.

Delaugerre, M., Brunstein, D. (1987): Observations sur la flore et la faune de plusieurs îlots du sud de la Corse (archipels des Lavezzi, des Cerbicale et côte sud-orientale). Travaux Scientifiques—Parc Naturel Régional et Réserves Naturelles de Corse **12**: 1-17.

Delaugerre, M., Cheylan, M. (1992): Atlas de répartition des batraciens et reptiles de Corse. Ajaccio Parc naturel régional de Corse: Ecole pratique des hautes études.

Delaugerre, M., Dubois, A. (1985): La variation géographique et la variabilité intrapopulationnelle chez *Phyllodactylus europaeus* (Reptilia, Sauria, Gekkonidae). Bulletin Du Muséum National d'histoire Naturelle. Section A, Zoologie, Biologie et Écologie Animales, **7**: 709-736.

Delaugerre, M., Ouni, R., Nouira, S. (2011). Is the European Leaf-toed gecko *Euleptes europaea* also an Afri-

can? Its occurrence on the Western Mediterranean landbrige islets and its extinction rate. Herpetol. Notes **4**: 127-137.

Delaugerre, M.-J. (2013). Going out tonight? When insular *Hierophis viridiflavus* breaks the Whip Snakes Rules. Acta Herpetol. **8**: 47-52.

Drake, D.R., Hunt, T.L. (2009): Invasive rodents on islands: integrating historical and contemporary ecology. Biol. Inv. **11**: 1483-1487.

Foufopoulos, J., Ives, A.R. (1999). Reptile extinctions on land-bridge islands: life-history attributes and vulnerability to extinction. Am. Nat. **153**: 1-25.

Gasc, A., Duryea, M.C., Cox, R.M., Kern, A., Calsbeek, R. (2010): Invasive predators deplete genetic diversity of island lizards. PloS One **5**: e12061.

Granjon, L., Cheylan, G. (1989): Le sort de rats noirs (*Rattus rattus*) introduits sur une île, relevé par radiotracking. Comptes Rendus de l'Académie Des Sciences. Série 3, Sciences de La Vie **309**: 571-575.

Harper, G.A., Bunbury, N. (2015): Invasive rats on tropical islands: Their population biology and impacts on native species. Global Ecol. Conserv. **3**: 607-627.

Harris, D.B., Gregory, S.D., Bull, L. S., Courchamp, F. (2011): Island prioritization for invasive rodent eradications with an emphasis on reinvasion risk. Biol. Inv. **14**: 1251-1263.

Hoare, J.M., Pledger, S., Nelson, N.J., Daugherty, C.H. (2007): Avoiding aliens: Behavioural plasticity in habitat use enables large, nocturnal geckos to survive Pacific rat invasions. Biol. Conserv. **136**: 510-519.

Howald, G., Donlan, C.J., Galván, J.P., Russell, J.C., Parkes, J., Samaniego, A., Wang, Y., Veitch, D., Genovesi, P., Pascal, M., Saunders, A., Tershy, B. (2007): Invasive Rodent Eradication on Islands. Conserv. Biol. **21**: 1258-1268.

Jones, H.P., Tershy, B.R., Zavaleta, E. ., Croll, D.A., Keitt, B.S., Finkelstein, M.E., Howald, G.R. (2008): Severity of the Effects of Invasive Rats on Seabirds: A Global Review: Effects of Rats on Seabirds. Conserv. Biol. **22**: 16-26.

Krebs, É, Abba, A, Gillet, P, Eudeline, R, Gauthier, J, Le Quilliec, P, Lorvelec, O, Martinerie, G, Vidal, É, Buisson, É. (2015): Réponses des populations de reptiles à l'éradication du Rat noir (*Rattus rattus*) sur l'île de Bagaud (Parc national de Port-Cros, Var, France). Rev. Ecol.-Terre Vie. **70**: 99-109.

Kuris, A.M. (2003): Evolutionary ecology of trophically transmitted parasites. J. Parasitol. **89**: S96–S100.

Lafferty, K.D., Hathaway, S.A., Wegmann, A.S., Shipley, F.S., Backlin, A.R., Helm, J., Fisher, R.N. (2010): Stomach Nematodes (*Mastophorus Muris*) in Rats (*Rattus rattus*) Are Associated with Coconut (*Cocos nucifera*) Habitat at Palmyra Atoll. J. Parasitol. **96**: 16-20.

Legendre, P., Legendre, L.F. (2012): Numerical ecology. Elsevier B.V., Amsterdam.

Martin, J.-L., Thibault, J.-C., Bretagnolle, V. (2000): Black rats, island characteristics, and colonial nesting birds in the Mediterranean: consequences of an ancient introduction. Conserv. Biol. **14**: 1452-1466.

McArdle, B.H., Anderson, M.J. (2001): Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecology **82**: 290-297.

Mertens, R. (1970): Ueber die Lebensdauer einiger Amphibien und Reptilien in Gefangenschaft. Zool. Gart. **39**: 13-209

Mooney, H.A., Cleland, E.E. (2001): The evolutionary impact of invasive species. P. Natl. Acad. Sci. USA, **98**: 5446-5451.

Moser, D., Lenzner, B., Weigelt, P., Dawson, W., Kreft, H., Pergl, J., Pyšek, P., van Kleunen, M., Winter, M., Capinha, C., Cassey, P., Dullinger, S., Economo, E.P., García-Díaz, P., Guénard, B., Hofhansl, F., Mang, T., Seebens, H., Essl, F. (2018): Remoteness promotes biological invasions on islands worldwide. P. Natl. Acad. Sci. USA, **115**: 9270-9275.

Müller, J. (2001): A new fossil species of *Euleptes* from the early Miocene of Montaigu, France (Reptilia, Gekkonidae). Amphibia-Reptilia **22**: 341-348.

Narayan, E.J., Cockrem, J.F., Hero, J.-M. (2013): Sight of a Predator Induces a Corticosterone Stress Response and Generates Fear in an Amphibian. PLoS One **8**: e73564.

Pérez-Mellado, V., Hernández-Estévez, J.Á., García-Díez, T., Terrassa, B., Ramón, M.M., Castro, J., Picornell, A., Martín-Vallejo, J., Brown, R. (2008): Population density in *Podarcis lilfordi* (Squamata, Lacertidae), a lizard species endemic to small islets in the Balearic Islands (Spain). Amphibia-Reptilia **29**: 49-60.

Petralia, E., Messina, A., Petralia, A., Siracusa, A.M. (2010): Stato della popolazione di *Rattus rattus* (Linnaeus, 1758)(Rodentia, Muridae) nella Riserva Naturale Integrale "Isola Lachea e Faraglioni dei Ciclopi" di Acicastello (Catania, Italia). Bolletino Accademia Gioenia Sci. Nat., **43**: 12-22.

Pisanu, B., Caut, S., Gutjahr, S., Vernon, P., Chapuis, J.-L. (2011): Introduced black rats *Rattus rattus* on Ile de la Possession (Iles Crozet, Subantarctic): diet and trophic position in food webs. Polar Biol. **34**: 169-180.

Prenter, J., MacNeil, C., Dick, J.T., Dunn, A.M. (2004): Roles of parasites in animal invasions. Trends Ecol. Evol., **19**: 385-390.

R Core Team. (2018). R: A language and environment for statistical computing. https://www.r-project.org/

Ragionieri, L., Cutuli, G., Sposimo, P., Spano, G., Navone, A., Capizzi, D., Baccetti, N., Vannini, M., Fratini, S. (2013): Establishing the eradication unit of Molara Island: a case of study from Sardinia, Italy. Biol. Inv. **15**: 2731-2742.

Recher, H.F., Clark, S.S. (1974): A biological survey of Lord Howe Island with recommendations for the conservation of the island's wildlife. Biol. Conserv. **6**: 263-273.

Robertson, B.C., Gemmell, N.J. (2004): Defining eradication units in pest control programmes. J. Appl. Ecol. **41**: 1032-1041.

Rödl, T., Berger, S., Romero, L.M., Wikelski, M. (2007): Tameness and stress physiology in a predator-naive island species confronted with novel predation threat. P. Roy. Soc. B-Biol. Sci. **274**: 577-582.

Ruffino, L., Bourgeois, K., Vidal, E., Duhem, C., Paracuellos, M., Escribano, F., Sposimo, P., Baccetti, N., Pascal, M., Oro, D. (2009): Invasive rats and seabirds after 2,000 years of an unwanted coexistence on Mediterranean islands. Biol. Inv. **11**: 1631-1651.

Ruffino, L., Russell, J.C., Pisanu, B., Caut, S., Vidal, E. (2011): Low individual-level dietary plasticity in an island-invasive generalist forager. Popul. Ecol. **53**: 535-548.

Ruffino, L., Vidal, E. (2010): Early colonization of Mediterranean islands by *Rattus rattus*: a review of zooarcheological data. Biol. Inv. **12**: 2389-2394.

Russell, J.C., Beaven, B.M., MacKay, J.W.B., Towns, D.R., Clout, M.N. (2008): Testing island biosecurity systems for invasive rats. Wildlife Res. **35**: 215.

Russell, J.C., Miller, S.D., Harper, G.A., MacInnes, H.E., Wylie, M.J., Fewster, R.M. (2010): Survivors or reinvaders? Using genetic assignment to identify invasive pests following eradication. Biol. Inv. **12**: 1747-1757.

Salvidio, S., Delaugerre, M. (2003): Population dynamics of the European leaf-toed gecko (*Euleptes europaea*) in NW Italy: implications for conservation. Herpetol. J. **13**: 81-88.

Salvidio, S., Lanza, B., Delaugerre, M.J. (2010): *Euleptes europaea* (Gené, 1839). In: Fauna d'Italia Vol. 45 (Reptilia), pp. 258-270. Corti, C., Capula, M., Luiselli, L., Razzetti, E., Sindaco, R., Eds, Edizioni Calderini de Il Sole 24 ORE, Milano.

Savidge, J.A., Hopken, M.W., Witmer, G.W., Jojola, S.M., Pierce, J.J., Burke, P.W., Piaggio, A.J. (2012): Genetic evaluation of an attempted *Rattus rattus* eradication on Congo Cay, U.S. Virgin Islands, identifies importance of eradication units. Biol. Inv. **14**: 2343-2354.

Sax, D.F., Stachowicz, J.J., Brown, J.H., Bruno, J.F., Dawson, M.N., Gaines, S.D., Grosberg, R.K., Hastings, A., Holt, R.D., Mayfield, M.M. (2007): Ecological and evolutionary insights from species invasions. Trends Ecol. Evol. **22**: 465-471.

Silva-Rocha, I., Montes, E., Salvi, D., Sillero, N., Mateo, J.A., Ayllón, E., Pleguezuelos, J.M., Carretero, M.A. (2018): Herpetological History of the Balearic Islands: When Aliens Conquered These Islands and What to Do Next. In: Histories of Bioinvasions in the Mediterranean, pp. 105–131. Queiroz, A.I., Pooley, S., Eds., Springer International Publishing, Cham, Switzerland.

Silva-Rocha, I., Salvi, D., Carretero, M.A., Ficetola, G.F. (2019): Alien Reptiles on Mediterranean Islands: A Model for Invasion Biogeography. Divers. Distrib. **25**: 995-1005.

Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M. (2013): Impacts of biological invasions: what's what and the way forward. Trends Ecol. Evol. **28**: 58-66.

Simberloff, D.S. (1974): Equilibrium theory of island biogeography and ecology. Annu. Rev. Ecol. Syst. **5**: 161-182.

Sposimo, P., Spano, G., Navone, A., Fratini, S., Ragionieri, L., Putzu, M., Capizzi, D., Baccetti, N. (2012): Rodent eradication on Molara Island and surrounding islets (NE Sardinia): from success to the riddle of reinvasion. Aliens **32**: 33-38.

Strauss, S.Y., Lau, J.A., Carroll, S.P. (2006): Evolutionary responses of natives to introduced species: what do introductions tell us about natural communities? Ecol. Lett. **9**: 357-374.

Stuart, Y.E., Campbell, T.S., Hohenlohe, P.A., Reynolds, R.G., Revell, L.J., Losos, J.B. (2014): Rapid evolution of a native species following invasion by a congener. Science **346**: 463-466.

Thibault, J.C. (1992): Eradication of the Brown Rat from the Toro Islets (Corsica): remarks about an unwanted colonizer. Avocetta **16**: 114-117.

Thibault, J.C., Delaugerre, M., Cheylan, G., Guyot, I., Miniconi, R. (1987): Les vertébrés terrestres non domestiques des îles Lavezzi (sud de la Corse). Bulletin de La Société Linnéenne de Lyon **56**: 73-103.

Thibault, M., Brescia, F., Jourdan, H., Vidal, E. (2016): Invasive rodents, an overlooked threat for skinks in a tropical island hotspot of biodiversity. New Zealand J. Ecol. **41**: 74-83.

Towns, D.R., Atkinson, I.A.E., Daugherty, C.H. (2006): Have the harmful effects of introduced rats on islands been exaggerated? Biol. Inv. **8**: 863-891.

Traveset, A., Richardson, D. (2006): Biological invasions as disruptors of plant reproductive mutualisms. Trends Ecol. Evol. **21**: 208-216.

Trompeter, W.P., Langkilde, T. (2011): Invader danger: Lizards faced with novel predators exhibit an altered behavioral response to stress. Horm. Behav. **60**: 152-158.

Vigne, J.-D., Valladas, H. (1996): Small mammal fossil assemblages as indicators of environmental change in northern Corsica during the last 2500 years. J. Archaeol. Sci. **23**: 199-215.

Whitaker, A.H. (1973): Lizard populations on islands with and without Polynesian rats, *Rattus exulans* (Peale). Proc. New Zeal. Ecol.Soc. **20**: 121-130.

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PIT-Tags as a technique for marking fossorial reptiles: insights from a long-term field study of the amphisbaenian *Trogonophis wiegmanni*

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Abstract. Many field studies of ecology or conservation require individual identification of the animals, and for this, several marking techniques have been developed. However, no specific labeling technique has been tested for fossorial reptiles, such as amphisbaenians. We describe the use of Passive Integrated Transponder (PIT) tags as a long-term labeling method of the amphisbaenian *Trogonophis wiegmanni*. We present the details of the marking procedure and examine the benefits and drawbacks of the technique considering the fossorial environment. After marking many individuals in a long-term field study, we can ensure that the marks were easily applicable and were not lost over a period of at least four years. Moreover, PIT tags did not negatively affect the body condition of amphisbaenians. We conclude that PIT tags are useful for doing field studies of this and similar fossorial species.

Keywords. Amphisbaenians, *Trogonophis wiegmanni*, PIT-tagging, body condition, fossorial reptiles.

INTRODUCTION

Many field studies of ecology, behavior or conservation require individual recognition of the subjects that make up a population. Being able to distinguish individuals allows the assessment of diverse ecological traits such as the size and dynamics of the population, survivorship, movements, home ranges, activity patterns, social interactions, etc. (reviewed in Plummer and Ferner, 2012; Ferner and Plummer, 2016). For that reason, labeling individuals is often necessary, and diverse tagging techniques have been developed depending on the species and/or the traits that are the object of study. Ideally, these marks should allow a correct identification and be easily applicable, but without causing suffering to the animals, and they should last for at least the duration of the entire field study, but without affecting survival or behavior of the marked animals (reviewed in Ferner and Plummer, 2016).

Diverse methods of marking individuals have been described for reptiles. Some are intended for short-term studies, such as external painting marks, beads, adhesive tapes, elastic bands, metal or plastic discs, buttons, etc. (Gibbons and Andrews, 2004; Ribeiro and Sousa, 2006; Ferner and Plummer, 2016). While others are focused on long-term studies, such as toe clipping, scale clipping, shell notching on turtles, heat/freeze branding, photo identification based on natural markings, Visible Implant Elastomer (VIE) tags and/or Passive Integrated Transponder (PIT) tags (Daniel et al., 2006; Hutchens et al., 2008; Ekner et al., 2011; Ferner and Plummer, 2016).

Several groups of reptiles and amphibians, comprising as much as 20% of the global herpetofauna, or nearly 3,000 species, are fossorial (Measey, 2006). However, as is the case with other fossorial animals, their ecology and conservation status are much less well understood than those of their epigeal relatives (Copley, 2000; Wolters, 2001; Böhm et al., 2013). This may be explained because

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of the difficulty of doing field studies of fossorial animals (Measey, 2006; Henderson et al., 2016), which includes difficulties in individually marking these animals, given their burrowing habits. Although several marking techniques have been tested in fossorial caecilians (Measey et al., 2001) and *Ambystoma* salamaders (Connette and Semlitsch, 2012), to our knowledge, no specific labeling technique has been tested for limbless fossorial reptiles such as amphisbaenians (Henderson et al., 2016).

Due to the morphology of most amphisbaenian species (i.e., elongated body without limbs in most species), it is obviously not possible to use many types of marking methods. Further, given the fossorial habits of amphisbaenians, most external markings (painting, beads, adhesive tapes, etc.) may be incompatible with the burrowing behavior of these animals and will be quickly lost by repeated contact of the body with the soil. Therefore, potential methods that could be used for long-term marking of amphisbaenians might be restricted to scale clipping, heat/freeze branding, VIE tags and/or PIT tags (Camper and Dixon, 1988; Jemison et al., 1995; Hutchens et al., 2008; Ferner and Plummer, 2016). Here, we describe the use of PIT tags as a labeling method for long-term field studies of the checkboard amphisbaenian *Trogonophis wiegmanni*, Kaup 1830. A PIT tag is a microchip with an electromagnetic coil encased in a biocompatible glass cylinder, encoded alphanumerically in an unique way, that is implanted in the animal (Gibbons and Andrews, 2004). We present here the detailed marking procedure that we applied to amphisbaenians, examine the potential benefits and drawbacks of the technique, considering the peculiar characteristics of the fossorial environment, and discuss its utility for doing ecological studies of this and similar fossorial species.

MATERIALS AND METHODS

Study species

The checkboard amphisbaenian *T. wiegmanni*, Kaup 1830 is a representative of the family Trogonophidae (Gans, 2005) (Fig. 1a) that inhabits arid areas from southwest Morocco to northeast Tunisia (Bons and Geniez, 1996). These amphisbaenians live all their life buried in the soil, but they are frequently found under rocks (Civantos et al., 2003; Martín et al., 2013a). Little research has been carried out on this species, as on other amphisbaenians, but there is now a growing body of information on aspects such as its thermal biology (López et al., 2002), microhabitat and soil selection (Civantos et al., 2003; Martín et al., 2013a), reproduction (Bons and Saint Girons, 1963), social behavior and population structure (Martín et al., 2011b, c) or diet (Martín et al., 2013b; Baeckens et al., 2017). However, all these studies have been made by randomly sampling unmarked amphisbaenians. More detailed studies would require to individually identify the amphisbaenians that are being examined. This is important, not only because of the scientific interest in understanding the ecology and behavior of amphisbaenians, but because several conservation problems that may potentially affect their populations have been noted (Martín et al., 2011a, 2015, 2017), and a detailed long-term monitoring of these populations require the ability to individually identify and follow the study subjects.

Field study and marking procedure

We have carried out field and laboratory studies of *T. wiegmanni* amphisbaenians on the Chafarinas Islands (Spain) for almost twenty years. This is an archipelago, formed by three small islands, located in the southwestern area of the Mediterranean Sea (35°10'N, 02°25'W), 2.5 nautical miles to the north of the Moroccan coast (Ras el Ma, Morocco) and 27 miles to the east of the Spanish city of Melilla. The islands have a dry, warm, Mediterranean climate, and vegetation is dominated by bushy plants (*Suaeda, Salsola*, *Lycium* and *Atriplex*) adapted to salinity and drought. *Trogonophis wiegmanni* is very common and is represented by very large populations on these islands (Martín et al., 2011a).

During the years 2015-2018, we made field campaigns twice a year, during two weeks in spring (March-April) and two weeks in Autumn (September-October), to capture, mark and recapture *T. wiegmanni*. We delimited three study plots (surface area = 0.14 Ha, 0.40 Ha and 0.58 Ha) on different islands, which we walked systematically and intensively during the morning and afternoon of different days. Amphisbaenians were found by carefully lifting almost all rocks located inside the study plots. Individuals were captured by hand, measured and immediately after marked in the field with PIT tags. We used one of the smallest available PIT tags (Biomark MiniHPT8; Biomark, Inc., Boise, Idaho, USA), with a length of 8.4 mm, 1.4 mm in diameter and a weigh of 0.03 g. This weigh represents 0.6 % of the mean body mass (i.e., around 5 g) of a typical adult amphisbaenian in our population (Martín et al., 2011c). We gently implanted PIT tags subcutaneously in the upper right side of the body of amphisbaenians (Fig. 1). For this, we made a small puncture at around 3 cm from the snout (mean SVL of adult amphisbaenians is around 14 cm) using a stainless steel needle (Biomark N165 needle; length = 5.1 cm, needle diameter = 1.49 mm), disinfected with alcohol before and after puncturing each individual, which was fitted to a specially designed syringe style implanter (Biomark MK165 syringe). We gently lifted the skin from the underlying muscle and then inserted the transponder subcutaneously using the implanter. During the insertion of the PIT tag, the needle was maintained parallel to the body to ensure that the tag remained under the skin and did not enter the coelomic cavity (Fig. 1). The injection site was immediately disinfected with alcohol after the implant. According to Brown (1997), losses of PIT tags may occur immediately after the implant is done, while the wound is still open. To avoid this, incisions may be sealed with medical grade suture glue. However, in our case, this was not needed as the incision was

Fig. 1. An adult amphisbaenian (*Trogonophis wiegmanni*) as it was found under a stone (left); PIT tag implantation procedure (right).

very small and the tags showed no evidence of becoming displaced. Further, at least in lizards, the glue may slow the healing process (Le Galliard et al., 2011). Moreover, the long needle pushed up the tag under the skin towards the posterior part of the animal. Thus, the tag was implanted at least 2 cm posterior of the small puncture point, which precluded tits loss when the amphisbaenian burrowed forward. All the marking procedure could be easily made by a single experienced researcher, holding the amphisbaenian with one hand and the implanter with the other. However, the presence of an additional researcher, who prepared the equipment and took notes, made the process easier and quicker, decreasing the manipulation time and disturbance to the animals.

This marking technique is particularly appropriate for amphisbaenians, as their skin attachment is quite loose and leaves a subcutaneous space where the pit-tag is inserted. The skin is connected to the axial mass by costocutaneous and vertebrocutaneous muscles, that allow the skin to move independently from the body, mainly in rectilinear locomotion (Gans, 1978; Gasc, 1981; see illustrations in Smalian, 1884), As those muscles are numerous and redundant, the insertion of a strange body (or even the damage of some muscle fibers) should not interfere with the normal locomotion or excavation.

Although amphisbaenians obviously "felt" and showed a small aversive response to the puncture with the needle, we did not observe any subsequent additional negative behavioral responses (e.g., stress, immobility, forced unnatural movements, or attempts to remove the tag) (Warwick et al., 2013). Amphisbaenians behaved normally when they were released at their capture points a few minutes after being captured and marked. The implant procedure very rarely resulted in a small drop of blood, but in that case the wound was cleaned with alcohol and bleeding stopped rapidly. We avoided the use of local anesthesia, because the duration of the recovery time from anesthesia could be much longer than the natural recovery from the implanting procedure. Moreover, the administration of anesthesia *per se* is an additional procedure that requires increasing manipulation time and careful control of conditions, and it could have negative physiological side effects for small reptiles (Heard, 2001; Chatigny et al., 2017).

A hand-held portable reader (Biomark 601 Reader) was used to read the individual unique code of the tag (the tags have a 134.2 kHz, ISO FDX-B, frequency), The code can be provided either as a hexadecimal or as a decimal number (15 digits). In the practice, the four last digits were enough for a reliable identification of individuals in each study population. The reader works in the field with AA rechargeable batteries but it may be also used in the lab with an AC power supply.

To test the long-term effect of PIT tags in amphisbaenians, we compared the body condition of individuals at first capture, when they were untagged, and when they were recaptured one year after being implanted with a PIT tag. Body condition was assessed as the residuals of an ordinary least squares linear regression of log-transformed mass (measured with an electronic balance to the nearest 0.1 g) against log-transformed total length (measured with a metallic ruler to the nearest 1 mm). To ensure that amphisbaenians had empty stomach and intestines before being weighed, we gently compressed their vents to force the expulsion of feces (used for a study of diet). The small weight of the tag was considered negligible.

RESULTS AND DISCUSSION

In the four years of marking amphisbaenians, we have implanted PIT tags in a mean of 45 $\pm$ 4 amphisbaenians per study plot and campaign (3 plots and 7 campaigns of 15 days each), which so far leads to a grand total of 930 marked individuals in the four years. The number of individuals found and marked was significantly higher in spring than in autumn for a similar search effort ($F_{1,19} = 11.82$, $P = 0.003$).

Recapture rate was, however, relatively low; only around 15% of individuals found had already been marked. This is likely attributable to the difficulty of find-

ing the same individual on several occasions in a relatively short field campaign (i.e., each study plot is surveyed only during 3-4 days per campaign) and the high density of amphisbaenians, rather than to the fact that the marking procedure might affect survivorship or that the tags were lost and we were not be able to identify previously marked individuals. In fact, when we captured an unmarked individual, we always ensured that it had no scars at the usual injection point, which may indicate that it had been marked previously but had no tag inside. Such scars are typical of marked individuals, but they have never been observed in unmarked individuals. Also, we have not noted a decrease of population size, as assessed from the number of individuals usually found in a working day, which might reflect low survivorship of marked animals. Moreover, nearby populations on the islands, where we sampled amphisbaenians without marking them, show similar trends to the marked populations (unpublished data). Therefore, we are confident that the marking procedure is effective and it is not adversely affecting the populations.

Several authors have considered that PIT tags are not always permanent (Brown, 1997; Ott and Scott, 1999), while others claim permanence for more than 20 (Germano and Williams, 1993) or 70 years (Ferner and Plummer, 2016). In our study, we have recaptured individuals marked in the first year of the fieldwork after four years and we are confident that the mark will persist during the entire life of the amphisbaenian. Gibbons and Andrews (2004) postulated that tag migration may complicate code checking when it is not possible to find the tag, and can also lead to health problems when migrating through the digestive or urinary systems (Jemison et al., 1995). This problem may be greater in fossorial burrowing animals due to the constant friction with the substrate (Measey et al., 2001). In our study, although tag migration occurred in several individual amphisbaenians, in all cases, the tag had stayed just under the skin and was relocated posteriorly of the injection point, reaching a point close to the cloaca in the longest observed migrations. This movement of the tag seems to be along the subcutaneous space typical of amphisbaenians (see above) (Gans, 1978; Gasc, 1981). We did not detect injuries or health problems (e.g., infection, sores, bleeding, low body condition, etc) in any case. Besides, we did not encounbter any problems in reading the tag, even in cases where its exact location was not easily detected at first sight, probably because the small size of *T. wiegmanni* allowed us to scan the entire body surface under the reader at the same time.

On the other hand, long-term effects of PIT tags have been described for several species. However, Lobos et al. (2013) did not find significant impacts on growth rates, mating, or risk of predation when PIT tagging different species of *Liolaemus* lizards. Brown (1997) also concluded that PIT tags did not make any difference in survivorship nor body condition of diverse amphibians, and Keck (1994) obtained similar results for growth rates and mobility in several snake species. Nevertheless, females of the newt *Ichthyosaura alpestris* laid significantly more eggs when marked, which seems to be related to a stress response (Perret and Joly, 2002). Also, measures of corticosterone in blood have shown that the PIT tag implanting procedure can be stressful for small skinks at least 14 days after the implant (Langkilde and Shine, 2006), but have no effects on stress five days after in common lizards (Le Galliard et al., 2011). Our data show that PIT tags do not have a negative long-term impact on the body condition of *T. wiegmanni* (body condition of the same individuals, initial vs. recapture: 0.05 ± 0.03 vs. 0.06 ± 0.04; one-way repeated measures ANOVA: $F_{1,96} = 0.34$, $P = 0.56$). This lack of change of the body condition is a good indication of the absence of long-term negative effects of the PIT tag on health of individuals, as it is known that natural and anthropomorphic alterations of the soil are reflected in a low body condition of these amphisbaenians (Martín et al., 2015, 2017).

Another disadvantage associated with PIT tagging may be related to the price of the reader and each transponder (Gibbons and Andrews, 2004). In our case, the current price of each PIT tag is $2.58 (they are provided in packs of 100 units), the implanters cost $5 each (each one is useful for many markings), the needles costs $2 each (for an optimal functioning, we used a different needle for every 20 punctures), and the reader costs $595. These costs may be normally easily assumed by research or conservation projects financed by the government or other institutions.

With respect to the invasiveness of the procedure, it has been recommended that it should not be used for individuals smaller than 8 cm (Camper and Dixon, 1988; Gibbons and Andrews, 2004; Ferner and Plummer, 2016). In our study, the small size of the PIT tags allowed us to mark amphisbaenians as small as 90 mm SVL without problems, the suitability for being marked depending more on the diameter of the body than on the length. These "small" amphisbaenians are second year young subadult individuals (Martín et al., 2001c). Only newborn individuals (SVL<70 mm when they born in autumn) seem unsuitable for marking with these PIT tags, considering the size of the currently available tags. This can be a problem that precludes the study of aspects of population dynamics, such as growth rates and survivorship of juveniles in their first year. However, given the low movement rate of amphisbaenians, is still possible to assess the

number of individuals born in a population if we control for the geographic location of the newborn individuals found, to ensure that we do not repeat the same individual on different days. We expect that the future development of smaller PIT tags will allow them to be used in all individuals.

In contrast to PIT tags, scale clipping and heat branding may be cheaper (Winne et al., 2006; Ekner et al., 2011), whereas VIE tags can be seen without capturing the subject (Daniel et al., 2006; Hutchens et al., 2008), and none of these techniques are as invasive as PIT tagging (Gibbons and Andrews, 2004; Ferner and Plummer, 2016). However, PIT tags offer numerous advantages compared with the other long-term techniques potentially useful for marking amphisbaenians. PIT tags are permanent and marks are unmistakable, while brands made by scale clipping or heating/freezing procedures may be confounded with natural marks or become unreadable as time passes due to external agents (Winne et al., 2006; Ekner et al., 2011). Similarly, VIE tags might be hard to detect in darkly pigmented tissues (Hutchens et al., 2008; Petit et al., 2012). Also, PIT tags are able to provide data even after the death of the marked individual, and/or can be even reused (Gibbons and Andrews, 2004). Finally, PIT-tag telemetry (i.e., detecting the radiofrequency signal of the tag at distance; e.g., Connette and Semlitsch, 2012; Ousterhout and Semlitsch, 2014) may allow the detection and relocation of fossorial animals burrowed underground without physically contacting them. However, for this technique a special, and more expensive, detector with an attached antenna is needed to carefully scan the soil surface, and the detection range for the smallest 8 mm PIT tags is only 16 cm in depth increasing to 30 cm for a 12 mm PIT tag (Ousterhout and Semlitsch, 2014).

In conclusion, although further and more specific studies are needed to test the usefulness and effectiveness of PIT tagging in fossorial reptiles, it seems to be a valid procedure for individual recognition in long-term field studies of amphisbaenians such as *Trogonophis wiegmanni*, and, therefore, we suggest that it may be also applied to other similar limbless fossorial species.

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REFERENCES

Baeckens, S., García-Roa, R., Martín, J., Ortega, J., Huyghe, K., Van Damme, R. (2017): Fossorial and durophagous: implications of molluscivory for head size and bite capacity in a burrowing worm lizard. J. Zool. **301**: 193-205.

Böhm, M., Collen, B. Baillie, J.E.M., Bowles, P., Chanson, J. et al. (2013): The conservation status of the world's reptiles. Biol. Cons. **157**: 372-385.

Bons, J., Geniez, P. (1996): Amphibians and reptiles of Morocco. Asociación Herpetológica Española, Barcelona.

Bons, J., Saint Girons, H. (1963): Ecologie et cycle sexuel des amphisbeniens du Maroc. Bull. Soc. Sci. Nat. Phys. Maroc. **43**: 117-158.

Borges-Landáez, P.A., Shine, R. (2003): Influence of toe-clipping on running speed in *Eulamprus quoyii*, an australian scincid lizard. J. Herpetol. **37**: 592-595.

Brown, L.J. (1997): An evaluation of some marking and trapping techniques currently used in the study of anuran population dynamics. J. Herpetol. **31**: 410-419.

Camper, J.D., Dixon, J.R. (1988): Evaluation of a microchip marking system for amphibians and reptiles. Texas Parks and Wildlife Department, Research Publication, 7100-159. Austin, Texas.

Chatigny, F., Kamunde, C., Creighton, C.M., Stevens, E.D. (2017): Uses and doses of local anesthetics in fish, amphibians, and reptiles. J. Am. Assoc. Lab. Anim. Sci. **56**: 244-253.

Civantos, E., Martín, J., López, P. (2003): Fossorial life constrains microhabitat selection of the amphisbaenian *Trogonophis wiegmanni*. Can. J. Zool. **81**: 1839-1844.

Connette, G.M., Semlitsch, R.D. (2012): Successful use of a passive integrated transponder (PIT) system for below-ground detection of plethodontid salamanders. Wildlife Res. **39**: 1-6.

Copley, I. (2000): Ecology goes underground. Nature **406**: 452-454.

Daniel, J.A., Baker, K.A., Bonine, K.E. (2006): Retention rates of surface and implantable methods in the medi-

terranean house gecko (*Hemidactylus turcicus*), with notes on capture methods and rates of skin shedding. Herpetol. Rev. **37**: 319-321.

Ekner, A., Sajkowska, Z., Dudek, K., Tryjanowski, P. (2011): Medical cautery units as a permanent and non-invasive method of marking lizards. Acta Herpetol. **6**: 229-236.

Elbin, S., Burguer, J. (1994): In my experience…Implantable microchips for individual identification in wild captive populations. Wildl. Soc. Bull. **22**: 677-683.

Ferner, J.W., Plummer M.V. (2016): Marking and measuring reptiles. In: Reptile ecology and conservation. A handbook of techniques, pp. 45-59. Dodd, C.K., Ed, Oxford University Press, Oxford.

Gans, C. (2005): Checklist and bibliography of the Amphisbaenia of the world. Bull. Am. Mus. Nat. Hist. **280**: 1-130.

Gasc, J.P. (1981): Axial musculature. In: Biology of the Reptilia, vol. 11. Morphology F, pp. 355-435. Gans, C., Parsons, T.S., Eds. Academic Press, London.

Germano, D.J., Williams, D.F. (1993): Field evaluation of using Passive Integrated Transponder (PIT) tags to permanently mark lizards. Herpetol. Rev. **24**: 54-56

Gibbons, J.W., Andrews, K.M. (2004): PIT Tagging: simple technology as its best. BioSci. **54**: 447-454.

Henderson, R.W., Powell, R., Martín, J., López, P. (2016): Sampling techniques for arboreal and fossorial reptiles. In: Reptile ecology and conservation. A handbook of techniques, pp. 139-153. Dodd, C.K., Ed, Oxford University Press, Oxford.

Heard, D.J. (2001): Reptile anesthesia. Veter. Clin. N. Amer.: Exot. Anim. Pract. **4**: 83-117.

Hutchens, S.J., Deperno, C.S., Matthews, C.E., Pollock, K.H., Woodward, D.K. (2008): Visible Implant Fluorescent Elastomer: a reliable marking alternative for snakes. Herpetol. Rev. **39**: 301-303.

Jemison, S.C., Bishop, L.A., May, P.G., Farrell, T.M. (1995): The impact of PIT-tags on growth and movement of the rattlesnake *Sistrurus miliarus*. J. Herpetol. **29**: 129-132.

Keck, M.B. (1994): Test for detrimental effects of PIT tags in neonatal snakes. Copeia **1994**: 226-228.

Langkilde, T., Shine, R. (2006): How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. J. Exp. Biol. **209**: 1035-1043.

Le Galliard, J., Paquet, M., Pantelic, Z., Pret, S. (2011): Effects of miniature transponders on physiological stress, locomotor activity, growth and survival in small lizards. Amphib.-Rept. **32**: 177-183.

Lobos, G., Méndez, C., Alzamora, A. (2013): Utilización de marcas electrónicas "PIT tags" en *Liolaemus* y descripción de una técnica de implante para especies de pequeña y mediana talla. Gayana **77**: 26-34.

López, P., Civantos, E., Martín, J. (2002): Body temperature regulation in the amphisbaenian *Trogonophis wiegmanni*. Can. J. Zool. **80**: 42-47.

Martín, J., Polo-Cavia, N., Gonzalo, A., López, P., Civantos, E. (2011a): Distribución, abundancia y conservación de la culebrilla mora (*Trogonophis wiegmanni*) en las Islas Chafarinas. Bol. Asoc. Herpetol. Esp. **22**: 107-112.

Martín, J., Polo-Cavia, N., Gonzalo, A., López, P., Civantos, E. (2011b): Social aggregation behaviour in the North African amphisbaenian *Trogonophis wiegmanni*. Afr. J. Herpetol. **60**: 171-176.

Martín, J., Polo-Cavia, N., Gonzalo, A., López, P., Civantos, E. (2011c): Structure of a population of the amphisbaenian *Trogonophis wiegmanni* in North Africa. Herpetologica **67**: 250-257.

Martín, J., López, P., García, L.V. (2013a): Soil characteristics determine microhabitat selection of the fossorial amphisbaenian *Trogonophis wiegmanni*. J. Zool. **290**: 265-272.

Martín, J., Ortega, J., López, P., Pérez-Cembranos, A., Pérez-Mellado, V. (2013b): Fossorial life does not constrain diet selection in the amphisbaenian *Trogonophis wiegmanni*. J. Zool. **291**: 226-233.

Martín, J., López, P., Gutiérrez, E., García, L.V. (2015): Natural and anthropogenic alterations of the soil affect body condition of the fossorial amphisbaenian *Trogonophis wiegamnni* in North Africa. J. Arid Environ. **122**: 30-36.

Martín, J., Gutiérrez, E., García, L.V. (2017): Alteration effects of ornamental whitewashing of rocks on the soil properties and body condition of fossorial amphisbaenians that live under them. Herpetol. Conserv. Biol. **12**: 367-372.

Measey, G.J. (2006): Surveying biodiversity of soil herpetofauna: towards a standard quantitative methodology. Eur. J. Soil Biol. **42**: S103-S110.

Measey, G.J., Gower, D.J., Oommen, O.V., Wilkinson. M. 2001. Permanent marking of a fossorial caecilian, *Gegeneophis ramaswamii* (Amphibia: Gymnophiona: Caeciildae). J. South Asian Nat. Hist. **5**: 141-147.

Ott, J.A., Scott, D.E. (1999): Effect of toe-clipping and PIT-tagging on growth and survival in metamorphic *Ambystoma opacum*. J. Herpetol. **33**: 344-348.

Ousterhout, B.H., Semlitsch, R.D. (2014): Measuring terrestrial movement behavior using passive integrated transponder (PIT) tags: effects of tag size on detection, movement, survival, and growth. Behav. Ecol. Sociobiol. **68**: 343-350.

Perret, N., Joly, P. (2002): Impacts of tattooing and PIT-

tagging on survival and fecundity in the alpine newt (*Triturus alpestris*). Herpetologica **58**: 131-138.
Petit, S., Waudby, H.P., Walker, A.T., Zanker, R., Rau, G. (2012): A non-mutilating technique for marking small wild mammals and reptiles. Austr. J. Zool. **60**: 64-71.
Plummer, M.V., Ferner, J.W. (2012): Marking reptiles. In: Reptile biodiversity: standard methods for inventory and monitoring, pp. 143-150. McDiarmid, R.W., Foster, M.S., Guyer, C. et al., eds., University of California Press, Berkeley, California.
Ribeiro, L.B., Sousa, B.M. (2006): Elastic hair bands: an effective marking technique for lizards in mark-recapture studies. Herpetol. Rev. **37**: 434-435.
Smalian, C. (1884): Beitrage zur Anatomie der Amphisbaeniden. Z. Wiss. Zool. **42**: 126-202.
Warwick, C., Arena, P.C., Lindley, S., Jessop, M., Steedman, C. (2013): Assessing reptile welfare using behavioural criteria. In Pract. **35**: 123-131.
Winne, C.T., Willson, J.D., Andrews, K.M., Reed, R.N. (2006): Efficacy of marking snakes with disposable medical cautery units. Herpetol. Rev. **37**: 25-54.
Wolters, V. (2001): Biodiversity of soil animals and its function. Eur. J. Soil Biol. **37**: 221-227.

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Occurrence of *Batrachochytrium dendrobatidis* in the Tensift region, with comments on its spreading in Morocco

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Abstract. The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a generalist pathogen that affects many amphibian species and is responsible of chytridiomycosis onset, considered as the main causes of species extinctions and populations declines worldwide. The chytrid fungal pathogen has been first described in North Africa in 2011. The present work reported the first survey on *Bd* prevalence and intensity in the Tensift region of Morocco. The survey has been conducted on 11 different localities by collecting skin swabs and tissue samples of 97 individuals. Using a quantitative Polymerase Chain Reaction (qPCR) protocol, low-intensity of *Bd* infection has been detected in the area of study. In fact, the chytrid fungal pathogen has been identified in 10 individuals distributed in six of the 11 sites investigated, placing the 95% confidence interval for overall prevalence at 5.5-19.6%. The survey confirmed the occurrence of *Bd* at both high and low altitude localities, on four species out of seven known to inhabit the region and added two additional species (*Pelophylax saharicus* and *Sclerophrys mauritanica*) to the list of *Bd* susceptible amphibians in Morocco. The present records extended *Bd* distribution more than 400 km in the South of Morocco, indicating that the chytrid fungal pathogen is more widespread in the country than previously thought.

Keywords. *Batrachochytrium dendrobatidis,* amphibians, Tensift, prevalence, intensity.

INTRODUCTION

Habitat degradation and overexploitation are the main large-scale factors causing loss of biodiversity worldwide (Hoffmann et al., 2010). While these two factors are to blame for the rapid declines of many vertebrate species, amphibians continue to decline also in undisturbed habitats, due essentially to chytridiomycosis, an emerging infectious skin disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger, 1998; Longcore and Pessier, 1999; Bosch and Martínez-solano, 2006; Lips, 2016). *Bd* is considered as the first wildlife fungal pathogen to have caused widespread species extinctions (Skerratt et al., 2007), and has been implicated in rapid population declines and extinction of several amphibians (Retallick et al., 2004; Schloegel et al., 2006; Skerratt et al., 2007; Kolby and Daszak, 2016; Lips, 2018). It has been detected in about 48% of tested amphibian species (Olson et al., 2013), and is recently known to be rapidly expanding its global range of distribution (Fisher et al., 2009; O'Hanlon et al., 2018). In North Africa, *Bd* has been detected for the first time in Morocco at three (out of 51) tested localities, and was found in only three species (*Discoglos-*

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sus scovazzi, *Hyla meridionalis* and *Pelobates varaldii*), with a total prevalence of 6% (El Mouden et al., 2011). The sites where *Bd* has been detected are in the northern part of the country (Tingitana peninsula and its surroundings) near South of Spain. El Mouden et al. (2011) explained this presence through a possible arrival from Spain where chytrid fungus is largely distributed, and it's responsible for severe decline of amphibian populations in mountain areas (Bosch et al., 2001; Bosch et al., 2007; Walker et al., 2010; Lips, 2016). These findings suggest further investigations in other areas were needed to collect more information on the potential extent of the spread of this fungal pathogen in Morocco, and to elucidate its potential source. Morocco is home to many endangered amphibian populations that are already threatened by habitat alteration and destruction, pollution, and climatic changes (Fahd et al., 2015; Ben Hassine and Nouira, 2012; Escoriza and Ben Hassine, 2017). The aim of this study is to evaluate the chytrid fungal presence and prevalence using qPCR test for amphibians naturally occurred in Tensift region, an area located around 400 km South from the localities where *Bd* was detected for the first time in 2011. This region is characterized by a high diversity of habitats from wetlands (High Atlas Mountains) to arid environments (Jbilets), with seven amphibians species known to inhabit the area, two of which are Moroccan endemics (Bons and Geniez, 1996; Beukema et al., 2013).

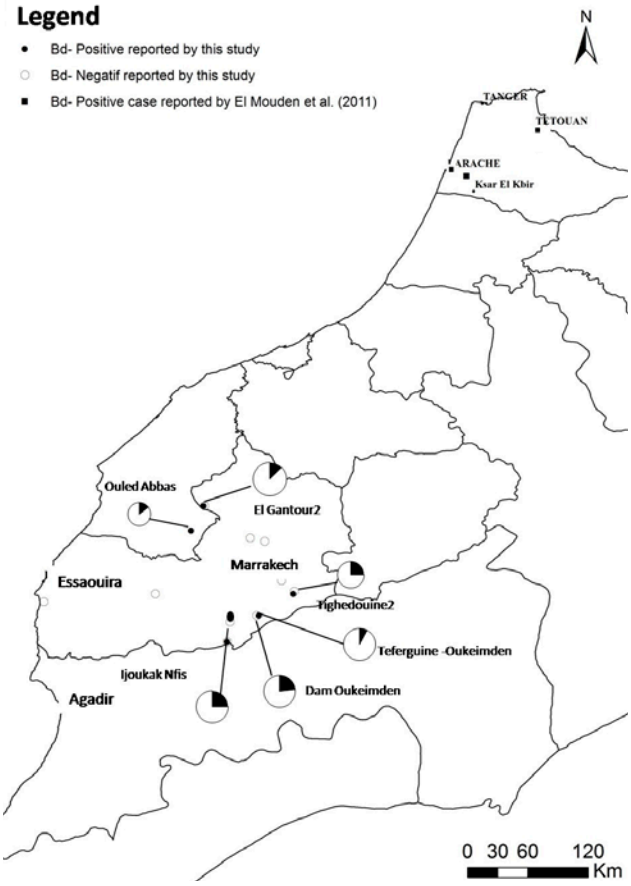

Fig. 1. Map of the Northern Morocco showing the result of *Batrachochytrium dendrobatidis* (*Bd*) sampling sites realized during the present study (circles); dark circles indicate populations with *Bd*-positive samples and open circles indicate populations with *Bd*-negative samples. Within *Bd* positive sites, pie charts indicate the proportion of infected individuals in black and uninfected individuals in white. Squares indicate infected populations as reported by El Mouden et al. (2011).

MATERIALS AND METHODS

Surveys were conducted between October 2013 and October 2017 in the Tensift region (central Morocco). The study area covers about 20,000 km^2, surrounded by the southern crest of the High Atlas Mountains (with an altitude up to 4000 m a.s.l.) and the northern Jbilet hills (separated by the Haouz plain), while the coastline of Essaouira extends in the East (Fig. 1). The climate of the region is characterized by strong annual variability, with mean temperatures ranging between a maximum of 37.7 °C and a minimum of 4.9 °C. The rainfall is generally irregular, with occasional prolonged droughts. The mean annual rainfall varies from 800 mm in the mountain to 190 mm in the plain, with significant snowfall between December and March at high elevation (up to 2000 m a.s.l.) (Alaoui Haroni et al., 2009). The semi-arid environment dominates throughout the region, while the sub-humid zones appear at high altitude (up to 1500 m a.s.l.). The Tensift region is characterized by a complex landscape and topography, including escarpments, floodplains, and a great variety of aquatic ecosystems (rivers, permanent ponds and a large network of Mediterranean temporary ponds). This diversity of water ecosystems provides a wide availability of breeding sites for seven native amphibian species: *Hyla meridionalis* Boettger, 1874, *Bufotes boulengeri* (Lataste, 1879), *Sclerophrys mauritanica* (Schlegel, 1841), *Bufo spinosus* Daudin, 1803 (Near threatened in Morocco), *Barbarophryne brongersmai* (Hoogmoed, 1972) (endemic to Morocco and Algeria), *Pelophylax saharicus* (Boulenger, 1913) (endemic to North Africa) and *Discoglossus scovazzii* Camerano, 1878 (endemic to Morocco).

Amphibians were searched opportunistically in 11 sites (Table 1; Fig. 1), during the rainy season (when detectability of amphibians is higher), during nocturnal and occasional diurnal surveys. We extensively searched in all water bodies available in the area. After capture, animals were swabbed (using Medical Wire Sterile Dryswab™ - MW100) and released immediately after sampling to the place of capture. To prevent the transmission of diseases by animals, each individual was handled with disposable gloves. In total, 86 individuals were swabbed. Swabs were then air-dried and stored at cool temperature (4 °C) until processing. Tissues samples have been collected from 11 specimens that were found dead in two sites

Table 1. Surveyed localities of the Tensift region (Morocco) with date and approximate coordinates and altitude (meters a.s.l.) of each site. Species names are abbreviated as follows: Sm, *Sclerophrys mauritanica*; Bb, *Bufotes boulengeri*; Br, *Barbarophryne brongersmai*; Ds, *Discoglossus scovazzi*; Hm, *Hyla meridionalis*; Ps, *Pelophylax saharicus*. Life history stage (LHS): Ad, adult; Juv, juvenile. P/S: number of individuals testing positive / Number of analyzed individuals. *Bd* load in genomic equivalents of zoospores. (*) Indicate individual found dead in the field and tested positive for *Bd*.

Date	Locality	Coordinates		Altitude	Species	LHS	P/S	*Bd* load
Oct 2013	Sidi Bouathman	31.9	-7.92	~ 517	Ps	Ad	0/1	
Nov 2013	**Ijoukak N'fis**	31.059	-8.164	~ 1042	**Ps**	Juv	0/1	
						Ad	1/3	26.8
Dec 2013	Sidi Bouathman	31.9	-7.92	~ 517	Ps	Ad	0/3	
Dec 2013	**Dam d'Ouled Abbas**	31.966	-8.447	~ 468	**Sm***	Ad	1/7	0.3
Dec 2013	Dam-Oukaimeden	31.208	-7.851	~ 2623	Ps	Ad	0/7	
Dec 2013	Tighedouine 1	31.4	-7.532	~ 1145	Ps	Ad	0/6	
Avr 2014	**Tighedouine 2**	31.423	-7.524	~ 1066	**Ps**	Ad	1/4	0.3
					Hm	Ad	1/7	0.4
Avr 2014	Ijoukak Tizgui	30.971	-8.125	~ 1142	Ds	Ad	0/2	
					Bb	Ad	0/1	
					Ps	Ad	0/5	
Dec 2014	El Gantour 1	32.189	-8.335	~ 402	Ps	Ad	0/5	
Dec 2014	**El Gantour 2**	32.187	-8.329	~ 398	Ds	Ad	0/2	
					Ps	Juv	0/1	
					Ps	Ad	1/8	1.3
Jan 2015	Jaidate	31.87	-7.79	~ 623	Bb	Ad	0/9	
					Br	Ad	0/3	
					Ps	Ad	0/2	
Avr 2015	**Tighedouine 2**	31.423	-7.524	~ 1066	**Ps**	Ad	1/1	1.7
Jun 2016	**Tifergine-Oukaimeden**	31.207	-7.84	~ 2565	**Ds**	Ad	1/13	1.3
Oct 2017	**Dam-Oukaimeden**	31.208	-7.851	~ 2623	**Ps***	Ad	3/6	0.6, 3.0, 0.3

(Dam-Oukaimden and Dam d'Ouled Abbas). These 11 tissue samples were preserved in ethanol (Brem et al., 2007; Hyatt et al., 2007). In total, 97 amphibian specimens were sampled belonging to four families and six species. Of these, 56 individuals were sampled in the high Atlas Mountains, 16 in the arid area of Jbilets, 23 in the El Gantour region and two in the Haouz plain (Table 1; Fig. 1).

DNA was extracted from both swabs and tissue samples using PrepMan Ultra reagent and extractions were diluted 1:10 before real-time PCR amplification, performed in duplicate with a CFX96 thermocycler (Bio-Rad), following Boyle et al. (2004). Each 96-well assay plate included samples, a negative control and standards of 100, 10, 1, and 0.1 *Bd* zoospore genome equivalents in duplicate. Samples were considered positives when both replicates were ≥ 0.1 and the amplification curves had the typical sigmoidal shape. When only one replicate from any sample amplified, we ran this sample a third time. If the third amplification did not result in an amplification profile, we considered sample as negative for infection. Samples that showed signs of inhibition (non-sigmoidal amplification) were further diluted to 1:100 and re-analyzed. If signs of inhibition remained, the samples were excluded.

RESULTS

The numbers of screened and testing positive individuals from each population and sampling event, along with geographic information on the sampling site and year of capture are reported in Table 1. The results show that 10 out of 97 screened individuals were *Bd* infected (10.3%), corresponding to a 95% confidence interval of overall *Bd* prevalence of 5.5-19.6%. Across the four species that tested positive, the infection prevalence was 11.9% (95% CI: 5.5-19.6%). This study confirmed *Bd* occurrence in both the northern and southern parts of the Tensift watershed at elevations between 468 and 2625 m a.s.l (Fig. 1).

In the study area, *Bd* was detected in six out of 11 investigated sites (Table 1), with prevalence values ranging between 0 and 25%, and lower GE (genomic equivalent) values that varied between 0.3 and 26.8. These results indicate a significant presence of *Bd* in the Tensift region, with low infection prevalence and intensities across all sites that tested positive for *Bd*. However,

Table 2. *Bd* prevalence with 95% Clopper-Pearson binomial confidence intervals for individuals grouped by species and elevation.

Group	sample size	*Bd* prevalence (%)	95% CI
Family/Species			
Ranidae			
Pelophylax saharicus	53	13.2	5.6 - 25.8%
Alytidae			
Discoglossus scovazzi	17	5.9	0.1 - 28.7%
Bufonidae			
Bufotes boulengeri	10	0	0 - 30.8 %
Sclerophrys mauritanica	7	14.3	0.4 - 57.9%
Barbarophryne brongersmai	3	0	0 - 70.8%
Hylidae			
Hyla meridionalis	7	14.3	0.4 - 57.9%
Elevation (for all species)			
Less than 700 m	41	4.9	0.6 - 16.2%
More than 700 m	56	14.3	6.1 - 25.4%

in October 2017, 50% prevalence was recorded in Dam-Oukaimden, with three testing positive samples out of the six screened individuals, which represents the maximum prevalence obtained in this study.

It seems that *Bd* prevalence can vary among species and elevations (Table 2). Consequently, 95% confidence intervals for *Bd* prevalence for individuals grouped according to these two parameters were determined. All confidence intervals were overlapping. Thus, no significant taxonomic and altitudinal difference in *Bd* prevalence has been detected given our sample sizes. However, the obtained results tend towards a more significant prevalence at higher elevations (14.3% vs 4.9%). Likewise, species infection rate varied between 0 and 14.3%. The higher percentage was observed in three species. *Bd* was found in seven out of 53 *P. saharicus* across five sampling sites. In the Dam-Oukaimden site, where a large numbers of dead *P. saharicus* were found in October 2017, we detected *Bd* on three out of six tested individuals (95% CI: 11.8-88.2%), which represents the highest prevalence recorded by species and by site during this study. For both *H. meridionalis* (Tighedouine region; April 2014) and *S. mauritanica* (Culinary dam of Ouled Abbas; December 2013), *Bd* has been detected in one out of seven individuals, corresponding to a confidence interval for the two species of 0.40-57.9%. During our investigation at Ouled Abbas dam, numerous dead specimens of *S. mauritanica* were observed in the water. Seven tissue samples were collected and screened. The results showed that one sample tested positive for *Bd*.

DISCUSSION

In our study area, four out of six amphibian species have been tested as *Bd* positive, although in comparison with other regions in Marocco, they have lower infection rate. Lower GE values are probably the result of unfavorable conditions, such as changes in the abiotic environment (Ron, 2005; Thorpe et al., 2018). It has been reported that *Bd* is temperature sensitive, with optimal growth ranging between 17 and 25 °C (Piotrowski et al., 2004; Pounds et al., 2006), with higher temperature (more than 30 °C) reported as unfavorable for its development (Watve, 2013). Previous studies have shown that lower temperature regime resulted in extended zoospore longevity and in such conditions zoospores numbers in water bodies could be expected to be greater than in warmer water (Voyles et al., 2012; Thorpe et al., 2018). The higher temperatures (more than 30 °C) recorded in the study area expected to have a negative impact on the development of this fungal pathogen. Additionally, optimum rainfall for *Bd* development has been reported to range between 1500 and 2500 mm/year (Thorpe et al., 2018), which are much higher values of the one observed in the Tensift region. Only, Oukaimden approach these rainfall values, but in general remains below to optimum parameters for the development of *Bd*.

In Morocco, *Bd* surveys were previously conducted only in the northern Tingitana area. El Mouden et al. (2011) reported *Bd* occurrence in three sampling sites. Through this study, *Bd* occurrence has been reported in six additional Moroccan localities. Considering all these data, 14.5% of the screened Moroccan localities tested positive to *Bd*. The finding that *Bd* was recorded in two separated areas suggested a possible wider distribution across the country. Similarly, *Bd* was detected in different sites ranging from 400 to over 2600 m a.s.l, indicating a probable wide distribution also along the altitudinal gradient. Sample size is still limited and should be extended to have a more robust overview of the pattern of *Bd* presence across Morocco.

The report on the occurrence of *Bd* in the Tingitana peninsula (North of Morocco) was potentially explained by the extensive commercial trade of products and animals across the straits of Gibraltar with a possible introduction of *Bd* from Spain (El Mouden et al., 2011). This hypothesis is in agreement with the explanations of O'Hanlon et al. (2018) who found *Bd* spreading out of Asia and dated its emergence on the early 20th century, coinciding with the international expansion of commercial trade in amphibians for exotic pet, medical, and food purposes. The presence of *Bd* in the Tensift region can be explained by a dissemination process from the North

of the country to the South, but also by a possible independent episode of introduction through other commercial routes, such as airports and harbors. The spreading of *Bd* through the country can be carried out by potential natural vectors, including waterfowl on their feathers or feet (Johnson and Speare, 2005; Garmyn et al., 2012; Burrowes and De La Riva, 2017), water (Johnson and Speare, 2003), and non-susceptible to chytridiomycosis amphibians acting as carrier (Kolby et al., 2015).

In Morocco, as in most regions around the world, the detected *Bd* belongs to the Global Panzootic Lineage (J. Bosch, unpublished data). This is a highly virulent and highly transmissible chytrid fungus, which is currently infecting more that 700 amphibian species worldwide (Olson and Ronnenberg, 2014; Lips, 2016). We detected a mass mortality of amphibians in two localities (*S. mauritanica* in Dam d'Ouled Abbas and *P. saharicus* in Dam-Oukaimden) and some of the sampled individuals tested positive for *Bd* infection. However, in the absence of a detailed study, we have no evidence of *Bd*-associated amphibian mortality especially because mortality of individuals generally occurred with higher infection rates than those found in the present study. Other biotic and abiotic factors can be the cause of the witnessed mass mortality (e.g., Croteau et al., 2008; Hayes et al., 2010; Relyea et al., 2012; Whittaker et al., 2013; Budzik et al., 2014; De Wijer et al., 2018). However, it is worth noting that the two new species found infected by *Bd* (*P. saharicus* and *S. mauritanica*) have a wide distribution and abundance in the southern Mediterranean region (Bons and Geniez, 1996; Schleich et al., 1996; Mateo et al., 2013), which can make *Bd* dissemination faster.

A systematic survey to determine the impact of *Bd* occurrence in Morocco is crucial to better characterize its impact on the amphibian's population dynamic. In the mean time, other *Bd* positive sites located in Tensfit region have been recorded and helped extending the known *Bd* occurrence area in Morocco, both at the high and the low altitudes (486-2625 m a.s.l.). In addition, having detected *Bd* at multiple sites and in two new amphibian species contributes to the growing knowledge on the global pattern of *Bd* distribution in Morocco and North Africa.

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REFERENCES

Alaoui Haroni, S., Alifriqui, M., Simonneaux, V. (2009): Recent dynamics of the wet pastures at Oukaimeden plateau (High Atlas). Biodivers. Conserv. **18**: 167-189.

Ben Hassine, J., Nouira, S. (2012): Répartition géographique et affinités écologiques des Amphibiens de Tunisie. Rev. Écol. (Terre Vie) **67**: 437-457.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H. (1998): Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America, Proc. Natl. Acad. Sci. USA **95**: 9031-9036.

Beukema, W., De Pous, P., Donaire-Barroso, D., Bogaerts, S., Garcia-Porta, J., Escoriza, D., Arribas, J.O., El Mouden, E.H., Carranza, S. (2013): Review of the systematics, distribution, biogeography and natural history of Moroccan amphibians. Zootaxa **3661**: 1-60.

Bons, J., Geniez, P. (1996): Amphibiens et reptiles du Maroc (Sahara Occidental compris): atlas biogéographique. Asociación Herpetológica Española, Barcelona.

Bosch, J., Carrascal, J.M., Durán, L., Walker, S., Fisher, M.C. (2007): Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? Proc. R. Soc. B **274**: 253-260.

Bosch, J., Martínez-Solano, I. (2006): Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park , Spain. Oryx **40**: 84-89.

Bosch, J., Martínez-Solano, I., García-París, M. (2001): Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biol. Conserv. **97**: 331-337.

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A. D. (2004): Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis. Aquat. Org. **60**: 141-148.

Brem, F., Mendelson, J.R. III, Lips, K.R. (2007): Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians, using alcohol preserved

swabs. Version 1.0. Electronic document accessible at http://www.amphibians.org Conservation International, Arlington.

Budzik, K.A., Budzik, K.M., Kukiełka, P., Łaptaś, A., Bres, E.E. (2014): Water quality of urban water bodies – a threat for amphibians? Ecol. Q. **19**: 57-65.

Burrowes, P.A., De La Riva, I. (2017): Detection of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in museum specimens of Andean aquatic birds: Implications for pathogen dispersal. J. Wildl. Dis. **53**: 349-55.

Croteau, M.C., Hogan, N., Gibson, J.C., Lean, D., Trudeau, V.L. (2008): Toxicological threats to amphibians and reptiles in urban environments. In: Urban Herpetology, pp. 197-210. Mitchell, J.C., Brown, R.E.J., Bartholomew, B., Eds, Society for the Study of Amphibians and Reptiles, Salt Lake City, Utah.

De Wijer, P., Watt, P.J., Oldham, R.S. (2018): Amphibian decline and aquatic pollution: effects of nitrogenous fertiliser on survival and development of larvae of the frog *Rana temporaria*. Appl. Herpetol. **1**: 3-12.

El Mouden, E.H., Slimani, T., Donaire-Barroso, D., Fernández-Beaskoetxea, S., Fisher, M. C., Bosch, J. (2011): First record of the chytrid fungus *Batrachochytrium dendrobatidis* in North Africa. Herpetological Review **42**: 71-75.

Escoriza, D., Ben Hassine, J. (2017): Diversity of guilds of amphibian larvae in north-western Africa. PLoS ONE **12**: 1-18.

Fahd, S., Mediani, M., Ohler, A.O., Denys, C.D., Santos, X. (2015): Diversité et conservation de la faune batrachologique du bassin versant d'oued Laou (Rif, nord-ouest du Maroc), Trav. Inst. Sci. Sér. Gén. **8**: 69-84.

Fisher, M.C., Garner, T.W.J., Walker, S.F. (2009): Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu. Rev. Microbiol. **63**: 291-310.

Garmyn, A., Van Rooij, P., Pasmans, F., Hellebuyck, T., Van Den Broeck, W., Haesebrouck, F., Martel, A. (2012): Waterfowl: Potential environmental reservoirs of the chytrid fungus *Batrachochytrium dendrobatidis*. PLoS ONE **7**: e35038.

Hayes, T.B., Falso, P., Gallipeau, S., Stice, M. (2010): The cause of global amphibian declines: a developmental endocrinologist 's perspective. J. Exp. Biol. **213**: 921-933.

Hoffmann, M., Hilton-Taylor, C., Angulo, A., Bohm, M., Brooks, T.M., et al. (2010): The impact of conservation on the status of the world 's vertebrates. Science **330**: 1503-1509.

Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F., Colling, A. (2007): Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis. Aquat. Org. **73**: 175-192.

Kolby, J.E., Daszak, P. (2016): The emerging amphibian fungal disease, chytridiomycosis: a key example of the global phenomenon of wildlife emerging infectious diseases, Microbiol. Spectr. **4**: 1-17.

Kolby, J.E, Ramirez, S.D., Berger, L., Richards-Hrdlicka, K.L., Jocque, M., Skerratt, L.F. (2015): Terrestrial dispersal and potential environmental transmission of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). PLoS ONE **10**: e0125386.

Johnson, M.L., Speare, R. (2003): Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. Emerg. Infect. Dis. **9**: 922-925.

Johnson, M.L., Speare, R. (2005): Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Dis. Aquat. Organ. **65**: 181-186.

Lips, K.R. (2016): Overview of chytrid emergence and impacts on amphibians. Phil. Trans. R. Soc. B **371**: 20150465.

Lips, K.R. (2018): Witnessing extinction in real time. PLoS Biol **16**: e2003080.

Longcore, J., Pessier, A. (1999): *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia **91**: 219-227.

Mateo, J., Geniez, P., Pether, J. (2013): Diversity and conservation of Algerian amphibian assemblages. Chapter 26. Basic Appl. Herpetol. **27**: 51-83

O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., et al. (2018): Recent asian origin of chytrid fungi causing global amphibian declines. Science **360**: 621-627.

Olson, D.H., Aanensen, D.M., Ronnenberg, K.L., Powell, C.I., Walker, S.F., et al. (2013): Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PloS ONE **8**: 1-13.

Olson, D.H., Ronnenberg, K.L. (2014): Mapping Project: 2014 Update. FrogLog **22**: 17-21.

Piotrowski, J.S., Annis, S.L., Longcore, J.E. (2004): Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia **96**: 9-15.

Pounds, J.A., Bustamante, M.R., Coloma, L.A., Consuegra, J.A., Fogden, M.P.L, Foster, P.N., La Marca, E., Masters, K.L., Merino-Viteri, A., Puschendorf, R., Ron, S.R., Sanchez-Azofeifa, G.A., Still, C.J., Young, B.E. (2006): Widespread amphibian extinctions from epidemic disease driven by global warming. Nature **439**: 161-167.

Relyea, R.A., Tejedo, M., Torralva, M. (2012): Understanding of the impact of chemicals on amphibian : a meta-analytic review. Ecol. Evol. **2**: 1382-1397.

Retallick, R.W.R., Mccallum, H., Speare, R. (2004): Endemic infection of the amphibian chytrid fungus in a frog community post-decline, PLoS Biol. **2**: e351.

Ron, S.R. (2005): Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the New World. Biotropica **37**: 209-221.

Schloegel, L.M., Hero, J., Berger, L., Speare, R., Mcdonald, K., Daszak, P. (2006): The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): the first documented case of extinction by infection in a free-ranging wildlife species? EcoHealth **3**: 35-40.

Skerratt, L.F., Berger, L., Speare, R., Cashins, S., Mcdonald, K.R., Phillott, A.D., Hines, H.B., Kenyon, N. (2007): Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth **4**: 125-134.

Thorpe, C.J., Lewis, T.R., Fisher, M.C., Wierzbicki, C.J., Kulkarni, S., Pryce, D., Davies, L., Watve, A., Knight, M.E. (2018): Climate structuring of *Batrachochytrium dendrobatidis* infection in the threatened amphibians of the northern Western Ghats, India. R. Soc. Open Sci. **5**: 180211.

Voyles, J., Johnson, L.R., Briggs, C.J., Cashins, S.D., Alford, R.A., Berger, L., Skerratt, L.F., Speare, R., Rosenblum, E.B. (2012): Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. Ecol. Evol. **2**: 2241-2249.

Walker, S.F., Bosch, J., Gomez, V., Trenton, W.J., Andrew, A., Schmeller, D.S., Ninyerola, M., Henk, D.A., Ginestet, C., Arthur P.A., Fisher, M.C. (2010): Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecol. Lett. **13**: 372-382.

Watve, A. (2013): Status review of rocky plateaus in the northern Western Ghats and Konkan region of Maharashtra, India with recommendations for conservation and management. J. Threat. Taxa **5**: 3935-3962.

Whittaker, K., Koo M.S., Wake D.B., Vredenburg V.T. (2013): Global Declines of Amphibians. In: Encyclopedia of Biodiversity, second edition, pp. 691-699. Levin S.A., Ed, Academic Press, Cambridge.

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Ontogenetic and interspecific variation in skull morphology of two closely related species of toad, *Bufo bufo* and *B. spinosus* (Anura: Bufonidae)

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Abstract. Using micro-CT and 3D landmark-based geometric morphometrics, I investigated postmetamorphic shape variation in the skull of *Bufo bufo* and *Bufo spinosus*, two widespread European toad species with small phenotypic differences. Two ontogenetic series were compared, for a total of 58 individuals. They exhibited similar allometric growth patterns, characterised by cranial widening with relative shortening and dorsoventral compression. However, some interspecific shape divergence was observed, particularly among adults: a relatively shorter skull and a more dorsally extended snout distinguished *B. spinosus* from *B. bufo*. This disparity, which gives further support to species separation, can probably be ascribed to changes in the allometric trajectories, and seen in light of the evolutionary history of the two lineages.

Keywords. Allometry, *Bufo bufo*, *Bufo spinosus*, divergence, geometric morphometrics, landmark, ontogeny, skull shape.

INTRODUCTION

Divergent traits can be expected to be found in closely related species with a broad geographical distribution, relatively to ecological and climatic variation, but developmental constraints may also play an important role in restricting or channelling phenotypic evolution (Cvijanović et al., 2014; Ivanović and Arntzen, 2018). The Common toad, *Bufo bufo* (Linnaeus, 1758), and the Spined toad, *Bufo spinosus* Daudin, 1803, are members of the Common toad species group of the western Palaearctic (Arntzen et al., 2013b). *B. bufo* has a wide Eurasian distribution that comprises northern and eastern France, central and southern Europe (including Sardinia; Cossu et al., 2018), and stretches northwards into Scandinavia and eastwards deep into Russia; *B. spinosus* is found in the Iberian Peninsula, western and southern France, and North Africa, from Morocco to Tunisia (Arntzen et al., 2013a). Their lineages have diverged around 9 Ma (million years ago; Recuero et al., 2012), but in contrast to a deep genetic differentiation, *B. bufo* and *B. spinosus* appear phenotypically similar (Arntzen et al., 2013a). Whereas a few diagnostic characters were described for the external morphology, virtually nothing is known about interspecific osteological differences, neither in the postcranial nor in the cranial skeleton. Such a complex structure as the skull is of particular interest in a wide range of studies, due to its fundamental biological functions and the fact that it often undergoes adaptive variation (Ivanović et al., 2012). Substantial changes are

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known to occur in the anuran skull after metamorphosis (Ponssa and Candioti, 2012).

The purpose of this investigation was to highlight potential interspecific differences in the skull morphology of *B. bufo* and *B. spinosus*, in the context of postmetamorphic development, using a geometric morphometrics approach. The analysis of ontogenetic shape variation in these two species is interesting not only for a taxonomic evaluation based on morphology; it can provide insights into their morphological evolution, to be interpreted in the context of their evolutionary history and past distribution. Moreover, this analysis could contribute to a better understanding of the interplay between ontogeny and morphological differentiation among anuran species.

MATERIAL AND METHODS

A total of 58 alcohol-stored specimens, including freshly metamorphosed, juvenile, and adult (male and female) toads, were analysed (Table S1). These toads had been collected from various populations in different localities of the Iberian Peninsula (*B. spinosus*), France (*B. spinosus*, *B. bufo*), and the Netherlands (*B. bufo*). They were subdivided into two ontogenetic series, made up of 28 *Bufo bufo* and 30 *Bufo spinosus* individuals, respectively. Body size in the whole sample ranged from 16.0 mm to 78.0 mm SUL (snout-urostyle length).

Mature *B. spinosus* individuals were larger on average (mean SUL 71.5 mm) than *B. bufo* ones (mean SUL 60.4 mm), reflecting size disparity between western European Common toads and Spined toads (Cvetković et al., 2009).

Three-dimensional imaging

Skulls were CT-scanned with two x-ray machines: SkyScan 1172 (Bruker, Kontich, Belgium) and ZEISS Xradia 520 Versa (Carl Zeiss XRM, Pleasanton, CA, USA). The former was used for the smaller toads, with 0.5 mm Aluminium filter, 2K resolution (2000 × 1336), pixel size of 13.17 μm, voltage of 29-54 kV, exposure time of 420-750 ms, 0.4 rotation step, averaging of four frames. Voltage and exposure time were modified when scanning specimens of different sizes (which showed differences in skull density), in order to keep image quality consistent. Xradia scanner was employed for adult individuals, mainly due to its larger sample holder; resolution was set at 1K (1000 × 1024), with pixel size of 34.18 μm, and voltage of 80 kV.

Scanning was followed by two-dimensional reconstruction of raw image data into stacks, which were processed with Avizo 9 software (FEI SAS, France): the segmentation editor was used to segregate homogeneous volumes (corresponding to skull bones), with manual adjustment of masking. Notable variation in the extent of cranial ossification, not merely restricted to small juveniles, was observed at this point. A 3D surface model of the skull was then generated, applying a variable degree of unconstrained smoothing according to ossification extent.

Landmarks

Thirty-one homologous landmark points were collected on each 3D surface model to describe overall skull shape (Fig. 1), using Landmark editor 3.6 (Institute for Data Analysis and Visualization, University of California, Davis, 2007). Fifteen points were bilateral and symmetric, while one was median. Anatomical description of points is provided in Table S2. Incomplete skull ossification of small juveniles made it challenging, at times, to perform an accurate placement of homologous landmarks (Zelditch et al., 2004).

Geometric morphometrics

Morphometric and statistical analyses based on the landmark coordinates, and qualitative observation of the associated shape changes, were carried out with MorphoJ 1.06 software (Klingenberg, 2011). A generalized Procrustes analysis (GPA), consisting of a full Procrustes superimposition for object symmetry, was applied: the symmetric components of shape variation, as a measure of skull shape, and centroid size, as a measure of skull size, were computed for each individual (Klingenberg, 2016). The covariance matrix of shape variables was then generated and used to perform a principal component analysis (PCA), in order to explore overall patterns of shape variation.

Shape changes in relation to growth were evaluated with a multivariate regression of shape (symmetric components, i.e. dependent variables) on size (log-transformed centroid size, i.e. independent variable), one for each ontogenetic series (Monteiro, 1999), in association with a permutation test against independence between dependent and independent variables (made up of 10^4 randomization rounds). The angle between the two regression vectors was calculated for comparison, including a test against randomness of vector directions in shape tangent space.

Interspecific morphological distinction was assessed by performing a discriminant function analysis (DFA) with leave-one-out cross-validation (Lachenbruch, 1967; Webster and Sheets, 2010) on shape data of the 25 largest individuals, 12 *B. bufo* and 13 *B. spinosus* toads comprising adults and subadults. Such a subsample was selected in order to maximize interspecific differences, since the PCA had previously shown shape divergence in late stages of growth (Fig. 2). Results of this analysis were compared with those of a DFA on the remaining individuals of the sample, namely juveniles. Both analyses included a parametric T-square test against the null hypothesis of equal group means, and a permutation test for the T-square statistic with 10,000 randomization rounds.

RESULTS

The first two principal components (PC1, PC2) of the PCA accounted for 71.9% of total shape variation. PC1 alone grouped 65.4% of variation and was positively correlated with size: therefore, the scatter plot of PC2

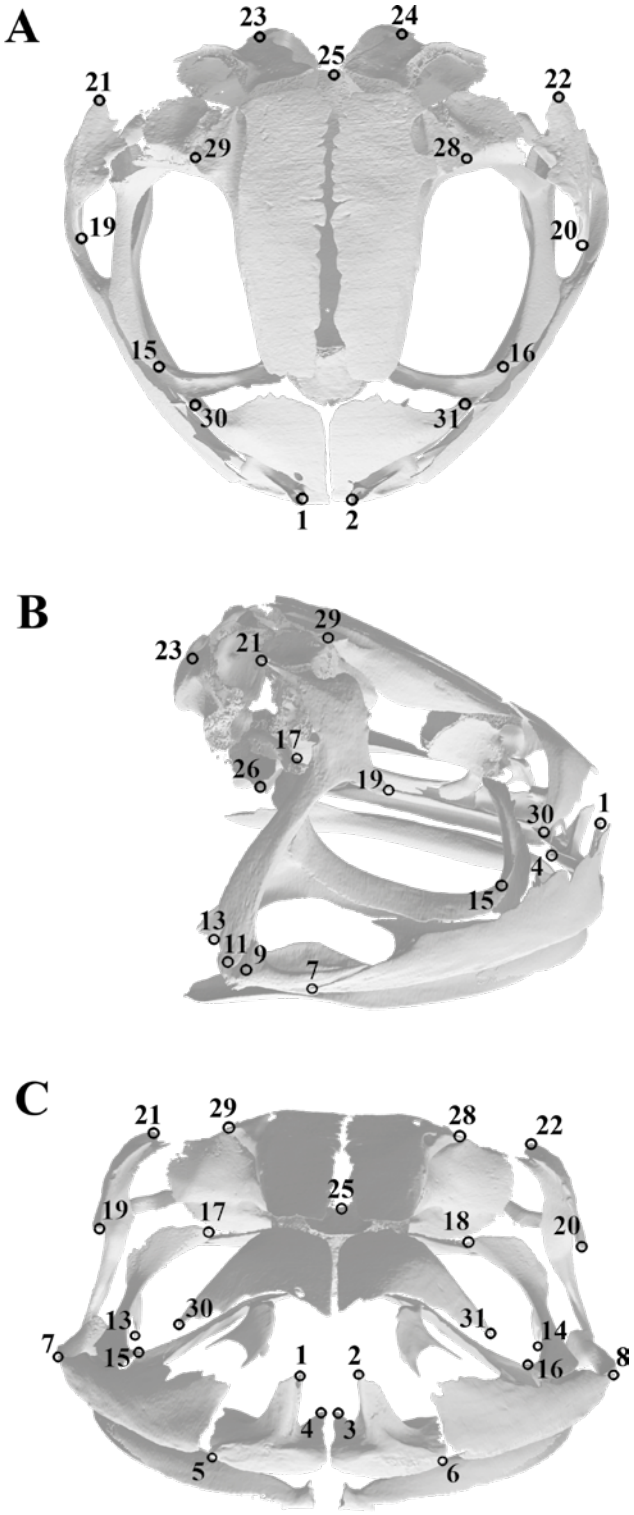

Fig. 1. Landmark positions on the digitized skulls, in dorsal (A), right lateral (B), and frontal (C) views. For the anatomical description of landmarks, see Table S2.

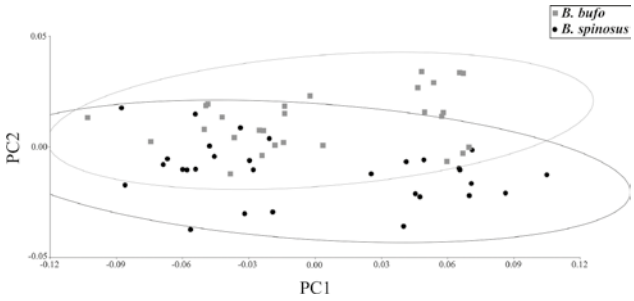

Fig. 2. Ontogenetic shape variation of *B. bufo* and *B. spinosus*, described by the scatter plot of the first two principal components (with 95% confidence ellipses). PC1 summarises allometric variation, while PC2 displays species divergence in late development.

vs. PC1 can be looked at as a morphospace that contains the ontogenetic shape trajectories of *B. bufo* and *B. spinosus* (Fig. 2). Cranial shape variation along PC1 was characterised, in the positive direction, by a broadening (at the level of the jaw joint), an overall shortening (at the level of both the exoccipital and the premaxilla), and dorsoventral compression. Some interspecific variation was comprised in PC2, especially for the largest toads; positive direction shape changes along this axis were an increase in skull length, dorsoventral compression, and a slight widening.

Both regression analyses found a significant association between shape and size ($P < 0.0001$ for both), indicating allometric growth: 63.8% of shape variation in *B. bufo* and 68.5% of shape variation in *B. spinosus* were predicted by regression on size (Fig. 3). The two vector directions formed an angle of 17.2° (whereas 0° denote complete correspondence, 90° maximum divergence) and were not random in the shape tangent space ($P < 0.00001$). The major shape changes associated with regression were corresponding between the two species and matched those related to the first principal component of the PCA, thus confirming the correlation of PC1 with skull size.

Discriminant function analysis applied to the largest toads showed a clear distinction between the two species (Fig. 4): after cross-validation, 10/12 *B. bufo* individuals were reassigned to their true group and 2/12 were allocated to the Spined toad group, while 13/13 *B. spinosus* individuals were reassigned to their own group (Cohen's $K = 0.84$). However, the T-square test for the difference between group means was not statistically significant ($T^2 = 281$, $P = 0.82$). Permutation produced a significant result instead ($P < 0.0001$). The major interspecific shape differences pointed out by the analysis concerned cranial length and height: *B. spinosus* exhibited a longer upper jaw (with a more posterior jaw joint), yet a shorter

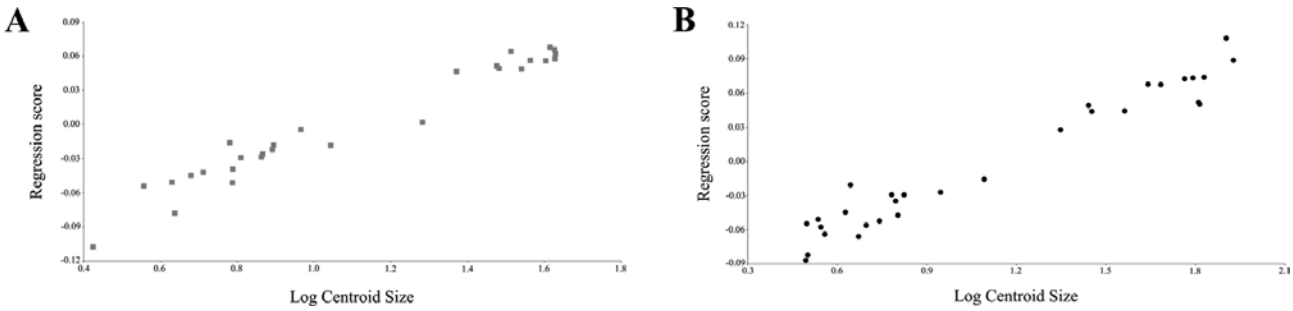

Fig. 3. Shape changes of *B. bufo* (A) and *B. spinosus* (B) in relation to size, described by the scatter plot of regression scores against log-transformed centroid size.

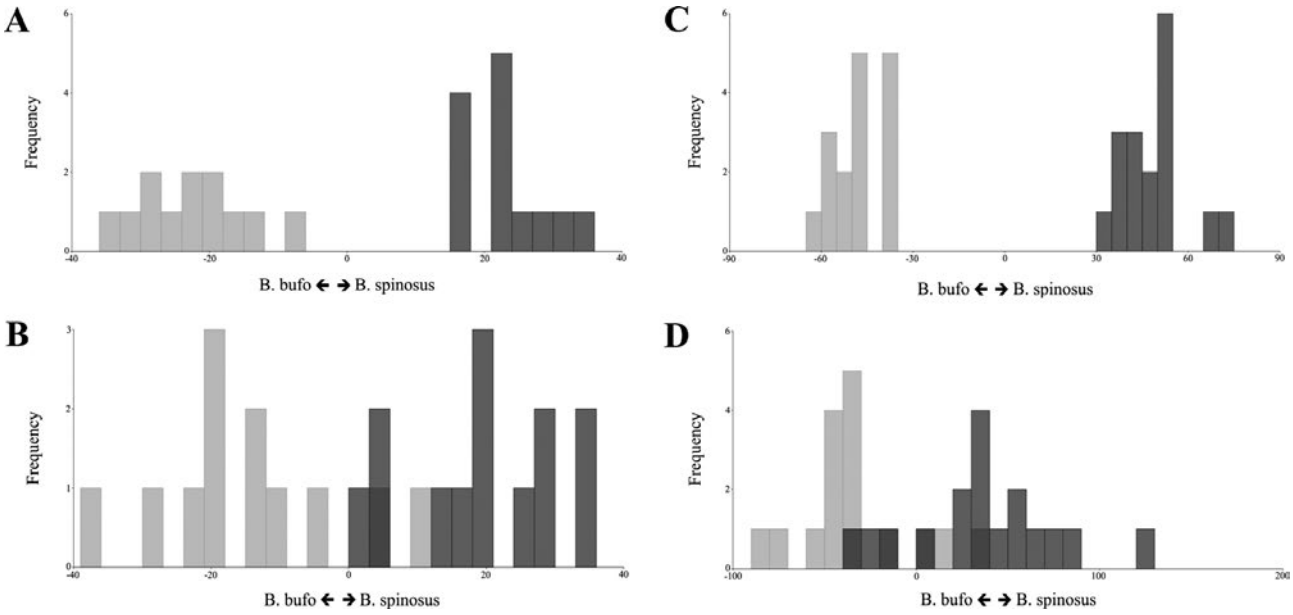

Fig. 4. Interspecific distinction, described by DFA histograms in which light grey bars represent *B. bufo* and dark grey bars represent *B. spinosus*. Species assignment and discriminant scores are shown before (A, C) and after (B, D) cross-validation, for the largest individuals (A, B) and juveniles (C, D).

skull (due to a shorter occipital region), with a dorsally expanded snout; skull width did not show noteworthy variation. DFA on juveniles yielded similar results, with a weaker interspecific distinction (Fig. 4): 13/16 *B. bufo* and 14/17 *B. spinosus* toads were reallocated to their group ($K = 0.63$); T-square test was not significant ($T^2 = 790.5$, $P = 0.72$), while permutation test was significant ($P < 0.0001$).

DISCUSSION

Most of the shape variation in the whole dataset was explained by differences in size, as shown by the concordant results of principal component and regression analyses. Therefore, I suggest that ontogenetic allometry (i.e., the association between morphological changes and ontogenetic growth; Klingenberg, 2016) characterises skull development of both *Bufo bufo* and *Bufo spinosus*, mainly in the form of a considerable widening (typical of skull growth in frogs; Ponssa and Candioti, 2012), and a relative shortening and dorsoventral flattening. Cranial allometry has already been recognized in other anuran species, both in larval (e.g., in *Rana sylvatica*; Larson, 2002) and post-metamorphic development (e.g. in *Rhinella marina* and the *Leptodactylus fuscus* group; Birch, 1999; Ponssa and Candioti, 2012). Allometric constraints are likely to limit phenotypic evolution of the skull, even in presence of selective pressure (Simon et al., 2016). This kind of scenario can be logically applied to the current study, which found shape disparities between

B. bufo and *B. spinosus* that are moderate if compared to the common allometric variation.

Nevertheless, there seems to be a shift in the ontogenetic trajectories of the two species, highlighted by both the principal component analysis (Fig. 2) and the angle between regression vectors (17.2°), and reflected in the morphological distinction provided by the discriminant function analyses (Fig. 4). Along with both latter analyses, T-square test against equal species mean shapes yielded significant results only after permutation, which probably compensated for the relatively small sample size. Interspecific divergence, however, resulted higher in the group of largest individuals: they showed greater agreement to true species membership, as described by a K coefficient of 0.84 against the 0.63 of juveniles. Consequently, it might be easier to discriminate between *B. bufo* and *B. spinosus* at mature stages, rather than among young individuals. This would be in contrast with the findings from other studies on amphibians, which pointed out a decrease in interspecific disparity over ontogeny (Adams and Nistri, 2010; Ponssa and Candioti, 2012).

DFA and, to some extent, PCA, indicate that *B. bufo* toads have on average a longer skull and a more dorsally compressed snout than *B. spinosus* toads. This morphological divergence could theoretically be placed within either the non-allometric or the allometric regime. Variation in non-allometric shape would imply some sort of relaxation of ontogenetic constraints, which seems very unlikely, as the present evidence suggests that these constraints are strong in both species. Alternatively, interpreting interspecific variation as comprised in the allometric framework should better reflect the results of the analyses. Allometric variation could have arisen in two ways. First, from changes in developmental timing of the ancestral shape trajectory, with a conserved shape-size relationship (ontogenetic scaling hypothesis; Strelin et al., 2016); however, this also seems unlikely, because the divergent skull shapes do not correspond to different stages of the same trajectory. Secondly – and more plausibly – divergent evolution of the ontogenetic programme in the two lineages may have occurred, with a conserved direction of early post-metamorphic shape trajectories. The latter hypothesis would imply that interspecific differences have arisen during the long-lasting separation of the lineages (about 9 Ma), and it could even be surprising that they are not more pronounced; as a possible explanation for this moderate divergence, a recent morphological evolution, following rapid postglacial European expansion of *B. bufo* from its Balkan refugium, cannot be excluded (Recuero et al., 2012; Arntzen et al., 2016).

Climate is thought to have an indirect influence on skull morphology, because it determines food type and availability (Simon et al., 2016), and changes in these ecological factors may lead to skull shape divergence – even in closely related species. Alternative climate-driven factors could also induce skull differentiation, such as reproduction sites (water pools) occurrence, and the ability of toads to detect them, which involves the olfactory capsules in the snout region (Trueb, 1993; Simon et al., 2016). Whether such factors are related to the interspecific differences found here – some of which concern the snout, more expanded in *B. spinosus* – it is not possible to say. Arntzen et al. (2013a) hypothesized that in European toads a larger body size, a more pronounced presence of keratinous spines on the cheek warts and a wider head shape might favour defence against predators, namely grass snakes. Although *B. spinosus* apparently meets these requirements more than *B. bufo*, relative cranial width did not show significant interspecific disparity in the current study. Thus, no link was found between widely divergent parotoid glands, a distinctive trait of *B. spinosus*, and a wider skull. Nevertheless, Spined toads could compensate by attaining a wider skull through their bigger size.

For further assessments of drivers and extent of morphological divergence between *B. bufo* and *B. spinosus*, it would be convenient to use a larger sample, especially for adult individuals, in order to properly account for the role of sexual dimorphism and benefit from the highest disparity. Different populations should be considered, since *B. bufo* exhibits remarkable variation across its wide range; for instance, Mediterranean Common toads appear to resemble Spined toads in body size – large – and skin texture – thick and warty (De Lange, 1973; Cvetković et al., 2009; Arntzen et al., 2013a).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 24709.

REFERENCES

Adams, D.C., Nistri, A. (2010): Ontogenetic convergence and evolution of foot morphology in European cave salamanders (Family: Plethodontidae). BMC Evol. Biol. **10**: 216.

Arntzen, J.W., McAtear, J., Recuero, E., Ziermann, J.M., Ohler, A., van Alphen, J., Martínez-Solano, I. (2013a): Morphological and genetic differentiation of *Bufo* toads: two cryptic species in Western Europe (Anura, Bufonidae). Contrib. Zool. **82**: 147-169.

Arntzen, J.W., Recuero, E., Canestrelli, D., Martínez-Solano, I. (2013b): How complex is the *Bufo bufo* species group? Mol. Phylogenet. Evol. **69**: 1203-1208.

Arntzen, J.W., Trujillo, T., Butôt, R., Vrieling, K., Schaap, O., Gutiérrez-Rodríguez, J., Martínez-Solano, I. (2016): Concordant morphological and molecular clines in a contact zone of the Common and Spined toad (*Bufo bufo* and *B. spinosus*) in the northwest of France. Front. Zool. **13**: 52.

Birch, J.M. (1999): Skull allometry in the marine toad, *Bufo marinus*. J. Morphol. **241**: 115-126.

Cossu, I.M., Frau, S., Delfino, M., Chiodi, A., Corti, C., Bellati, A. (2018): First report of *Bufo bufo* (Linnaeus, 1758) from Sardinia (Italy). Acta Herpetol. **13**: 43-49.

Cvetković, D., Tomašević, N., Ficetola, G.F., Crnobrnja-Isailović, J., Miaud, C. (2009): Bergmann's rule in amphibians: combining demographic and ecological parameters to explain body size variation among populations in the common toad *Bufo bufo*. J. Zool. Syst. Evol. Res. **47**: 171-180.

Cvijanović, M., Ivanović, A., Kalezić, M.L., Zelditch, M.L. (2014): The ontogenetic origins of skull shape disparity in the *Triturus cristatus* group. Evol. Dev. **16**: 306-317.

De Lange, L. (1973): A contribution to the intraspecific systematics of *Bufo bufo* (Linnaeus, 1758) (Amphibia). Beaufortia **21**: 99-116.

Ivanović, A., Arntzen, J.W. (2018): Evolution of skull shape in the family Salamandridae (Amphibia: Caudata). J. Anat. **232**: 359-370.

Ivanović, A., Sotiropoulos, K., Üzüm, N., Džukić, G., Olgun, K., Cogălniceanu, D., Kalezić, M.L. (2012): A phylogenetic view on skull size and shape variation in the smooth newt (*Lissotriton vulgaris*, Caudata, Salamandridae). J. Zool. Syst. Evol. Res. **50**: 116-124.

Klingenberg, C.P. (2011): MorphoJ: an integrated software package for geometric morphometrics. Mol. Ecol. Resour. **11**: 353-357.

Klingenberg, C.P. (2016): Size, shape, and form: concepts of allometry in geometric morphometrics. Dev. Genes Evol. **226**: 113-137.

Lachenbruch, P.A. (1967): An almost unbiased method of obtaining confidence intervals for the probability of misclassification in discriminant analysis. Biometrics **23**: 639-645.

Larson, P.M. (2002): Chondrocranial development in larval *Rana sylvatica* (Anura: Ranidae): morphometric analysis of cranial allometry and ontogenetic shape change. J. Morphol. **252**: 131-144.

Monteiro, L.R. (1999): Multivariate regression models and geometric morphometrics: the search for causal factors in the analysis of shape. Syst. Biol. **48**: 192-199.

Ponssa, M.L., Candioti, F.V. (2012): Patterns of skull development in anurans: size and shape relationship during postmetamorphic cranial ontogeny in five species of the *Leptodactylus fuscus* Group (Anura: Leptodactylidae). Zoomorphology **131**: 349-362.

Recuero, E., Canestrelli, D., Vörös, J., Szabó, K., Poyarkov, N.A., Arntzen, J.W., Crnobrnja-Isailovic, J., Kidov, A.A., Cogălniceanu, D., Caputo, F.P., Nascetti, G., Martínez-Solano, I. (2012): Multilocus species tree analyses resolve the radiation of the widespread *Bufo bufo* species group (Anura, Bufonidae). Mol. Phylogenet. Evol. **62**: 71-86.

Simon, M.N., Machado, F.A., Marroig, G. (2016): High evolutionary constraints limited adaptive responses to past climate changes in toad skulls. Proc. R. Soc. B Biol. Sci. **283**: 20161783.

Strelin, M.M., Benitez-Vieyra, S., Fornoni, J., Klingenberg, C.P., Cocucci, A.A. (2016): Exploring the ontogenetic scaling hypothesis during the diversification of pollination syndromes in *Caiophora* (Loasaceae, subfam. Loasoideae). Ann. Bot. **117**: 937-947.

Trueb, L. (1993): Patterns of cranial diversity among the Lissamphibia. In: The skull: patterns of structural and systematic diversity, pp. 255-343. Hanken, J., Hall, B.K., Eds, University of Chicago Press, Chicago.

Webster, M., Sheets, H.D. (2010): A practical introduction to landmark-based geometric morphometrics. Paleontol. Soc. Pap. **16**: 163-188.

Zelditch, M.L., Swiderski, D.L., Sheets, H.D., Fink, W.L. (2004): Geometric morphometrics for biologists: a primer. Elsevier/Academic Press, Amsterdam.

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Hematological parameters of the Bolson tortoise *Gopherus flavomarginatus* in Mexico

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Abstract. We present findings of our preliminary study to determine biometry and blood chemistry values of healthy wild individuals of the critically endangered Bolson tortoises (*Gopherus flavomarginatus*) in Mexico. Given the absence of previously published data regarding hematology parameters for this species, these results represent an important base for additional research. Hematocrit determination, stains, and cell counts were performed, as well as 18 parameters of blood chemistry. Values of biometry and blood chemistry for *G. flavomarginatus* were similar to reference values those already reported for *G. agassizii*, *G. polyphemus,* and *G. berlandieri*. These similarities reflect the phylogenetic relationships among these species. However, slight differences may point to particular adaptations that each has developed to their own habitat, and so point to questions to be addressed with future research.

Keywords. Chelonia, lymphocyte, desert, Mapimí Biosphere Reserve.

Hematological analysis is a common technique for evaluating the health status of wild animals (Campbell, 2004; Tavares-Dias et al., 2009). Blood biometry and chemistry of terrestrial, semiaquatic and marine tortoises have been described already (Stacy et al. 2011; Campbell, 2015). However, there are still species of ecological importance whose blood information is unavailable. This is the case of *Gopherus flavomarginatus*, a tortoise endemic to Mexico and considered the largest terrestrial tortoise in North America, with a carapace length of up to 40 cm (Morafka et al., 1989). This species is in danger of extinction according to the Official Mexican Standard 059 (SEMARNAT, 2010) and critically endangered according to the IUCN red list (Kiester et al., 2018). Its geographical distribution is restricted to the Mapimí Bolson in the Mexican Chihuahuan Desert, where it currently has protected status within the Mapimí Biosphere Reserve (CONANP, 2006). Tortoises of the genus *Gopherus* are keystone organisms for the ecosystems in which they live because, due to their feeding habits (herbivory), they perform the ecological function of seed dispersal (Carlson et al., 2003), and because the burrows that they excavate are deep and provide shelter for at least 300 species of invertebrates and 60 of vertebrates (Lips, 1991). Due to the importance of protecting this tortoise, we conducted this preliminary study to determine biometry and blood chemistry values of healthy wild individuals. This information will serve as a basis for future hematological studies carried out on this tortoise.

From May 2015 to September 2017, we captured 44 adult individuals of *G. flavomarginatus* (16 males and 28 females) within the Mapimí Biosphere Reserve in Mex-

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ico (26°00' and 26°10'N, and 104°10' and 103°20'W). Blood samples were collected from the subvertebral vein. Three milliliters of blood were collected from each specimen; one milliliter was placed in a Vacutainer® tube with lithium heparin as anticoagulant and the rest in a red Vacutainer® tube. The tubes were stored in a cooler at a temperature of approximately 4 °C. Each tortoise was determined to be healthy following the observation protocols of Jacobson (2014) and USFWS (2016). Tortoises were then released at the site of their capture. Biometric analysis of blood was performed following the protocols suggested by Thrall et al. (2006) and Turgeon (2012). The volume percentage of red cells (Hematocrit, Ht in%) was determined in the blood contained in the green Vacutainer® tube, using the microhematocrit technique. The total number (TR) and percentage (PR) of red cells were obtained for each blood sample. The formula used to obtain hematocrit values was Ht% = (PR/TR) × 100. Hemoglobin concentration was quantified with the Drabkin colorimetric method, using the Spinreact® commercial kit, in a VetTest® spectrophotometer. The reaction product was centrifuged (12,000 g × 5 min) to precipitate the nucleus of the cells and keep them from distorting the color to be measured (Thrall et al., 2006).

Erythrocyte and leukocyte counts were carried out using a Thoma pipette with red bead and a Neubauer hemocytometer with Natt and Herrick's stain (Thrall et al., 2006). The erythrocyte count was carried out under 10× magnification, on both sides of the Neubauer chamber, in the four corner squares and the center square within the large center square of the chamber. The following formula was applied (Kemal, 2014): erythrocytes ($\times 10^6$ ul) = mean erythrocytes × 10000. The leukocyte count was carried out under 40x magnification in the nine large squares on both sides of the Neubauer chamber. The following formula was applied: leukocytes ($\times 10^3$ ul) = mean leukocytes × 50 (Kemal, 2014). The erythrocyte indices were calculated according to the formulas described by Ball (2014) and Kemal (2014): mean corpuscular volume, MCV (fL) = Ht% × 10/erythrocytes ($\times 10^6$ ul); mean corpuscular hemoglobin, MCH (pg) = (Hg × 10)/erythrocytes ($\times 10^6$ ul); mean corpuscular hemoglobin concentration, MCHC (g/dl) = (Hg × 100)/Ht%. Two smears were prepared from each sample on glass slides using Wright's dye (Analytyka®). One hundred leukocytes were counted in the body of the smear of each of the two slides. The average of both slides was obtained, and the results were expressed as the proportion of each cell type (relative differential count). To obtain the absolute differential count, the total value of leukocytes ($\times 10^3$ cel/ul) was multiplied by the average percentage of each type of leukocyte and the result was divided by 100 (Thrall et al., 2006).

The H:L ratio was obtained by dividing the percentage of heterophiles by the percentage of lymphocytes in each sample (Davis et al., 2008). Blood from the red Vacutainer® tube was centrifuged at 12,000 g for five minutes; the serum was separated from the red cells and placed in a sterile plastic tube. No hemolysis was observed in any sample. The following parameters were analyzed: glucose, uric acid, urea, blood urea nitrogen (BUN), creatinine, total proteins, albumin, globulins, cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), chlorine, sodium, calcium, phosphorus and osmolality (mOsm/kg=1.86 (Na [mmol/L]) + glucose [mg/dL]/18 + BUN [mg/dL]/2.8 + 9). Human serum Spintrol H Spinreact® was used as control. These analyzes were performed in a VetTest® spectrophotometer with Spinreact® reagents. Descriptive statistics (mean, standard error, median, standard deviation, minimum, and maximum) were obtained in PAST 3.14 (Hammer et al., 2001).

Blood biometry results for *G. flavomarginatus* are shown in Table 1 and for blood chemistry in Table 2. A comparison among biometry and chemistry variables of *G. flavomarginatus* and other *Gopherus* species are shown in Tables 3 and 4, respectively. Blood biometric and chemistry values obtained for *G. flavomarginatus* were similar to those reported for *G. agassizii* (Christopher et al., 1999; Dickinson et al., 2000), *G. polyphemus* (Taylor and Jacobson, 1982), and *G. berlandieri* (Teare, 2013a) (Tables 3 and 4). Christopher et al. (1999) reported that the most abundant leukocytes in *G. agassizii* are heterophils, followed by lymphocytes, basophils, and finally eosinophils and monocytes. In our findings for *G. flavomarginatus*, the order of abundance of the leukocyte cells was similar to the one mentioned by Duguy (1970), with lymphocytes as the most abundant leukocytes (males = 53.43%, 4.79×10^3 cel/ul; females = 44.03%, 4.20×10^3 cel/ul). This agrees with the findings of Diaz-Figueroa (2005) for *G. polyphemus*, and other authors who indicate that lymphocytes are predominant in the peripheral blood of most reptile species (Davis et al.2008; Stacy et al., 2011).

Heterophils were the second most abundant leukocyte cells in *G. flavomarginatus*. In general, the abundance of this type of leukocyte in reptiles ranges from 30 to 45% (Duguy, 1970; Frye, 1991); however, in tortoises it can reach up to 50% (Alleman et al., 1992; Christopher et al. 1999). Basophils were the third most abundant leukocytes in the blood of the Bolson tortoise. Alleman et al., (1992), and Duguy, (1970) estimated that the percentage of basophils in healthy terrestrial and aquatic tortoises can be higher than 40%. In *G. flavomargi-*

Table 1. Descriptive statistics of blood biometric variables from male/female *Gopherus flavomarginatus*. SE = standard error, SD = standard deviation, Min = minimum, Max = maximum.

Variable	Mean	SE	Median	SD	Min	Max
Hematocit (%)	25.35/23.48	1.41/0.77	25.30/23.61	5.64/4.09	16.47/17.89	35.62/33.33
Hemoglobin (g/dl)	6.53/6.59	0.40/0.26	6.56/7.15	1.62/1.41	4.05/3.89	9.67/8.95
Erythrocytes ($\times 10^6$ cel/ul)	0.54/0.57	0.06/0.03	0.47/0.58	0.25/0.19	0.22/0.32	0.97/0.98
MCV (fl)	539.02/448.98	54.81/27.17	458.56/401.53	219.26/143.78	264.56/196.17	921.72/717.91
MCH (pg)	144.12/122.92	18.96/5.79	124.11/122.53	75.86/30.67	64.66/45.95	292.86/197.78
MCHC (g/dl)	26.37/28.49	1.64/1.23	25.62/28.84	6.56/6.52	17.69/16.20	39.36/44.66
Leukocytes ($\times 10^3$ cel/ul)	8.88/9.50	0.82/0.70	8.55/8.55	3.28/3.73	5.00/2.44	17.67/21.33
Heterophils (%)	25.43/29.32	1.99/1.68	26.00/29.00	7.99/8.89	14.00/13.00	39.00/45.00
Eosinophils (%)	2.75/3.17	0.57/0.51	2.00/2.50	2.29/2.74	0.00/0.00	7.00/11.00
Basophils (%)	17.68/21.42	2.11/1.67	15.50/18.50	8.47/8.86	6.00/8.00	29.00/46.00
Lymphocytes (%)	53.43/44.03	2.99/2.06	54.00/42.50	11.99/10.92	34.00/27.00	73.00/70.00
Monocytes (%)	0.68/2.03	0.17/0.40	1.00/1.00	0.70/2.16	0.00/0.00	2.00/9.00
H:L ratio	0.52/0.73	0.06/0.06	0.45/0.71	0.27/0.36	0.22/0.24	1.11/1.59
Heterophils ($\times 10^3$ cel/ul)	2.17/2.81	0.19/0.29	2.09/2.56	0.77/1.55	0.93/0.66	3.45/7.25
Eosinophils ($\times 10^3$ cel/ul)	0.21/0.34	0.04/0.08	0.18/0.21	0.17/0.45	0.00/0.00	0.66/2.35
Basophils ($\times 10^3$ cel/ul)	1.61/1.95	0.28/0.16	1.45/1.80	1.14/0.85	0.30/0.39	5.12/4.16
Lymphocytes ($\times 10^3$ cel/ul)	4.79/4.20	0.57/0.36	3.96/4.04	2.31/1.95	2.57/0.78	10.32/8.96
Monocytes ($\times 10^3$ cel/ul)	0.07/0.18	0.02/0.03	0.06/0.09	0.09/0.20	0.00/0.00	0.35/0.85

Table 2. Descriptive statistics of blood chemistry variables from male/female *Gopherus flavomarginatus*. SE = standard error, SD = standard deviation, Min = minimum, Max = maximum.

Variable	Mean	SE	Median	SD	Min	Max
Glucose (mg/dl)	79.19/58.34	7.97/8.28	68.33/40.47	31.90/43.82	43.32/13.34	134.67/165.30
Uric acid (mg/dl)	5.63/5.58	1.07/0.71	5.66/5.96	4.28/3.76	0.27/0.24	12.45/13.48
Urea (mg/dl)	25.60/31.22	0.82/0.76	25.39/31.75	3.30/7.86	20.64/14.67	32.86/51.34
BUN	11.96/14.37	0.38/0.82	11.86/14.22	1.54/4.35	9.64/6.86	15.36/23.99
Creatinine (mg/dl)	0.48/0.43	0.06/0.05	0.47/0.40	0.24/0.26	0.01/0.01	0.95/0.89
Total protein (g/dl)	4.12/3.95	0.53/0.24	3.76/3.78	2.13/1.27	1.29/2.00	8.96/7.13
Albumin (g/dl)	0.87/0.68	0.18/0.10	0.56/0.47	0.74/0.56	0.09/0.04	2.50/2.55
Globulins (g/dl)	3.24/3.27	0.42/0.24	3.23/3.09	1.70/1.30	0.99/1.37	6.46/6.50
Cholesterol (mg/dl)	280.55/214.94	45.28/23.50	269.50/167.43	181.13/124.36	41.46/78.32	692.27/508.20
Triglycerides (mg/dl)	207.85/177.57	13.79/16.18	213.80/164.06	55.18/85.62	114.08/42.35	283.63/402.30
ALT (UI/I)	7.74/8.15	1.06/1.06	7.53/6.48	4.25/5.65	2.04/1.74	15.93/22.43
AST (UI/I)	41.79/45.23	6.30/3.53	32.27/47.92	25.21/18.69	14.95/15.63	105.60/80.42
AP (UI/I)	60.30/66.53	8.08/4.58	56.91/70.05	32.32/24.24	14.11/24.86	102.60/99.32
Chlorine (mmol/l)	119.90/121.64	4.24/2.98	118.90/122.31	16.97/15.81	95.68/94.53	139.62/149.14
Sodium (mmol/l)	137.53/138.24	3.20/2.47	136.24/136.42	12.80/13.11	112.36/117.90	160.90/177.60
Calcium (mg/dl)	11.61/13.44	0.65/0.41	12.64/13.29	2.63/2.19	7.46/10.27	15.67/18.30
Phosphorus (mg/dl)	3.95/4.08	0.56/0.36	3.59/4.29	2.24/1.93	1.02/1.31	7.18/7.16
Osmolality (mOsm/kg)	270.50/273.87	5.92/4.73	268.60/272.56	23.70/23.85	225.43/240.81	316.39/345.66

natus, the average was 17.68% for males and 21.42% for females, with wide variation between individuals (min = 6%, max = 46%). Eosinophilic leukocytes were the fourth most abundant leukocytes. The seasons and type of diet can influence the amount of eosinophilic leukocytes in some species (Duguy, 1970; Deem et al., 2006). According to Frye (1991), the percentage of eosinophils in healthy reptiles ranges from 7 to 20%; in the case of

Table 3. Comparison among minimum-maximum blood biometry values reported for *Gopherus flavomarginatus* and reference values of other three species of the genus.

Variable	*G. flavomarginatus*[1]	*G.agassizii*[2]	*G. polyphemus*[3,4]	*G. berlandieri*[5]
Hematocit (%)	16.47-35.62	19.50-37.10	15.00-30.00	12.00-44.80
Hemoglobin (g/dl)	3.89-9.67	4.10-9.90	4.20-8.60	-
Erythrocytes ($\times10^6$ cel/ul)	0.22-0.98	0.36-1.08	0.24-0.91	-
MCV (fl)	196.17-921.72	254.00-638.00	200.10-838.60	-
MCH (pg)	45.95-292.86	74.00-186.00	-	-
MCHC (g/dl)	16.20-44.66	20.00-33.00	-	-
Leukocytes ($\times10^3$ cel/ul)	2.44-21.33	1.49-10.92	10.00-22.00	0.00-80.650
Heterophils (%)	13.00-45.00	-	10.00-57.00	-
Eosinophils (%)	0.00-11.00	-	-	-
Basophils (%)	6.00-46.00	-	2.00-11.00	-
Lymphocytes (%)	27.00-73.00	-	32.00-79.00	-
Monocytes (%)	0.00-9.00	-	3.00-13.00	-
H:L ratio males	0.22-1.59	-	-	-
Heterophils ($\times10^3$ cel/ul)	0.66-7.25	0.71-7.15	0.00-6.59	0.00-5.21
Eosinophils ($\times10^3$ cel/ul)	0.00-2.35	0.00-0.95	-	-
Basophils ($\times10^3$ cel/ul)	0.30-5.12	0.06-3.57	0.02-0.92	0.00-1.31
Lymphocytes ($\times10^3$ cel/ul)	0.78-10.32	0.63-2.74	0-4.15	0-3.12
Monocytes ($\times10^3$ cel/ul)	0.00-0.85	0.00-0.32	-	0.00-0.44

[1] Present study. [2] Christopher et al. (1999) in summer season. [3] Taylor and Jacobson (1982). [4] Teare (2013b). [5] Teare (2013a).

Table 4. Comparison among minimum-maximum blood chemistry values reported for *Gopherus flavomarginatus* and reference values of other three species of the genus.

Variable	*G. flavomarginatus*[1]	*G.agassizii*[2]	*G. polyphemus*[3,4]	*G. berlandieri*[5]
Glucose (mg/dl)	13.34-165.30	65.00-186.00	55.00-128.00	9.00-157.00
Uric acid (mg/dl)	0.24-13.48	1.70-9.20	0.90-8.50	0.00-8.60
Urea (mg/dl)	14.67-51.34	-	1.00-130.00	-
BUN	6.86-23.99	1.00-37.00	2.00-29.00	0.00-13.00
Creatinine (mg/dl)	0.01-0.95	0.20-0.40	0.10-0.40	-
Total protein (g/dl)	1.29-8.96	2.30-5.30	1.30-4.60	1.20-7.70
Albumin (g/dl)	0.04-2.55	0.80-1.90	0.50-2.60	0.50-2.70
Globulins (g/dl)	0.99-6.50	1.30-3.90	0.50-4.60	0.60-5.10
Cholesterol (mg/dl)	41.46-692.27	33.00-381.00	19.00-150.00	
Triglycerides (mg/dl)	42.35-402.30	7.00-603.00	-	-
ALT (UI/I)	1.74-22.43	1.00-5.00	2.00-57.00	-
AST (UI/I)	14.95-105.60	15.00-123.00	57.00-392.00	0.00-265.00
AP (UI/I)	14.11-102.60	25.00-114.00	11.00-71.00	-
Chlorine (mmol/l)	94.53-149.14	101.00-138.00	35.00-128.00	89.00-122.00
Sodium (mmol/l)	112.36-177.60	127.00-176.00	127.00-148.00	123.00-153.00
Calcium (mg/dl)	7.46-18.30	8.60-23.90	10.00-14.00	5.00-17.40
Phosphorus (mg/dl)	1.02-7.18	1.10-6.50	1.00-3.10	0.70-4.90
Osmolality (mOsm/kg)	225.43-345.66	252.00-352.00	-	-

[1]Present study. [2]Christopher *et al.* (1999) in summer season. [3]Taylor and Jacobson (1982). [4]Teare (2013b). [5]Teare (2013a).

G. flavomarginatus, the average percentage found in the present study was 2.75% for males and 3.17% for females, with a total variability of 0-11%. The average percentage of monocytes in the Bolson tortoise was 0.68% for males and 2.03% for females (min = 0, max = 9); these values are within the range of abundance reported for reptiles (Duguy, 1970).

The H:L ratio (heterophils/leukocytes) has been used as a reliable method to evaluate the exposure of vertebrates to chronic stress due to the relationship between the leukocyte profile and the adrenal response (production of cortisol). When the level of cortisol rises, the number of circulating heterophils increases, while the number of lymphocytes decreases (Davis et al., 2011). Davis (2009) indicated that blood biometry studies conducted with terrestrial tortoises in which the possibility of individuals being stressed was not considered (including studies with *G. agassizii*, *G. berlandieri,* and *G. polyphemus*), the H:L ratio was less than 2.0 (mean of 0.65, SD=0.34), which coincides with the values obtained in the present study for *G. flavomarginatus*.

Blood chemistry variables provide important information for establishing levels of hydration (BUN, uric acid, osmolality), nutrition (glucose, total protein, albumin, colesterol, phosphorus), and metabolic activity (alanine aminotransferas, aspartate aminotransferase, and alkaline phosphatase activities) of desert tortoises (Christopher et al., 1999). Christopher et al. (1999) showed that blood chemistry values in *G. agassizii* change due to the effect of physiological (reproductive cycle, hibernation) and environmental factors (precipitation patterns, availability of water and food). However, we observed similar blood values between *G. agassizii and G. flavomarginatus* at the same times of year (summer). In general, the phylogenetic relation among *G. flavomarginatus*, *G. agassizii*, *G. polyphemus*, and *G. berlandieri* (Reynoso and Montellano-Ballesteros, 2004) may be reflecting the similarity between reported hematology values for these species, and the slight differences may point to particular adaptation conditions that each one has developed in their own habitat.

Our small sample size (n = 44) precluded the calculation of formal reference intervals, a process that would require a minimum of 120 individuals (Geffre, et al., 2009). However, given the limited sample size available, and the lack of previously published data regarding hematology parameters in *G. flavomarginatus*, these results provide a useful starting point for researchers.

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REFERENCES

Alleman, A.R., Jacobs, E.R., Raskin, R.E. (1992): Morphologic and cytochemical characteristics of blood cells from the desert tortoise (*Gopherus agassizii*). Am. J. Vet. Res. **53**: 1645-1651.

Campbell, T.W. (2004): Hematology of lower vertebrates. In: 55th Annual Meeting of the American College of Veterinary Pathologists (ACVP) & 39th Annual Meeting of the American Society of Clinical Pathology, pp. 1103-1214. ASVCP, ACVP, ASVCP, Eds, Middleton WI, USA.

Campbell, T.W. (2015): Exotic Animal Hematology and Cytology. 4th ed. Wiley Blackwell, Oxford, UK.

Carlson, J.E., Menges, E.S., Marks, P.L. (2003): Seed dispersal by *Gopherus polyphemus* at Archbold Biological Station, Florida. Fla. Sci. **66**: 147-154.

Christopher, M.M., Berry, K.H., Wallis, I.R., Agy, K.A., Enen, B.T., Peterson, C.C., (1999): Reference intervals and physiologic alterations in hematologic and biochemical values of free-ranging desert tortoises in the Mojave Desert. J. Wildl. Dis. **35**: 212-238.

CONANP. (2006): Programa de Conservación y Manejo Reserva de la Biosfera Mapimí México. CONANP-SEMARNAT, Mexico.

Davis, A.K. (2009): The wildlife leukocytes webpage; the ecologists' source for information about leukocytes of wildlife species. Available at: http://wildlifehematology.uga.edu. August 17, 2017.

Davis, A.K., Maney, D.L., Maerz J.C. (2008): The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. **22**: 760-772.

Davis, A.K., Ruyle, L.E., Maerz, J.C. (2011): Effect of trapping method on leukocyte profiles of Black-Chested Spiny-Tailed Iguanas (*Ctenosaura melanosterna*): implications for zoologists in the field. ISRN Zool. **2011**: 1-8.

Deem, S.L., Dierenfeld, E.S., Sounguet, G.P., Alleman, A.R., Cray, C., Poppenga, R.H., Norton, T.M., Karesh, W.B. (2006): Blood values in free-ranging nesting leatherback sea turtles (*Dermochelys coriacea*) on the coast of the Republic of Gabon. J. Zoo. Wildl. Med. **37**: 464-471.

Dickinson, V.M., Jarchow, J.L., Trueblood, M.H. (2002): Hematology and plasma biochemistry reference range values for free-ranging desert tortoises in Arizona. J. Wildl. Dis. **38**: 143-153.

Diaz-Figueroa, O. (2005): Characterizing the health status of the Louisiana Gopher tortoise (*Gopherus polyphemus*). Unpublished doctoral dissertation. Louisiana State University and Agricultural and Mechanical College.

Duguy, R. 1970. Numbers of blood cells and their variation. In: Biology of Reptilia (vol 3), pp. 93-109. Gans, C., Pough, F.H., Eds, Academic Press, New York.

Frye, F.L. (1991): Infectious diseases. In: Biomedical and surgical aspects of captive reptile husbandry, pp. 113-123. Frye, F.L., Ed, Krieger Publishing Co., Malabar, Florida.

Geffré, A., Friedrichs, K., Harr, K., Concordet, D., Trumel, C., Braun, J.P. (2009): Reference values: a review. Vet. Clin. Path. **38**: 288-298.

Hammer, Ř., Harper, D.A.T., Ryan, P.D. (2001): PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. **4**: 9.

Jackson, D., Milstrey, E.G. (1989): The fauna of gopher tortoise burrows. In: Proceedings of the Gopher Tortoise Relocation Symposium, pp. 86-98. Diemer, J., Jackson, D., Landers, L., Layne, J., Wood, D., Eds, Florida Game and Freshwater Fish Comm. Nongame Wildlife Program, Technical Report No. 5. Tallahassee, Florida.

Jacobson, E.R. (2014): Health issues of North American tortoises. In: Biology and conservation of North American tortoises, pp. 60-76. Rostal, D.C., McCoy, E.D., Mushinsky, H.R., Eds, Johns Hopkins University Press. Baltimore, Maryland.

Kemal, J. (2014): Laboratory Manual and Review on Clinical Pathology. OMICS Group eBooks, California, EUA.

Kiester, A.R., Palomo-Ramos. R., Ríos-Arana, J., Goode, E.V. (2018). *Gopherus flavomarginatus*. The IUCN Red List of Threatened Species; e.T9402A112660985. Available at: http://dx.doi.org/10.2305/IUCN.UK.2018-2.RLTS.T9402A112660985.en. [accessed on 3 December 2018]

Lips, K.R. (1991): Vertebrates associated with tortoise (*Gopherus polyphemus*) burrows in four habitats in south-central Florida. J. Herpetol. **25**: 477-481.

Morafka, D.J., Aguirre, G., Adest, G.A. (1989): *Gopherus flavomarginatus* Bolson Tortoise. In: The Conservation Biology of Tortoises, pp. 10-13. Swingland, R., Klemens, M.W., Eds, Occasional Papers of the IUCN Species survival Commission (SSC) No. 5. IUCN, Gland, Switzerland.

Reynoso, V.H., Montellano-Ballesteros, M. (2004): A new giant turtle of the genus *Gopherus* (Chelonia: Testudinidae) from the Pleistocene of Tamaulipas, México, and a review of the phylogeny and biogeography of gopher tortoises, J. Vert. Paleontol. **24**: 822-837.

SEMARNAT. (2010): NORMA Oficial Mexicana NOM-059-SEMARNAT-2010, Protección ambiental-Especies nativas de México de flora y fauna silvestres-Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio-Lista de especies en riesgo. Diario Oficial, 30 Diciembre 2010, México.

Stacy, N.I., Alleman, A.R., Sayler, K.A. (2011): Diagnostic hematology of reptiles. Clin. Lab. Med. **31**: 87-108.

Tavares-Dias, M., Oliveira-Junior, A.A., Silva, M.G., Marcon, J.L., Barcellos J.F.M. (2009): Comparative hematological and biochemical analysis of giant turtles from the Amazon farmed in poor and normal nutritional conditions. Vet. Arh. **79**: 601-610.

Taylor, R.W., Jacobson, E.R. (1982): Hematology and serum chemistry of the gopher tortoise, *Gopherus polyphemus*. Comp. Biochem. Physiol. **72**: 425-428.

Teare, J.A. (2013a): *Gopherus berlandieri*, no selection by gender, all ages combined, Conventional American units 2013 CD.html. In: ISIS Physiological Reference Intervals for Captive Wildlife: A CD-ROM Resource. International Species Information System, Ed, Eagan, MN, USA.

Teare, J.A. (2013b): *Gopherus polyphemus*, no selection by gender, all ages combined, Conventional American units 2013 CD.html. In: ISIS Physiological Reference Intervals for Captive Wildlife: A CD-ROM Resource. International Species Information System, Ed, Eagan, MN, USA.

Thrall, M.A., Baker, D.C., Campbell, T.W., DeNicola, D., Fettman, M.J., Lassen, E.D., Rebar, A., Weiser, G. (2006): Veterinary hematology and clinical chemistry. Blackwell Publishing, Iowa, USA.

Turgeon, M.L. (2012): Clinical Hematology: Theory & Procedures. 5th ed. Wolters Kluwer, Lippincott Williams & Wilkins, Philadelphia, USA.

USFWS (2016): Health assessment procedures for the desert tortoise (*Gopherus agassizii*): A handbook pertinent to translocation. U.S. Fish and Wildlife Service, Desert Tortoise Recovery Office, Reno, Nevada, USA.

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Visible Implant Alphanumeric (VIA) as a marking method in the lesser snouted treefrog *Scinax nasicus*

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Abstract. In this study we assessed the efficacy of Visible Implant Alphanumeric (VIA) for marking adults and juveniles of the Neotropical treefrog *Scinax nasicus*. We evaluated the success of this technique in the identification of individuals and the prevalence of tags in the field. As a control, we marked the same individuals through toe-clipping. Of 196 marked individuals, 57 were recaptured in a 7-month study period. Only one mark was unreadable because it was located too deep in the skin. We found one case of tag expulsion and two inverted tags. Almost complete regeneration of the adhesive disk was observed by the fifth month of the study in all recaptured frogs. We suggest VIA tagging method as suitable for *S. nasicus* over long term studies. Even though, a hybrid method for marking (VIA + toe-clipping) is recommended for species with dark and/or loose skin, or large frogs.

Keywords. Amphibian, mark-recapture, fluorescent elastomer, toe-clipping.

The recognition of individuals in a population is a key aspect in many amphibian ecology and conservation studies (Seber, 1982; Campbell et al., 2009; Clemas et al., 2009). Their identification over time and space helps researchers to estimate individual and demographic parameters such as growth and mortality rates, dispersal, population size and habitat use (Osbourne et al., 2011). In cases where individuals of a species do not possess characteristics that distinguish them from each other, like coloration and color patterns, and photo-identification is not suitable, it is necessary to use marking techniques (Donelly et al., 1994). For amphibians, the most commonly used are toe-clipping, Passive Integrated Transponder (PIT), Visible Implant Elastomer (VIE) tags, and more recently Visible Implant Alphanumeric (VIA) tags, the last two manufactured by North-west Marine Technology, Shaw Island, USA (Heard et al., 2008; Branelly et al., 2014).

Toe-clipping is the most widely used technique for marking anurans and salamanders (Ferner, 2010). However, this method is highly criticized because a decrease in the probability of recapture has been detected in some amphibian species (Clarke, 1972; Mccarthy and Parris, 2004; Waddle et al., 2008). Another disadvantage of toe-clipping in long term studies is the possibility of tissue regeneration, although this has been observed only in a few species (Ferner, 2007; Ursprung et al., 2011). PIT tags consist of an electromagnetic capsule with an alphanumeric code, which is read by a scanner. Insertion is done subcutaneously or in a body cavity (Ferner,

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2010). However, loss of tags and deleterious effects related to survival have been detected using this technique (Scherer et al., 2005; Guimaraes et al., 2014). VIE tagging is done through the subcutaneous injection of an elastomer mixed with a curing agent. This technique allows the individualization of many animals by creating different fluorescent color codes as well as placing the marks in various body locations (Moosman and Moosman, 2006). When comparing these three techniques, Branelly et al. (2014) mention that the least efficient is the VIE tagging, due to the migration, darkening and in some cases expulsion of the tag.

VIA tags are compacted elastomers in rectangular shape with an alphanumeric code in fluorescent colors visible under UV light (Heard et al., 2008). The insertion is done with an injector provided by the manufacturer, but in some cases the results are better making a previous incision (Buchan et al., 2005; Gower et al., 2006; Heard et al., 2008; Clemas et al., 2009; Kaiser et al., 2009). Despite being considered a reliable technique due to the capacity of individual identification, time of marking, handling of the individuals, durability of the marks and relatively low cost (Haw et al., 1990; Buchan et al., 2005; Gower et al., 2006), this technique also has disadvantages when used in amphibians: difficulty of tag insertion in species with loose skin, variation on tag retention among species and darkening of the tag when marking heavily pigmented species (Kaiser et al., 2009). Additionally, tag retention was low in studies of mark-recapture of tadpoles (Courtois et al., 2013).

We tested the VIA tags in the lesser snouted treefrog *Scinax nasicus* (Cope, 1862) in order to 1) evaluate the effectiveness of this technique in the identification of individuals, 2) determine the prevalence of tags in mark-recapture studies, and 3) provide some recommendations for further studies upon this species and others with similar morphology.

S. nasicus is a small sized hylid frog distributed in northern and central Argentina, Paraguay, Uruguay, eastern Bolivia, and central and southern Brazil (Frost, 2019). The species is commonly found in open areas of the Atlantic Forest, Cerrado, Chaco, Pampa, and Pantanal domains (Dalmolin et al., 2017). We captured individuals of *S. nasicus* using 173 PVC pipes as a refuge in an area of approximately 3 km^2 of wetland and associated riparian forest in Benjamín Aceval, Presidente Hayes department in Paraguay (-24.960522S, -57.359425W). Field work was done during the months of November 2017, January, March and May 2018.

The individuals were tagged both with VIA tags and toe-clipping. Additionally, measurements of the snout-vent length (SVL) were taken with a digital caliper Mitutoyo Absolute AOS Digimatic. The VIA tags used were of standard size (1.2 × 2.7 mm) in fluorescent green with black letters. The insertion site was the ventral region of the right thigh following Buchan et al. (2005) (Fig. 1). The implantation site was sterilized with ethanol 90%, then the tag was inserted under the skin using the injector provided by NMT following the manufacturer protocol and no veterinary glue was used. All marking equipment was sterilized between frogs by immersion in 90% ethanol for several minutes. Toe-clipping was carried out as control following the method of Martof (1953). As suggested by Kinkead et al. (2006), no anesthesia was used in either both procedures. Frogs showed no signs of discomfort, e.g., emitting distress calls or abnormal movements of the affected limb and/or foot. Frogs were put under observation for 24 hours in plastic bags filled with air to observe presence of redness, edema or bleeding on the treated foot or at the injection site. Subsequently they were released on the same PVC pipe where they were captured. We marked 196 individuals of *S. nasicus* (55 females, 46 males, and 95 juveniles). Frogs averaged 25.9 mm (SD = 5.34 mm) of SVL. We obtained a total of 57 (29%) recaptures, of which 2 individuals were recaptured three times, 14 individuals twice, and 41 individuals only once. The recapture rates in juveniles was 47%, males 16% and females 37% (Fig. 2). We did not find significant differences in the recapture rates of juveniles and adults ($t = 0.3487$, d.f. = 4, $P = 0.7449$); therefore, it can be suggested that the VIA implants did not have negative effects on the survival of juveniles.

Fig. 1. Location of the VIA tag in the inner thigh of *Scinax nasicus*. VIA tag was injected just below the skin.

Every time the label was observed, the code could be identified without ambiguity with the help of the UV light lantern provided by the kit, in 98% of the times. In one case, the label was located too deep under the skin and could not be read properly. At the same time, by January 2018, we observed tissue regeneration in recaptured

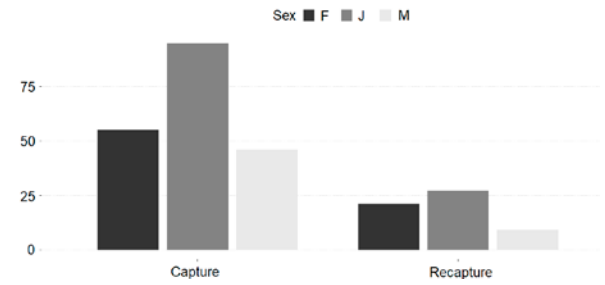

Fig. 2. Number of capture and recapture males, females and juveniles of *Scinax nasicus*.

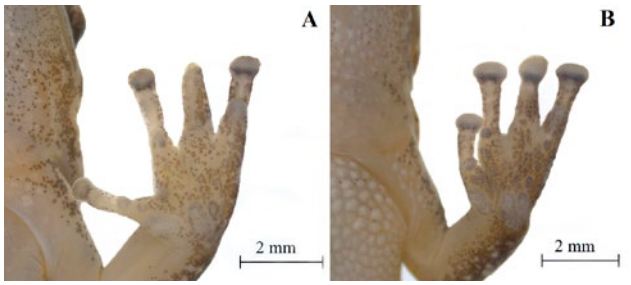

Fig. 3. Toe pad regeneration in *Scinax nasicus*. A: after a week of clipping, B: after two months of clipping.

frogs where the beginning of the growth of the adhesive discs was noticeable, and by March 2018 the discs had similar diameters to those that were not clipped (Fig. 3). In general, discs presented similar shape and coloration to those not clipped and no cases of aberrant growth were observed, such as those described by Hoffman et al. (2008).

Kaiser et al. (2009) studied the possibility of using VIA tags as a marking method that does not require the recapture of animals (by placing the tags on the back of a small frog species). They concluded that it is not possible to read the codes on the labels without having the animal in hand. When comparing the handling times between VIA tags and toe-clipping, Clemas et al. (2009) found that toe-clipping took slightly less amount of initial time of handling and marking than time with the VIA tags, but the last one took less time during identification. Despite results showed by Clemas et al. (2009), by using VIA tags in adults and juveniles, we had success identifying individuals of *S. nasicus* through this methodology.

Regarding the second objective of this work, we observed the retention of tags on 97% of the cases. Only on one occasion the label was expelled and, in another opportunity, as a result of an inflammation caused in a frog's leg, the tag had to be removed when the animal was recaptured. Also, in two cases the tags were inverted but could be easily rotated by prodding with a finger, and on one occasion a small piece of the tag broke when removed from the tag block but was not discarded.

When marking individuals at the caudal end of the dorsum, Kaiser et al. (2009) noted that very often the labels were turned over and migrated ventrally, but they do not mention if this affects frog's survivor or movement capacity. They also mention that when tags were not easily detected they remarked animals, and this constituted an extra effort and a waste of resources. Contrarily, Heart et al. (2008) compared tag migration placing them in the thigh and dorsolateral region of the thorax, finding that tag retention was higher in the latter site. Although, movement of tags in dorsal regions of the body is less common, this area tends to be more pigmented, making reading difficult (Moosman and Moosman, 2006). We opted for the ventral thigh location because this area is not heavily pigmented in *S. nasicus*, being almost translucid, thus we did not observe any noticeable migration, which may arise when the target species is large and allows movements of the tag in the interfemoral sac (Clemas et al., 2009). We also consider that the use of veterinary glue is not necessary since only in one case we observed the expulsion of the tag. When we purchased the product, manufacturers mentioned that the injection needle was re-designed and that it should not be necessary to make an initial incision. However, for marking large or thick-skinned species as well as when marking a large number of individuals this may be necessary, in order to maintain the instrument sharp and in shape to not cause discomfort to the animals.

The estimation of population and demographic parameters in ecological studies are based on assumptions that depend on the marking technique; these are: (1) no loss of marks; (2) no misidentification of marks; and (3) marking procedures do not alter survival or capture probabilities (Seber, 1986; Pollock et al., 1990). This last assumption is the most controversial because in most techniques' negative effects on both survival and recapture probability have been observed, being toe-clipping the most deleterious technique and VIE and VIA tagging the least, although this last one has been scarcely studied (Heard et al., 2008; Schmidt and Schwarzkopf, 2010; Sapsford et al., 2014, 2015).

VIA tags are an interesting method to test in amphibians due to its relatively low cost, lower invasiveness when compared to other techniques such as toe-clipping, and straightforward code interpretation. In our study, the rate of success of the VIA tag method suggests that it is suitable for *S. nasicus*, as it was easy, safe, rapid and effective to carry out, as well as easy to detect. Moreover, the method is well advised for the species

if long term studies are made, due to the pad regeneration observed in clipped toes. Still, we suggest keeping a hybrid marking method (i.e., VIA tags + toe-clipping) when working with other species than *S. nasicus*, since VIA tags can have a distinct rate of success depending on several factors, such as the degree of stretch in the skin, size of the leg or other body part, transparency. We also advise to take time to inspect carefully the animals to detect VIA tag migration or loss, so there is no resource waste and unnecessary animal stress.

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REFERENCES

Brannelly, L.A., Berger, L., Skerratt, L.F. (2014): Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii* alpine. Herpetol. Conserv. Biol. **9**: 428-435.

Buchan, A., Sun, L., Wagner, R.S. (2005): Using alphanumeric fluorescent tags for individual identification of amphibians. Herpetol. Rev. **36**: 43-44.

Campbell, T.S., Irvin, P., Campbell, K.R., Hoffmann, K., Dykes, M.E., Harding, A.J., Johnson, S.A. (2009): Evaluation of a new technique for marking anurans. Appl. Herpetol. **6**: 247-256.

Clarke, R.D. (1972): The effect of toe clipping on survival in Fowler's toad (*Bufo woodhousei fowleri*). Copeia: 182-185.

Clemas, R.J., Germano, J.M., Speare, R., Bishop, P.J. (2009): Use of three individual marking methods in Australian frogs (Genus: *Litoria*) with notes on placement of Visible Implant Alphanumeric tags. New Zeal. J. Sci. **34**: 1-7.

Courtois, E.A., Lelong, C., Calvez, O., Loyau, A., Schmeller, D.S. (2013): The use of visible implant alpha tags for anuran tadpoles. Herpetol. Rev. **44**: 230-233.

Dalmolin, D.A., Rosa, F.O., Freire, M.D., Fonte, L.F.M., Machado, I.F., Paula, C.N., Loebmann, D., Périco, E. (2017): First record of the Lesser Snouted Treefrog *Scinax nasicus* (Cope, 1862) in Brazilian coast and new species records for the state of Rio Grande do Sul. Braz. J. Biol. **77**: 659-661.

Donnelly, M.A., Guyer, C., Juterbock, J.E., Alford, R.A. (1994): Techniques for marking amphibians. In: Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians, pp. 277-284. Heyer, W.R., Donnelly, M.A., McDiarmid, R., Hayek, L.A.C., Foster M.S., Eds, Smithsonian Institution Press, Washington, DC, USA.

Ferner, J.W. (2007): A review of marking and individual recognition techniques for amphibian and reptiles. Herpetological Circular 35, Society for the Study of Amphibians and Reptiles, Atlanta.

Ferner, J.W. (2010): Measuring and marking post-metamorphic amphibians. In: Amphibian Ecology and Conservation: A Handbook of Techniques, pp. 123-141. Dodd, C.K. Jr., Ed, Oxford University Press, USA.

Frost, D.R. (2019): Amphibian Species of the World: An Online Reference. Version 6.0 (Date of access). Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

Gower, D.J., Oommen, O.V., Wilkinson, M. (2006): Marking amphibians with alpha fluorescent tags: Caecilians lead the way. Herpetol. Rev. **37**: 302.

Guimaraes, M., Correa, D.T., Filho, S.S., Oliveira, T.A.L., Doherty, P.F.J. Sawaya, R.J. (2014): One step forward: contrasting the effects of Toe clipping and PIT tagging on frog survival and recapture probability. Ecol. Evol. **4**: 1480-1490.

Haw, F., Bergman, P.K., Fralick, R.D., Buckley, R.M., Blankenship, H.L. (1990): Visible implanted fish tag. Am. Fish. Soc. Symp. **7**: 311-315.

Heard, G.W., Scroggie, M.P. Malone, B. (2008): Visible implant alphanumeric tags as an alternative to toe-clipping for marking amphibians: a case study. Wild. Res. **35**: 747-759.

Hoffmann, K.E., McGarrity, M.E., Johnson, S.A. (2008): Technology meets tradition: A combined VIE-C technique for individually marking anurans. Appl. Herpetol. **5**: 265-280.

Kaiser, K., Alloush, M., Jones, R.M., Marczak, S., Martineau, K., Oliva, M. (2009): Use of visual implant Alpha (VIAlpha) fluorescent Tags in a small hylid frog with a new technique for application. Herpetol. Rev. **40**: 421-422.

Kinkead, K.E., Lanham, J.D., Montanucci, R.R. (2006): Comparison of anesthesia and marking techniques on stress and behavioral responses in two *Desmognathus* Salamanders. J. Herpetol. **40**: 323-328.

Martof, B.S. (1953): Territoriality in the green frog, *Rana clamitans*. Ecology **34**: 165-174.

McCarthy, M.A., Parris, K.M. (2004): Clarifying the effect of toe clipping on frogs with Bayesian statistics. J. Appl. Ecol. **41**: 780-786.

Moosman, D.L., Moosman, P.R.J. (2006): Subcutaneous movements of Visible Implant Elastomers in wood frogs (*Rana sylvatica*). Herpetol. Rev. **37**: 300-301.

Osbourn, M.S., Hocking, D.J., Conner, C.A., Peterman, W.E., Semlitsch, R.D. (2011): Use of fluorescent visible implant alphanumeric tags to individually mark juvenile Ambystomatid Salamanders. Herpetol. Rev. **42**: 43-47.

Pollock, K.H., Nichols, J.D., Brownie, C., Hines, J.E. (1990). Statistical inference for capture recapture experiments. Wild. Monogr. **107**: 1-97.

Scherer, R.D., Muths, E., Noon, B.R., Corn, P.S. (2005): An evaluation of weather and disease as causes of decline in two populations of boreal toads. Ecol. Appl. **15**: 2150-2160.

Seber, G.A.F. (1982): The estimation of animal abundance and related parameters. Macmillan, New York.

Seber, G.A.F. (1986): A review of estimating animal abundance. Biometrics **42**: 267-292.

Ursprung, E., Ringler, M., Jehle, R., Hödl, W. (2011): Toe regeneration in the neotropical frog *Allobates femoralis*. Herpetol. J. **21**: 83-86.

Waddle, J.H., Rice, K.G., Mazzotti, F.J., Percival, H.F. (2008): Modeling the effect of toe clipping on treefrog survival: beyond the return rate. J. Herpetol. **42**: 467-473.

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Morphological variation of the newly confirmed population of the Javelin sand boa, *Eryx jaculus* (Linnaeus, 1758) (Serpentes, Erycidae) in Sicily, Italy

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Abstract. The presence of the Javelin sand boa in Sicily has recently been confirmed. Here the morphological characters and sexual dimorphism of the Sicilian population of *Eryx jaculus* are presented. Seven meristic and six metric characters in 96 specimens from Sicily were examined. The results show that tail length, snout-vent length, the distance between nostrils and the number of ventral and subcaudal scales are different between sexes. The characters found in the Sicilian population of the Javelin sand boa resemble those of the African population (ssp. *jaculus*) rather than the Eurasian population (ssp. *turcicus*), but biomolecular studies are necessary to understand its taxonomic identity.

Keywords. *Eryx jaculus*, Serpentes, morphological variation, folidosis.

The Javelin sand boa *Eryx jaculus* (Linnaeus, 1758), a member of the family Erycidae, is found in the southern Balkans, Middle East and North Africa (Sindaco et al., 2013). Two morphologically defined subspecies are usually recognized for this species. The nominate subspecies is present in North Africa and the ssp. *turcicus* (Olivier, 1801) is found in the Balkans and the Middle East (Tokar and Obst, 1993; Sindaco et al., 2013; Geniez, 2015). According to Tokar and Obst (1993), the Transcaucasian ssp. *familiaris* Eichwald, 1831 could be synonymous with *E. j. turcicus*. Morphological variations of *E. jaculus* have been reported both at a global (Tokar, 1991) and a local scale (Cattaneo, 1984, 2005, 2010; Rhadi et al., 2015; Zarrintab et al., 2017; Eskandarzadeh et al., 2018).

Recently, the presence of *E. jaculus* has been confirmed in a small area of southern Sicily, in Italy (Insacco et al., 2015). Knowledge of the Javelin sand boa in Sicily is currently limited to scant preliminary information on the population's distribution, morphology, habitat and diet (Insacco et al., 2015; Faraone et al., 2017a, b).

In this paper the first comprehensive report of the morphological characters and sexual dimorphism in the Sicilian population of *E. jaculus* is presented.

Sampling was carried out within the currently known geographic range of the Javelin sand boa in South Central Sicily, within an area between Palma di Montechiaro (province of Agrigento) and Butera (province of Caltanissetta) (see Insacco et al., 2015; Faraone et al., 2017b). A total of 55 adult (30 males; 25 females) and 41 juvenile (21 males;

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17 females; 3 undetermined) specimens were examined between August 2014 and September 2018. Among them, 47 specimens were collected during active searches at night, 12 specimens were rescued from abandoned cisterns and the tunnel greenhouse in which they were trapped, four were found dead inside cisterns, one had been killed by domestic cats and 32 specimens were roadkilled.

Sex was determined by examining external sexual features (spurs, tail shape) and, mainly in young snakes, also by cloacal popping. The reliability of external features for sexual determination was also confirmed by cloacal probing in dead specimens. In absence of detailed references, *E. jaculus* with a snout-vent length < 270 mm (see Itescu et al., 2018; Eskandarzadeh et al., 2018) were considered as juveniles.

Seven meristic characters were considered: the number of scales between the eyes (BE), around the eye (RE), posterior to the internasal (PIN), between the eye and the nasal (BEN), the rows of scales between the eye and the supralabial area (BES), the ventrals (VS) and the subcaudals (ScdS). Six metric characters were also recorded: snout-vent length (SVL), tail length (TL), the distance between the eyes (DBE), the distance between the posterior edge of the eye and the tip of the snout (DES), the distance between the nostrils (DBN) as well as body weight (W). For bilateral characters, only the right side was recorded. Biometric characters were measured using a digital caliper accurate to 0.01 mm, except for SVL, which was measured with a mm ruler, and body weight (W), which was measured only on live individuals using a digital scale with 1 g precision.

In order to remove the information related to size, each metric variable was transformed using the formula:

$$Z = Y_i (SVL/ SVL_i)^b$$

following Lleonart et al. (2000), where Z is the transformed value of the variable Y, which is the variable affected by size, represented as the snout-vent length (SVL), and b is the slope of the linear regression between logY and logSVL. The data were checked for parametric assumptions using Levene's test and the Shapiro-Wilk test. For each variable, parametric (independent t-test) or non-parametric (Mann-Whitney U test) analysis was conducted, with a level of significance at 0.05. Differences between the sexes were checked by using the Principal Component Analysis (PCA), applied to the correlation matrix. Body weight (W) was not included in PCA, because its value can vary greatly depending on the condition of each specimen (pregnancy, decay, freshly ingested prey, etc.). SVL and the characters with a P value greater than 0.05 were also excluded.

Color morph was classified according to the three types reported by Tokar (1991) and Tokar and Obst (1993). The 'discrete' morph is characterized by a reduced dorsal pattern, with narrow transverse dorsal bars and small scattered spots on the sides. In the 'standard' morph, the dorsal pattern is more extensive than in the 'discrete' morph and the dorsal blotches are spaced by light areas of similar thickness and are not in contact with each other. The 'negative' morph is characterized by a greater development of the dark pattern, while the light parts are reduced to narrow suboval blotches on the back.

The descriptive statistic and a comparison between sexes were made for adults (Table 1). Significant intersexual differences in five characters were found: the num-

Table 1. Descriptive statistics and univariate comparisons between sexes of the Sicilian population of *Eryx jaculus*. The first five characters are in millimetres, the sixth in grams and the others are meristic. Significant differences (P<0.05) are shown in bold. *= transformed values.

	Males			Females			T	U	P
	Mean ± SD	Range	n	Mean ± SD	Range	n			
SVL	342.1 ± 42.4	270.0 - 420.0	26	400.5 ± 86.1	270.0 - 580.0	23	3.2	-	**0.004**
*TL	40.7 ± 6.0	26.9 - 53.9	26	31.4 ± 6.2	20.7 - 45.9	23	**7.9**	-	**0.000**
*DES	7.1 ± 0.7	5.8 - 8.7	26	7.6 ± 1.2	5.9 - 10.5	21	1.8	-	0.086
*DBN	4.0 ± 0.4	3.3 - 4.7	26	4.2 ± 0.6	3.3 - 5.6	21	**2.4**	-	**0.019**
*DBE	7.0 ± 0.7	6.1 - 8.3	26	7.5 ± 1.1	5.5 - 10.0	21	-	165.0	0.194
W	36.9 ± 19.5	18 - 95	14	64.2 ± 49.6	15 – 204	16	-	76.0	0.135
VS	175.9 ± 2.8	170 - 182	26	181.9 ± 3.9	176 - 190	21	6.1	-	**0.000**
ScdS	25.2 ± 1.9	22 - 29	29	19.7 ± 1.4	17 - 22	23	11.8	-	**0.000**
PIN	2.6 ± 0.5	2 - 3	30	2.6 ± 0.5	2 - 3	24	-	357.0	0.958
BE	6.8 ± 0.6	6 - 8	30	6.8 ± 0.4	6 - 7	24	-	358.0	0.972
RE	9.4 ± 0.8	8 - 11	29	9.0 ± 0.7	8 - 10	22	-	238.5	0.126
BEN	3.0 ± 0	3 - 3	30	3.0 ± 0	3 - 3	24	-	-	-
BES	1.0 ± 0.2	1 - 2	30	1.0 ± 0	1 - 1	24	-	348.0	0.835

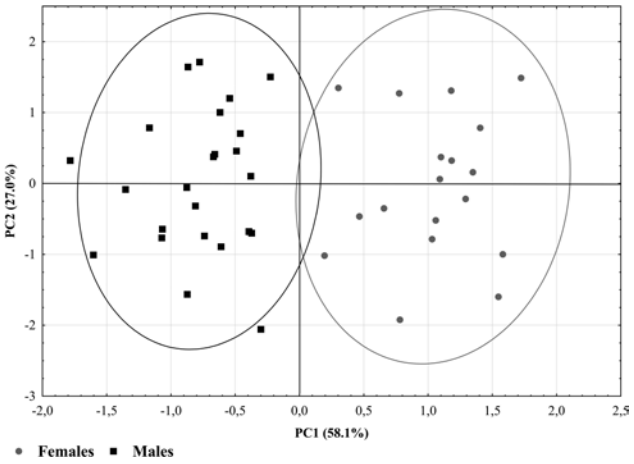

Fig. 1. Scatterplot of scores with the equiprobability ellipses at 95% projected on the first two principal components between sexes of the Sicilian population of *Eryx jaculus*.

Fig. 2. Examples of color and pattern variation in the Sicilian population of *Eryx jaculus*. (A) Adult, male (B) Adult, female (C) Juvenile, male (D) Ventral variation in three adult females.

ber of ventral scales and snout-vent length have higher values in females and the number of subcaudal scales, the distance between the nostrils and the tail length have higher values in males. The result of the Principal Components Analysis (PCA) shows that 85.1% of the variance is explained by the first two components (PC1 = 58.1%, PC2 = 27.0%). The scatterplot of scores with the equiprobability ellipses at 95% (Lagonegro and Feoli, 1985) projected on the first two principal components (Fig. 1) shows a clear separation between sexes on PC1, with males distributed within the negative values of the axis and the females within the positive values.

The correlation coefficients of the variables (Table 2) show that PC1 has a strong positive correlation with ventral scales (VS) and a negative correlation with subcaudal scales (ScdS) and tail length (TL).

The examined specimens showed different color patterns (Fig. 2). In most of the snakes, the 'standard' morph was identified, however in some individuals the dorsal dark blotches were so extended that they appeared similar to the 'negative' morph, yet these spots were not connected on the sides, so they were assigned to the 'standard' type. Seven snakes could generally be attributed to the 'negative' phenotype, but some small parts of their back looked like the 'standard' morph, similar to what was observed in some *E. jaculus* by Tokar and Obst (1993) and Werner (2016). Young individuals were generally lighter than adults, with a lower contrast in the dark pattern. No difference in dorsal color pattern was noticed between sexes. The dorsal base color is orange, sand or yellowish. The ventral, paraventral and some adjacent scales were usually pale yellowish orange (in only one large female it was a whitish-cream color). Ventral parts were adorned with small blackish spots. The orange color of the belly in a few cases extended to the subcaudal scales, which usually appeared whitish with a few scattered orange scales.

Table 2. Correlation coefficients of the variables for the first two factors used in the Principal Component Analysis (PCA).

Variables	PC 1	PC 2
TL	-0,81	0,52
DBN	-0,40	-0,88
VS	0,81	0,16
ScdS	-0,92	0,07

The results show clear intersexual differences in the Sicilian population of *E. jaculus*. The females usually reach a greater size in terms of snout-vent length and have a greater number of ventral scales than males, and males have a greater number of subcaudal scales, a longer tail and a greater distance between the nostrils. Significant intersexual differences in ventral scales have already been observed in this species (Tokar, 1991) but are not found in some Asian populations (Rhadi et al., 2015; Eskandarzadeh et al., 2018) nor in some other *Eryx* species (Al-Sadoon and Al-Otaibi, 2014; Eskandarzadeh et al., 2013).

The pattern of sexual dimorphism observed in the Sicilian population is also reported in other species of snakes (Marques et al., 2006, Pizzatto et al., 2007, Zanella and Cechin, 2010, Siqueira et al., 2013, Manjarrez et al., 2014). Larger females can provide more space for incubating eggs and embryos and more body reserves to

invest in reproduction (Bonnett et al., 1998) and generally, have a larger clutch size (Shine, 1993) and lower mortality in their offspring (Kissner and Weatherhead, 2005). Moreover, in various snake species the number of ventral scales corresponds to the number of dorsal vertebrae, which are generally more numerous in the larger sex (Shine, 2000). On the other hand, smaller males have greater agility during the search for females and a lower metabolic cost (Rivas and Burghardt, 2001). A larger tail and a greater number of subcaudal scales in males are linked to the positioning of the hemipenes within the tail and produce better performance in the courtship phase (King, 1989; Luiselli, 1996; Shine et al., 1999). The males of Sicilian *E. jaculus* also have a relatively larger internasal distance (DBN) than the females. Sexual dimorphism in head shape is a phenomenon known in various snake species and is often linked to a different trophic niche between males and females (Camilleri and Shine, 1990; Vincent et al., 2004). This result deserves to be investigated using appropriate tools (Geometric morphometric analysis) and its relation to the dietary habits of this population, which is currently being studied, should be taken into account.

The three dorsal patterns proposed by Tokar (1991) should be used with caution. It is sometimes difficult to apply these patterns to specimens with mixed phenotypes (see Tokar and Obst, 1993) that may be locally common (Werner, 2016).

The subspecies of *E. jaculus,* proposed by Tokar and Obst (1993), is currently accepted (Sindaco et al., 2013; Werner, 2016). The criteria proposed by Tokar and Obst (1993) are based on an in-depth revision of the morphological variation of *E. jaculus* within its geographical range (Tokar, 1991) and have been confirmed by recent research on a local scale (Eskandarzadeh et al., 2018; Rhadi et al., 2015; Zarrintab et al., 2017).

Comparing the characters reported by Tokar and Obst (1993) with those of the examined specimens in this study, a higher resemblance between the Sicilian population and the North African nominal subspecies, rather than with the Eurasian ssp. *turcicus*, was detected. In the Sicilian population, as well as in *E. j. jaculus*, there are usually seven scales between the eyes (74.7%; n = 95) (usually six in *E. j. turcicus*) and three postinternasal scales (64.6%; n = 96) (usually two in *E. j. turcicus*). Moreover, the Sicilian ventral scales range (170-190) overlaps with both the nominal subspecies (170-198) and ssp. *turcicus* (161-200).

Furthermore, a more frequent presence of the 'standard' phenotype (92.7%; n = 96) is observed with respect to the 'negative' and 'discrete' dorsal patterns, which are very common in European and Asian populations respectively (see also Tokar, 1991). The morphological affinities between the Sicilian and African populations reported here has only a preliminary value and, obviously, a biomolecular approach is necessary in order to properly assess the phylogeographic and taxonomic structure of the Javelin sand boa.

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REFERENCES

Al-Sadoon, M.K., Al-Otaibi, F.S. (2014): Ecology of the sand boa, *Eryx jayakari* in Riyadh Region of Saudi Arabia. Saudi J. Biol. Sci. **21**: 391-393.

Bonnet, X., Shine, R., Naulleau, G., Vacher-Vallas, M. (1998): Sexual dimorphism in snakes: Different reproductive roles favour different body plans. Proc. R. Soc. Lond. B. Biol. Sci. **265**: 179-183.

Camilleri, C., Shine, R. (1990): Sexual dimorphism and dietary divergence: differences in trophic morphology between male and female snakes. Copeia **3**: 649-658.

Cattaneo, A. (1984): *Podarcis erhardii naxensis* ad Antiparos (Cicladi centrali) e note di campagna sull'erpetocenosi dell'isola (Reptilia). Atti Soc. ital. Sci. nat. Mus. civ. Stor. nat. Milano **125**: 245-254.

Cattaneo, A. (2005): Osservazioni sull'erpetofauna dell'isola greca di Kos (Sporadi meridionali) con un inedito caso di simpatria microinsulare fra due specie affini di Colubridi: *Hierophis caspius* (Gmelin) e *Hierophis jugularis* (L.). Atti Mus. Stor. nat. Maremma, Grosseto **21**: 79-91.

Cattaneo, A. (2010): Note eco-morfologiche su alcune specie ofidiche egee, con particolare riferimento alle popolazioni delle Cicladi centro-orientali (Reptilia). Naturalista sicil., **34**: 319-350.

Eskandarzadeh, N., Darvish, J., Rastegar-Pouyani, E., Ghassemzadeh, F. (2013): Reevaluation of the taxonomic status of sand boas of the genus *Eryx* (Daudin, 1803) (Serpentes: Boidae) in northeastern Iran. Turk. J. Zool., **37**: 1-9.

Eskandarzadeh, N., Rastegar-Pouyani, N., Rastegar-Pouyani, E., Todehdehghan, F., Rajabizadeh, M. (2018): Sexual Dimorphism in the Javelin Sand Boa, *Eryx jaculus* (Linnaeus, 1758) (Serpentes: Erycidae), from Western Iran. Curr. Herpetol. **37**: 88-92.

Faraone, F.P., Barra, S.A., Giacalone, G., Chiara, R., Russotto, S., Lo Valvo, M. (2017a): First observations of oophagy in a wild population of the sand boa (*Eryx jaculus*). Herpetol. Bull. **142**: 48-49.

Faraone, F.P., Chiara, R., Barra, S.A., Giacalone, G., Lo Valvo, M. (2017b): Nuovi dati sulla presenza di *Eryx jaculus* (Linnaeus, 1758) in Sicilia. In: Atti XI Congresso Nazionale della Societas Herpetologica Italica, Trento 2016, pp. 75-79. Menegon, M., Rodriguez-Prieto, A., Deflorian, M.C., Eds, Ianieri Edizioni, Pescara.

Geniez, P. (2015): Serpents d'Europe, d'Afrique du Nord et du Moyen-Orient. Guide Delachaux. Paris, Delachaux et Niestlé S.A.

Insacco, G., Spadola, F., Russotto, S., Scaravelli, D. (2015): *Eryx jaculus* (Linnaeus, 1758): A new species for the Italian herpetofauna (Squamata: Erycidae). Acta Herpetol. **10**: 149-153.

Itescu, Y., Schwarz, R., Donihue, C.M., Slavenko, A., Roussos, S.A., Sagonas, K., Valakos E.D., Foufopoulos J., Pafilis P., Meiri, S. (2018): Inconsistent patterns of body size evolution in co-occurring island reptiles. Glob. Ecol. Biogeogr. **27**: 538-550.

King, R.B. (1989): Sexual dimorphism in snake tail length: sexual selection, natural selection, or morphological constraint? Biol. J. Linn. Soc. **38**: 133-154.

Kissner, K.J., Weatherhead, P.J. (2005): Phenotypic effects on survival of neonatal Northern Watersnakes *Nerodia sipedon*. J. Anim. Ecol. **74**: 259-265.

Lagonegro, M., Feoli, E. (1985): The use of ellipses of equal concentration to analyse ordination vegetation patterns. Studia Geobot. **5**: 143-165.

Lleonart, J., Salat, J., Torres, G.J. (2000): Removing allometric effects of body size in morphological analysis. J. Theoret. Biol. **205**: 85-93.

Luiselli, L. (1996): Individual success in mating balls of the grass snake, *Natrix natrix*: size is important. J. Zool. **239**: 731-740.

Manjarrez, J., Contreras-Garduño J., Janczur M.K. (2014): Sexual size dimorphism, diet, and reproduction in the Mexican garter snake, *Thamnophis eques*. Herp. Cons. Biol. **9**(1): 163-169.

Marques, O.A.V., Sawaya, R.J., Stender-Oliveira, F., Franca, F.G.R. (2006): Ecology of the colubrid snake *Pseudablabes agassizii* in southeastern south America. Herpetol. J. **16**: 37-45.

Pizzato, L.P., Almeida-Santos, S.M., Marques, O.A.V. (2007): Biologia reprodutiva das serpentes brasileiras. In: Herpetologia no Brasil II, p. 201-221. Nascimento, L.B., Oliveira, M.E., Eds, Sociedade Brasileira de Herpetologia, Belo Horizonte.

Rhadi, F.A., Rastegar-Pouyani, N., Karamiani, R., Mohammed, R.G. (2015): Taxonomic status of sand boas of the genus *Eryx* (Daudin, 1803) (Serpentes: Boidae) in Bahr Al-Najaf Depression, Al-Najaf Province, Iraq. Iran. J. Anim. Biosyst. **11**: 149-156.

Rivas, J.A., Boughardt, G.M. (2001): Understanding sexual size dimorphism in snakes: wearing the snake's shoes. Anim. Behav. **62**: F1-F6.

Shine, R., (1993): Sexual Dimorphism in Snakes. In: Snakes: Ecology & Behavior, pp. 49-86. Seigel, R.A., Collins J.T., Eds, McGrill-Hill, New York.

Shine, R. (2000): Vertebral numbers in male and female snakes: the roles of natural, sexual and fecundity selection. J. Evolution. Biol. **13**:455-465.

Shine, R.G., Olsson, M.M., Moore, I.T., LeMaster, M.P., Mason, R.T. (1999): Why do male snakes have longer tails than females? Proc. R. Soc. Lond. B. Biol. Sci. **266**: 2147-2151.

Sindaco, R., Venchi, A., Grieco, C. (2013): The Reptiles of the Western Palearctic. 2. Annotated checklist and distributional atlas of the snakes of Europe, North Africa. Middle East and Central Asia, with an update to the, Vol 1, Edizioni Belvedere, Latina.

Siqueira, D.M., Nascimento, L.P., Montingelli, G.G., Santos-Costa, M.C. (2013): Geographical variation in the reproduction and sexual dimorphism of the Boddaert's tropical racer, *Mastigodryas boddaerti* (Serpentes: Colubridae). Zoologia **30**: 475-481.

Tokar, A.A. (1991): A revision of the subspecies structure of Javelin sand boa, *Eryx jaculus* (Linnaeus, 1758) (Reptilia, Boidae). Herpetological Researches **1**: 18-41.

Tokar, A., Obst, F.J. (1993): *Eryx jaculus* - Westliche Sandboa. In: Handbuch der Reptilien und Amphibien Europas, Band 3/I., Schlangen (Serpentes) I, pp. 35-54. Böhme,W., Ed., Aula-Verlag, Wiesbaden.

Vincent, S.E., Herrel A., Irschick, D.J. (2004): Sexual dimorphism in head shape and diet in the cottonmouth snake (*Agkistrodon piscivorus*). J. Zool., Lond. **264**: 53-59.

Werner, Y.L. (2016): Reptile life in the land of Israel. Edition Chimaira, Frankfurt am Main.

Zanella, N., Cechin, S.Z. (2010): Reproductive biology of *Echinanthera cyanopleura* (Serpentes: Dipsadidae) in southern Brazil. Zoologia **27**: 30-34.

Zarrintab, M., Milto, K.D., Eskandarzadeh, N., Zangie, B., Jahane, M., Gholi Kamif, H., Rastegar-Pouyani, N., Rastegar-Pouyani, E., Rajabizadeh, M. (2017): Taxonomy and distribution of sand boas of the genus *Eryx* Daudin, 1803 (Serpentes: Erycidae) in Iran. Zool. Middle East **63**: 117-129.

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Variability in the dorsal pattern of the Sardinian grass snake (*Natrix natrix cetti*) with notes on its ecology

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Abstract. The Sardinian grass snake (*Natrix natrix cetti*) is a Critically Endangered snake endemic to Sardinia (Italy), for which information is still scarce. In the present work, we report information obtained from 36 observations of *N. n. cetti* performed in different areas of the Island. Three different colorations were mainly observed and darker snakes were in general males and big adults; the only juvenile found showed a complete different dorsal colouration. Snakes were observed active during day-time and often far from the aquatic habitats.

Keywords. Melanism, abundism, mimicry, predator avoidance, island.

The Sardinian grass snake (*Natrix natrix cetti*) is a Critically Endangered snake endemic to Sardinia, for which we still have very limited information (European Reptile & Amphibians Specialist Group, 1996; Vanni and Cimmaruta, 2010). A recent phylogenetic analysis performed on the *Natrix natrix* complex grouped the Sardinian grass snake together with the Corsican subspecies (*N. n. corsa*) into a distinct genetic group, which was likely promoted by their isolation occurred during glaciations (Fritz et al., 2012; Kindler et al., 2013). According to the most recent phylogenetic study, the western grass snakes belong to a new clade called *Natrix helvetica* (Kindler et al., 2017); however, in this study no samples from Sardinia have been analysed. Its elusiveness could be one of the causes of the few data available on its distribution, ecology and biology (Lunghi et al., 2016; Lunghi et al., 2018; Vanni and Cimmaruta, 2010). It is known that *N. n. cetti* can exploit different environments, from dry rocky areas to wetlands, rivers and even caves, and that the species is relatively widespread on the Island (Capula et al., 1994; de Pous et al., 2012; Lanza 1986; Mulargia et al., 2018; Salvi and Bombi, 2010). However, the presence of the Viperine snake (*Natrix maura*), as potential competitor, seems to affect its distribution (Stefani 1983; Vanni and Cimmaruta 2010).

Adults of *N. n. cetti* differ morphologically from the continental subspecies because of the lack of the typical light "collar", and the smaller size (Speybroeck et al., 2016; Vanni and Cimmaruta, 2010). Stefani (1983) reports for *N. n. cetti* a typical light greyish background

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colour, a characteristic that should be of help in distinguishing the Sardinian grass snake from the Corsican one, being the latter characterised by a dark greenish background. Recently, abundistic (i.e., dorsum characterized by enlarged dark stripes) and melanotic (i.e., nearly completely black coloration) individuals of *N. n. cetti* were observed (Lunghi et al., 2016), thus increasing the variability of the dorsal colouration known for this subspecies. Furthermore, as far as we know, no information exists on juveniles.

Here we report observations of *Natrix natrix cetti* gathered during three years fieldwork. All captured snakes were measured, and information on dorsal pattern and habitat were recorded. The observation of one juvenile is also reported.

From 2016 to 2018, we conducted herpetological field observations focusing on Eastern and Southern Sardinia (see Table 1; coordinates are not reported for species safeguard, see Lunghi et al., 2019). Repeated linear transects, based on VES (Visual Encounter Survey), were carried out. The surveys were performed by day (9 a.m. – 5 p.m.) throughout the year, but the most in spring (Jan-Mar = 9.1%; Apr-Jun = 70.6%; Jul-Sep = 14.4%; Oct-Dec = 5.9%; total surveys = 153). For each snake we recorded: locality, elevation and time of the observation, habitat typology and dorsal background colour pattern (greyish, greenish-brownish, melanotic) (Fig. 1). Captured snakes were measured (total length) and sexed. Total length was measured photographing the snakes on a plasticised millimetre paper; snakes' length was extrapolated using the program ImageJ. Sex was assessed combining visual inspection of the morphology of the snake (proportion of the head and tail) and number of sub-caudal scales (Vanni and Cimmaruta, 2010). Head pattern was used for individually snake recognition (Sacchi et al., 2016; Vaughan, 1999). A Generalized Linear Model (R software with nlme package; Pinheiro et al., 2016; R Development Core Team 2018) was used to assess whether snake coloration is related to sex, elevation, time of survey and total length (TL). We used colouration as dependent variable, while sex, TL, time of survey and elevation as independent variables; year and transect identity as random factors. To use col-

Fig. 1. The four different dorsal colourations of *Natrix natrix cetti* reported in this study: A) greyish, B) greenish-brownish, C) black, D) the juvenile. (Photos A and B by M. Di Nicola; C by E. Lunghi; D by S. Giachello).

Table 1. Data of the captured individuals of *Natrix natrix cetti*: sex, total length (TL), dorsal background colour (1 = greyish, 2 = greenish-brownish, 3 = melanotic), elevation (m a.s.l.), time at which snakes were observed (24h), province, year, transect identity and the mountain complex. *individuals captured twice.

Sex	TL (cm)	Colour	Elevation	Time	Province	Year	Transect	Mountain
Female	65.7	2	341	16:15	Carbonia-Iglesias	2017	Barega1	Barega
Female	44	1	474	14:32	Cagliari	2017	Cagliari3	Sette Fratelli
Female	72.1	2	515	10:30	Cagliari	2016	Cagliari2	Sette Fratelli
Female	74	1	534	10:08	Cagliari	2018	Cagliari2	Sette Fratelli
Female	53.1	1	535	11:10	Cagliari	2017	Cagliari2	Sette Fratelli
Female	62.3	2	539	12:00	Cagliari	2017	Cagliari2	Sette Fratelli
Female	61.8	2	541	14:01	Cagliari	2017	Cagliari2	Sette Fratelli
Male	54.3	2	633	11:02	Cagliari	2017	Cagliari2	Sette Fratelli
Female	59	2	634	10:36	Cagliari	2017	Cagliari2	Sette Fratelli
*Female	63	2	638	16:21	Cagliari	2017	Cagliari2	Sette Fratelli
Female	57.9	2	670	15:45	Cagliari	2016	Cagliari2	Sette Fratelli
Male	64.7	2	689	14:05	Cagliari	2017	Cagliari2	Sette Fratelli
Male	60.1	3	706	13:45	Cagliari	2016	Cagliari2	Sette Fratelli
Female	58.8	2	730	16:42	Cagliari	2016	Cagliari2	Sette Fratelli
*Female	63	2	736	16:40	Cagliari	2016	Cagliari2	Sette Fratelli
Female	63	1	792	15:58	Cagliari	2018	Cagliari2	Sette Fratelli
Male	46.4	2	830	15:50	Cagliari	2017	Cagliari2	Sette Fratelli
Female	59.8	2	863	13:20	Cagliari	2017	Cagliari3	Sette Fratelli
Male	55.5	1	1029	14:45	Nuoro	2018	M_albo3	Monte Albo

ouration as dependent variable, we ascribed an ascending order to the different colourations, going from the lightest (greyish = 1) to the darkest (greenish = 2) (Table 1). In this analysis we excluded the juvenile, the single melanotic individual, and, for the individual captured twice (see below) only the first observation.

In total we performed 153 surveys on 3 different areas (Barega, no. of transects = 1, total surveys = 5; Monte Albo, no. of transects = 11, total surveys = 102; Sette Fratelli, no. of transects = 2, total surveys = 46) and we observed 19 *Natrix natrix cetti*; seventeen were adults. Most of the observations (17) were performed on the Sette Fratelli Mountain and only an adult female was captured twice. The greenish-brownish (n = 12) coloration resulted to be the most common, followed by the greyish (n = 5) and the melanotic (n = 1) (Fig. 1A-C). The observed juvenile (TL = 22 cm) showed a complete different dorsal colouration: the background colour was black, the stripes white and a white collar was also present (Fig. 1D). Snakes were observed at an elevation between 341 and 1029 m a.s.l. Two individuals were found in small temporary water bodies, one in a mine, while the others 15 in rocky areas sometimes even more than 1 km far from the nearest water body.

Snakes' coloration significantly correlated only with total length ($F_{1,8}$ = 8.94, P = 0.017); the darker (greenish) coloration was most frequently observed in the longer snakes (Fig. 2). No significant effect was observed for other variables (sex, $F_{1,8}$ = 2.00, P = 0.195; elevation, $F_{1,8}$ = 2.90, P = 0.127; time of survey, $F_{1,8}$ = 1.69, P = 0.229).

During our study, a female with a wounded eye was captured twice, the first on May 2016 and the second in April 2017; in both occasions this female was active at about the same time (16:41 and 16:21 respectively). This individual was recaptured almost 90 m far from the site of first encounter (difference in altitude 98 m).

The Sardinian grass snake is one of the least studied Italian species. Elusiveness, rather than its potential rarity, could be the reasons for the few existing studies on this species (Vanni and Cimmaruta, 2010); indeed, although being made of just 19 records, our dataset is one of the richest available. The dorsal coloration of *Natrix natrix cetti* seems to be more variable than previously reported; indeed, most of our observations highlighted the high frequency of the greenish-brownish coloration, a characteristic that should be typical of the Corsican grass snake (Stefani, 1983).

Because of our dataset paucity, we cannot provide any assumptions on the causes supporting the high pattern differentiation observed in this subspecies (but see also Lunghi et al., 2016). Further studies are needed to assess whether factors are influencing the observed vari-

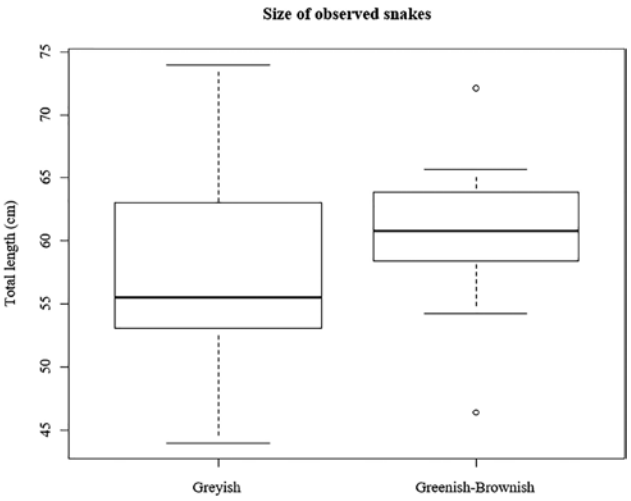

Fig. 2. Boxplots indicating differences in *Natrix natrix cetti* body size (total length) per dorsal coloration (only for greyish and greenish background colorations). Individuals used here are the same used in the GLM analysis (see Table 1). Dark bar inside boxes represents the median, boxes represent the range between 1st and 3rd quartile, whiskers represent the variability outside the upper/lower quartile (box), circles represent outliers.

ability and, if darker coloration may be beneficial for snakes' longevity (Castella et al., 2013; Clusella Trullas et al., 2007; Fulgione et al., 2015; Stevens et al., 2009; Zuffi 2008). The juvenile highlighted ontogenetic variability in dorsal coloration in *N. n. cetti*.

Our study agrees with Stefani (1983) and Lanza (1986) considering *N. n. cetti* not strictly related to water bodies. This likely allows the snake to exploit further habitats, increasing prey availability and lowering competition with *Natrix maura* (Lunghi et al., 2018; Stefani, 1983; Vanni and Cimmaruta, 2010). Our study was carrried out in day-time (see also Lanza, 1986) but we cannot exclude nocturnal activity as reported by Capula et al. (1994).

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REFERENCES

Capula, M., Rugiero, L., Luiselli, L. (1994): Ecological observations on the Sardinian grass snake, *Natrix natrix cetti*. Amphibia-Reptilia **15**: 221-227.

Castella, B., Golay, J., Monney, J.-C., Golay, P., Mebert, K., Dubey, S. (2013): Melanism, body condition and elevational distribution in the asp viper. J. Zool. **290**: 273-280.

Clusella Trullas, S., van Wyk, J.H., Spotila, J.R. (2007): Thermal melanism in ectotherms. J. Therm. Biol. **32**: 235-245.

de Pous, P., Speybroeck, J., Bogaerts, S., Pasmans, F., Beukema, W. (2012): A contribution to the atlas of the terrestrial herpetofauna of Sardinia. Herpetol. Notes **5**: 391-405.

European Reptile & Amphibians Specialist Group (1996): *Natrix natrix* ssp. *cetti*. The IUCN Red List of Threatened Species **1996**: e.T14364A4436077.

Fritz, U., Corti, C., Päckert, M. (2012): Mitochondrial DNA sequences suggest unexpected phylogenetic position of Corso-Sardinian grass snakes (*Natrix cetti*) and do not support their species status, with notes on phylogeography and subspecies delineation of grass snakes. Org. Divers. Evol. **12**: 71-80.

Fulgione, D., Lega, C., Trapanese, M., Buglione, M. (2015): Genetic factors implied in melanin-based coloration of the Italian wall lizard. J. Zool. **296**: 278-295.

Kindler, C., Böhme, W., Corti, C., Gvoždík, V., Jablonski, D., Jandzik, D., Metallinou, M., Široký, P., Fritz, U. (2013): Mitochondrial phylogeography, contact zones and taxonomy of grass snakes (*Natrix natrix*, *N. megalocephala*). Zool. Scr. **42**: 458-472.

Kindler, C., Chèvre, M., Ursenbacher, S., Böhme, W., Hille, A., Jablonski, D., Vamberger, M., Fritz, U. (2017): Hybridization patterns in two contact zones of grass snakes reveal a new Central European snake species. Sci. Rep. **7**: 7378.

Lanza, B. (1986): I Rettili e gli Anfibi. In: L'ambiente naturale in Sardegna (Elementi di base per la conoscenza e la gestione del territorio), pp. 289-321. Camarda, I., Falchi, S. Nudda, G., Eds, Carlo Delfino Editore, Sassari.

Lunghi, E., Corti, C., Manenti, R., Ficetola, G.F. (2019): Consider species specialism when publishing datasets. Nat. Ecol. Evol. **3**: 319.

Lunghi, E., Deschandol, F., Cornago, L., Cogoni, R. (2016): Dark coloration in Sardinian grass snakes (*Natrix natrix cetti*). Herpetol. Bull. **137**: 28-29.

Lunghi, E., Mascia, C., Mulargia, M., Corti, C. (2018): Is the Sardinian grass snake (*Natrix natrix cetti*) an active hunter in underground environments? Spixiana **41**: 160.

Mulargia, M., Corti, C., Lunghi, E. (2018): The herpetofauna of the Monte Albo, Sardinia (Italy). Russ. J. Herpetol. **25**: 172-176.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Team, R.C. (2016): nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-128.

R Development Core Team (2018): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/. [*accessed 28 September 2018*]

Sacchi, R., Scali, S., Mangiacotti, M., Sannolo, M., Zuffi, M.A.L. (2016): Digital identification and analysis. In: Reptile Ecology and Conservation, pp. 59-72. Kenneth Dodd, C.J., Eds, Oxford University Press, Oxford.

Salvi, D., Bombi, P. (2010): Reptiles of Sardinia: updating the knowledge on their distribution. Acta Herpetol. **5**: 161-177.

Speybroeck, J., Beukema, W., Bok, B., Van Der Voort, J., Velikov, I. (2016): Field guide to the Amphibians & Reptiles of Britain and Europe, Bloomsbury, London.

Stefani, R. (1983): La Natrice del Cetti. Biogeographia **8**: 745-755.

Stevens, M., Winney, I.S., Cantor, A., Graham, J. (2009): Outline and surface disruption in animal camouflage. Proc. R. Soc. Biol. Sci. Ser. B **276**: 781-781.

Vanni, S., Cimmaruta, R. (2010): *Natrix cetti* Gené, 1839. In: Fauna d'Italia. Reptilia, pp. 538-545. Corti, C., Capula, M., Luiselli, L., Razzetti, E. Sindaco, R., Eds, Edizioni Calderini de Il Sole 24 Ore Editoria Specializzata S.r.l., Bologna.

Vaughan, R. (1999): Provisional results from study of facial features as a means of individual identification in *Natrix natrix*. Br. Herpetol. Soc. Bull. **68**: 39-46.

Zuffi, M.A.L. (2008): Colour pattern variation in populations of the European Whip snake, *Hierophis viridiflavus*: does geography explain everything? Amphibia-Reptilia **29**: 229-233.

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Estimating abundance of the Stripeless tree-frog *Hyla meridionalis* by means of replicated call counts

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Abstract. The Stripeless tree-frog *Hyla meridionalis* reaches its eastern-most European distributional limit in NW Italy, and specifically in the Cinque Terre National Park. Here for two consecutive years, we estimated tree-frog population abundance by call surveys at 24 sites. Data were analysed in the framework of N-mixture open population models based on repeated counts of calling males. The results obtained by this statistical approach were effective in estimating population size together with annual recruitment and survival. The tree-frog male population size remained constant between years and site abundance was inversely related with altitude. On the bases of these findings, our application of N-mixture models to tree-frog calling males was successful and is a promising cost-effective method to obtain long-term monitoring data on this species over large geographic areas.

Keywords. Abundance estimation, call surveys, Cinque Terre National Park, detection probability, N-mixture models.

The Stripeless tree-frog *Hyla meridionalis* Boettger, 1874 is found in North-western Africa (Algeria, Morocco and Tunisa), South-western Europe (Portugal, Spain, South France and North-Western Italy) and the Canary and Balearic Islands (Sillero, 2010). On a portion of its distribution range the species is considered introduced, i.e., Canary and Balearic Islands (Sillero et al., 2014), and it is also possibly introduced for other European regions (Recuero et al., 2007). In Italy, the Stripeless tree-frog is common along the Mediterranean coast of Liguria (NW Italy), from the Province of Imperia to the province of La Spezia (Salvidio, 2007). Apart from morphometric and distributional data (Salvidio, 2007), little is known about the abundance and dynamics of Stripeless tree-frog populations in Italy, and quantitative data on populations size should be obtained to assess the species status and its ecological requirements, in particular near the species distribution limits, where a high population fragmentation is expected (Gaston, 2003).

Although photo-identification of Stripeless tree-frogs is possible (Crovetto unpublished data), the animals are arboreal and highly secretive during daytime. The use of PVC pipes may increase the probability of detection of tree-frogs (do Vale et al., 2018), however, in the CTNP the majority of the species' reproductive habitats are on private lands, and thus are not freely accessible (Romano et al., 2014). Therefore, the monitoring technique selected to estimate population size was based on nocturnal auditory surveys of calling males, because of the species highly distinctive mating call (Schneider, 1974; Márquez et al., 2005). Call survey is a relatively efficient technique for evaluating the distribution and diversity of anurans (Dorcas et al., 2009). Therefore, calling surveys are frequently used in large-scale amphibian monitoring programmes (e.g., Anthony, 2002; Weir and Mossman, 2005; Weir et al., 2005, 2009). However, the use of call surveys for estimating population abundances and trends suffers of the same problematic issues recognized in the case of repeated counts of individuals, because the detectability of anuran calling males is < 1 (i.e., not all males are calling in the same night; Schmidt and Pellet, 2005. Moreo-

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ver, anuran mating call activities display high variation in response to biotic and abiotic factors, that usually remain unknown and difficult to model (Royle and Link, 2005; Droege and Eagle, 2009). In fact, using raw counts of calling males or even scores derived from abundance indexes (i.e., indexes that group calling males by classes of relative abundance; Weir and Mossman, 2005) without accounting for detection probability may lead to relevant bias in abundance and trend estimates (Schmidt, 2004; Mazerolle et al., 2007). Therefore, to reliably estimate population abundance, the information derived from raw counts of calling males should always be corrected for species-specific detection probabilities (Schmidt and Pellet, 2005; Royle and Link, 2005). Recently, specific modelling approaches have been proposed for estimating anuran population abundances from the count of anuran calling indexes taking into account detection probabilities (Royle, 2004a; Royle and Link, 2005).

This study aimed to estimate the abundance of Stripeless tree-frog males together with some demographic parameters and ecological requirements in Italy, at the eastern limit of the species distribution. Moreover, we tried to establish a cost-effective monitoring protocol to provide future population trends. Because of the relatively small number of tree-frog males recorded per site, we had the opportunity to apply the open population generalization of Royle's (2004b) N-mixture model (Dail and Madsen, 2011) to count data derived from call surveys.

The eastern-most limit of the species' range in Europe is the village of Riomaggiore (Province of La Spezia), in the Cinque Terre National Park (CTNP), a protected area where the Stripeless tree-frog reproduces in streams and in artificial water tanks used for irrigation (Salvidio, 2007; Romano et al., 2014). In this area water streams display short and steep courses, with relatively long summer drying periods, due to the lack of precipitations (Olivari et al., 2013). Among many possible land use of rural areas, agriculture is the only one in the CTNP, and from the sea level up to the hill tops vineyards and orchards are cultivated on strips of arable land, or "terraces", sustained by dry-stone walls. Irrigation is provided by means of water stored in tanks, often colonised by amphibians (Olivari et al., 2013; Romano et al., 2014). The survey sites were selected during both daytime and nocturnal preliminary surveys. During the day, streams and water reservoirs were inspected and selected as potential reproductive sites if adults, larvae or eggs of some amphibian species were observed. During the night, sites were located by perceiving the calls of Stripeless three-frog males. In total 24 sites were surveyed in the municipalities of Levanto, Monterosso and Riomaggiore (from West to East): 6 streams and 18 artificial water reservoirs in agricultural lands or urban settings (Table 1; Fig. S1).

All surveys began after sundown and after hearing the first tree-frog calls. In 2017 three nocturnal surveys were performed, from the end of March to May, by two operators that counted the number of males calling at each site during a two minute period. In 2018, three nocturnal surveys were performed, from the beginning of May to the beginning of June, with the same observers and procedure of 2017. In addition in 2018, a fourth survey was performed by a single operator that tallied calling males for 4 minutes. The asynchrony and the different tonalities of calls permitted to count with confidence the minimum number of males per site that, in all cases, was ≤ 6 (Table S1). All sites were surveyed during the same overcast or rainy night, but never during heavy showers that could hinder a clear hearing of frog calls. Four climatic variables were obtained from the meteorological station of Levanto: rainfall during the 24 h preceding the survey (RAIN), air temperature (TEMP), relative humidity (RH) and wind speed (WIND), recorded during the last hour of survey. These weather variables were selected, because they are known to influence anuran calling behaviour (e.g., Walls et al., 2011). Finally, for each site three variables were considered: altitude above the sea level (ELEV), a categorical variable for the municipality of the site (CITY) and if the water body was a stream or an artificial site (SITE).

Repeated count data were analysed using the Dail-Madsen (2011) model, which is a generalization of the Royle's (2004b) N-mixture model, capable of relaxing the closure assumption by considering the population closed to immigration/births and emigration/deaths during a short period (i.e., three/four survey nights performed each year), while considering the population demographically open between years, in a robust design-similar approach. This model estimates four parameters, two of which are in common with the Royle's (2004b) N-mixture original formulation: individual detection prob-

Table 1. Continuous variables included in the N-mixture open population models (Dail and Madsen, 2011) used to estimate *Hyla meridionalis* abundance, in the Cinque Terre National Park.

Variable	Description	Sample size (N)	Mean (SD)	min	max
ELEV	Site altitude (m) -	24	35.04 (29.66)	8	83
TEMP	Air temperature (°C)	7	17.14 (3.44)	12	21
WIND	Wind speed (m/s)	7	3.57 (1.27)	2	5
RH	Relative humidity (%)	7	66 (13.55)	45	85

Table 2. Candidate N-mixture open population models (Dail and Madsen, 2011) used to estimate *Hyla meridionalis* abundance, ranked by AICc. γ = recruitment rate; λ= initial site abundance; p = individual detection probability; ω = survival; AICc*WT* = model weights. In model list t stands for time dependence. For covariate abbreviations see table 1.

Model	Parameters	AICc	ΔAICc	AICc*WT*
λ(ELEV) p(.) ω(.) γ(.)	5	451.35	0.00	0.40
λ(ELEV) p(.) ω(ELEV) γ(.)	6	453.45	2.1	0.14
λ(CITY) p(.) ω(.) γ(.)	6	453.98	2.63	0.11
λ(ELEV) p(.) ω(CITY) γ(.)	6	454.95	3.61	0.07
λ(.) p(.) ω(.) γ(.)	4	454.96	3.61	0.07
λ(SITE) p(.) ω(.) γ(.)	5	455.05	3.70	0.06
λ(ELEV) p(.) ω(SITE) γ(.)	7	456.15	4.80	0.04
λ(.) p(TEMP) ω(.) γ(.)	5	456.81	5.46	0.03
λ(.) p(.) ω(SITE) γ(.)	5	456.93	5.59	0.02
λ(.) p(RH) ω(.) γ(.)	5	457.14	5.80	0.02
λ(.) p(WIND) ω(.) γ(.)	5	475.54	6.20	0.02
λ(.) p(RAIN) ω(.) γ(.)	5	458.03	6.69	0.01
λ(.) p(.) ω(ELEV) γ(.)	5	459.15	7.80	0.01
λ(.) p(.) ω(CITY) γ(.)	6	459.35	8.01	0.01
λ(.) p(t) ω(.) γ(.)	11	483.07	31.73	0.00

ability (p) and mean initial abundance for each site (λ). The Dail-Madsen (2011) model estimates two additional parameters: the recruitment rate (γ), comprehensive of births and immigrations, and the apparent survival probability (ω), comprehensive of deaths and emigrations. In our study, we built models with Poisson error distribution, since Negative Binomial distribution could lead to identifiability issues and may produce infinite abundance estimates (Barker et al., 2017; Link et al., 2018). Furthermore, in order to avoid truncated estimates of abundance (Knape et al., 2018), we set the upper limit for integration (K) to 50 (i.e., we checked estimate stability at incremental values of K). We then began the model building procedure by fitting a global model (i.e., the most complex model on which other models are nested) and assessing the fit of this model in two ways: i) by means of a Pearson chi-square test (MacKenzie and Bailey, 2004), using a parametric bootstrap procedure (5000 re-samplings), ii) by inspecting residuals (Knape et al., 2018). In order to avoid overfitting and creating too many models, deriving from the combinations of covariates for each of the four parameters of the Dail-Madsen (2011) model, which can lead to uninformative and biologically unsound models, we preferred to build fewer models in a stepwise approach, considering one parameter at a time, and building biologically informative models.

We proceeded modelling the detection probability, considering it to be constant, time-dependent, or to

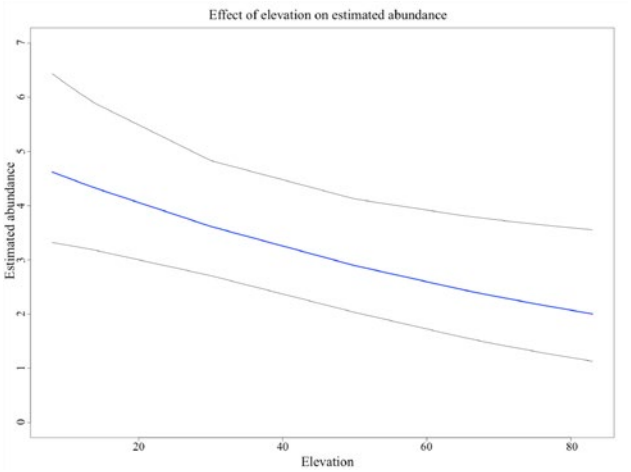

Fig. 1. Effect of elevation on site specific abundance of *Hyla meridionalis* in the Cinque Terre National Park, with 95% confidence intervals, obtained by N-mixture open population modelling (Dail and Madsen, 2011).

be affected by climatic variables. Then we modelled the initial abundance as a function of site specific covariates (ELEV, CITY and SITE) or constant over sites. Finally, we considered the survival to be influenced by the same site covariates as abundance, or constant over sites. For each model we considered recruitment as constant. We ranked all models with Akaike's Informative Criterion corrected for small samples (AICc). We conducted model selection and considered only models with ΔAICc > 2 (Burnham and Anderson, 2002). Modelling was conducted in the R environment with package Unmarked (Fiske and Chandler, 2011) and AICcmodavg (Mazerolle, 2017).

In 2017 we counted a total of 131 male frog calls during three surveys (44; 45; 42; respectively), while in 2018 we counted 129 male frogs during four surveys (33; 37; 32; 27; respectively, Table S1). The global model had a good fit (goodness-of-fit, P = 0.34; c-hat overdispersion = 1.12, and visual inspection of residuals). Model building procedure produced a total of 15 models (Table 2). The most supported model included elevation as a covariate on the initial abundance, highlighting a negative effect of elevation (β_{ELEV} = -0.331; 95% CI = -0.59 to -0.08; Figure 1). The estimated mean frog abundance per site was 3.4 (95% CI = 2.5 – 4.6). Individual detection probability for this model was constant, and estimated as p = 0.53 (95% CI = 0.42 – 0.63). Survival probability between years was considered constant among sites and resulted ω = 0.71 (95% CI = 0.50 – 0.86). Finally, the recruitment rate was constant across sites γ = 0.11 (95% CI = 0.00 – 12.10). From this best model we also obtained, as a derived parameter from the posterior distribution of the latent abundance, the total abundance of surveyed sites, which

resulted of 89 frogs in 2017 (95% CI = 81 – 147) and 64 frogs in 2018 (95% CI = 60 – 109).

Our study showed that N-mixture modelling applied to individual frog calls can be successfully used to estimate male population size together with demographic parameters and ecological understandings. In the CTNP, where *H. meridionalis* is a species of high conservation concern, the male tree-frog population size showed no significant change between years, and site abundance was negatively related with altitude (Salvidio, 2007; Sillero, 2010). Moreover, the usefulness of N-mixture approach may be appreciated by comparing population estimated corrected by detectability to raw counts that, in the present case, underestimated the total number of males by about 45%, in both years. Another important application of N-mixture population open models (Dail and Madsen, 2011) relies on the possibility of estimating temporal variations in inter-annual population size, this information being of interest in conservation and management programmes concerning protected species characterised by low or variable detection probabilities (Ficetola et al., 2018). Conversely, the major limits of our study were that the occurrence of calling males does not always assure for the presence of a breeding site, while no data on population structure (i.e., population sex ratio and proportion of juveniles) can be provided (Dorcas et al., 2009). In any case, N-mixture models are cost-effective alternatives to mark-recapture and removal sampling methods (Kéry and Royle, 2015; Kéry, 2018), and they have been used to estimate population size and temporal trends of many species in very different ecological contexts (e.g., Priol et al., 2014; Romano et al., 2017; Kéry, 2018; Costa et al., 2019). However, to our knowledge there are few applications of N-mixture modelling to anuran call counts, because of the difficulties in correctly counting calling males in large frog choruses when dozens of calls are synchronous (Weir and Mossman, 2005). Nevertheless, when few individual males are calling at each site the application of the N-mixture modelling seems useful and can be preferred to other methods that estimate population abundance because there is no need to mark and recapture the focal individuals (Royle, 2004a; Royle and Link, 2005).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 25342.

REFERENCES

Anthony, B.P. (2002): Results of the first batrachian survey in Europe using road call counts. Alytes **20**: 55-66.

Barker, R.J., Schofield M.R., Link W.A., Sauer, J.R. (2017): On the reliability of *N*-mixture models for count data. Biometrics **74**: 369-377.

Burnham, K.P., Anderson, D.R. (2002): Model selection and multimodel inference: a practical information-theoretic approach. Springer-Verlag, New York, USA.

Costa, A., Oneto, F., Salvidio,S. (2019): Time-for-space substitution in N-mixture modelling and population monitoring. J. Wildl. Manage. **83**:737-741.

Dail, D., Madsen, L. (2011): Models for Estimating Abundance from Repeated Counts of an Open Metapopulation. Biometrics **67**: 577-587.

Dorcas, M.E., Price, S.J., Walls, S.C., Barichivich, W.J. (2009): Auditory monitoring of anuran populations. In: Ecology and conservation of amphibians: a handbook of techniques, pp. 281-298. Dodd, C.K. Jr., Ed., Oxford University Press, New York, USA.

do Vale, R.L., Torres, I., Gomes, S., Fonseca, C., Ferreira, E. (2018): Ecological preferences of *Hyla molleri* in the colonisation of arboreal refuges in a human-shaped wetland. Amphibia-Reptilia **39**: 51-61.

Droege, S., Eagle, P. (2009): Evaluating calling surveys. In: Declining amphibians: A United States response to the global phenomenon, pp. 314-325. Lannoo, M.J., Ed., University of California Press, Berkeley, California, USA.

Ficetola F., Salvidio S., Romano A., Sindaco R. (2018): Optimizing monitoring schemes to detect trends in abundance over broad scales. Anim. Cons. **21**: 221-231.

Fiske, I., Chandler, R., (2011): Unmarked: An R package for fitting hierarchical models of wildlife occurrence and abundance. J. Stat. Softw. **43**: 1-23.

Gaston, K.J. (2003): The structure and dynamics of geographic ranges. Oxford University Press, Oxford, UK.

Kéry, M. (2018): Identifiability in N-mixture models: a large-scale screening test with bird data. Ecology **99**: 281-288.

Kéry, M., Royle, J.A. (2015): Applied hierarchical modeling in ecology - modeling distribution, abundance and species richness using R and BUGS. Volume 1:

Prelude and static models. Elsevier/Academic Press, Amsterdam, The Netherlands.

Knape, J., Arlt, D., Barraquand, F., Berg, Å., Chevalier, M., Pärt, T., Ruete, A., Żmihorski, M. (2018): Sensitivity of binomial N-mixture models to overdispersion: the importance of assessing model fit. Met. Ecol. Evol. **9**: 2102-2114.

Link, W.A., Schofield, M.R., Barker, R.J., Sauer, J.R. (2018): On the robustness of N-mixture models. Ecology **99**: 1547-1551.

MacKenzie, D.I., Bailey, L.L. (2004): Assessing the fit of site-occupancy models. J. Agric. Biol. Environ. Stat. **9**: 300-318.

Márquez, R., Moreira, C., do Amaral, J.P.S., Pargana, J.M., Crespo, E.G. (2005): Sound pressure level of advertisement calls of *Hyla meridionalis* and *Hyla arborea*. Amphibia-Reptilia **26**: 391-395.

Mazerolle, M.J., Bailey, L.L., Kendall, W.L., Royle, J.A., Converse, S.J., Nichols, J.D. (2007): Making great leaps forward: accounting for detectability in herpetological field studies. J. Herpetol. **41**:672-689.

Mazerolle, M.J. (2017): AICcmodavg: model selection and multimodel inference based on (Q)AIC(c). R package version 2.1-1. https://cran.r-project.org/package=AICcmodavg. [*Accessed on 22 January 2019*]

Olivari, S., Romano, A., Salvidio, S. (2013): Anfibi e habitat acquatici nel Parco Nazionale delle Cinque Terre - Censimento e indirizzi per la conservazione. Edizioni Belvedere, Latina.

Priol, P., Mazerolle, M., Imbeau, L., Drapeau, P., Trudeau, C., Ramière, J. (2014): Using dynamic N-mixture models to test cavity limitation on northern flying squirrel demographic parameters using experimental nest box supplementation. Ecol. Evol. **4**: 2165-2177.

Recuero, E., Iraola, A., Rubio, X., Machordom, A., García-París, M. (2007): Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): An unusual phylogeographical pattern. J. Biogeogr. **34**: 1207-1219.

Romano, A., Costa, A. Basile, M. Raimondi, R., Posillico, M., Scinti Roger, D., Crisci, A., Piraccini, R., Raia, P., Matteucci, G., De Cinti, B. (2017): Conservation of salamanders in managed forests: Methods and costs of monitoring abundance and habitat selection. For. Ecol. Manage. **400**: 12-18.

Romano, A., Salvidio, S., Mongillo, D., Olivari, S. (2014): Importance of a traditional irrigation system in amphibian conservation in the Cinque Terre National Park (NW Italy). J. Nat. Cons. **22**: 445-452.

Royle, A.J. (2004a): Modeling abundance index data from anuran calling surveys. Cons. Biol. **18**: 1378-1385.

Royle, A.J. (2004b): N-mixture models for estimating population size from spatially replicated counts. Biometrics **60**: 108-115.

Royle, J.A., Link; W.A (2005): A general class of multinomial mixture models for anuran calling survey data. Ecology **86**: 2505-2512.

Salvidio, S. (2007): *Hyla meridionalis* Boettger, 1874. In: Fauna d'Italia – Amphibia. XLII, pp. 338-346. Lanza, B., Andreone, F., Bologna, M.A., Corti, C., Razzetti, E., Eds, Calderini, Bologna.

Schmidt, B.R., (2004): Declining amphibian populations: the pitfalls of count data in the study of diversity, distributions, dynamics, and demography. Herpetol. J. **14**: 167-174.

Schmidt, B.R., Pellet, J. (2005): Relative importance of population processes and habitat characteristics in determining site occupancy of two anurans. J. Wildlife Manage. **69**: 884-893.

Schneider, H. (1974): Structure of the mating calls and relationships of the European tree frogs (Hylidae, Anura). Oecologia **14**: 99-110.

Sillero, N. (2010): Modelling suitable areas for *Hyla meridionalis* under current and future hypothetical expansion scenarios. Amphibia-Reptilia **31**: 37-50.

Sillero, N., Campos, J., Bonardi, A., Corti, C., Creemers, R., Crochet, P-A., Crnobrnja Isailovic, I., Denoël, M., Ficetola, G.F., Gonçalves, J., Kuzmin, S., Lymberakis, P., de Pous, P., Rodríguez, A., Sindaco, R., Speybroeck,J., Toxopeus, B., Vieites, D.R., Vences, M. (2014): Updated distribution and biogeography of amphibians and reptiles of Europe. Amphibia-Reptilia **35**: 1-31.

Walls, S., Waddle, J.H., Dorazio, R.M. (2011): Estimating occupancy dynamics in an anuran assemblage from Louisiana, USA. J. Wildlife Manage. **75**: 751-761.

Weir, L., Fiske, I.J., Royle, J.A. (2009): Trends in anuran occupancy from northeastern states of the North American Amphibian Monitoring Program. Herpetol. Cons. Biol. **4**: 389-402.

Weir, L.A., Mossman M.J. (2005): North American Amphibian Monitoring Program. In: Declining amphibians: A United States response to the global phenomenon, pp. 307-313. Lannoo, M.J., Ed, University of California Press, Berkeley, California, USA.

Weir, L.A., Royle, J.A., Nanjappa, P., Jung, R.E. (2005): Modeling anuran detection and site occupancy on North American Amphibian Monitoring Program (NAAMP) routes in Maryland. J. Herpetol. **39**: 627-639.

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AT-rich microsatellite loci development for *Fejervarya multistriata* by Illumina HiSeq sequencing

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Abstract. In our study, a total of 2561 sequences that contained microsatellite loci were found potentially to be used for primer design. Furthermore, Illumina HiSeq sequencing technology identified trinucleotide repeats and AT-rich repeats with the the highest proportion in our genomic DNA sequence library of *Fejervarya multistriata*. Eighteen new microsatellite loci of *F. multistriata* were isolated and we characterize these loci genotyping 48 individuals sampled from 3 populations in Lishui City, Zhejiang Province, China. Seventeen loci were polymorphic, with the number of alleles ranging from 2 to 11 within each population. The polymorphic information content, observed and expected heterozygosity ranged 0-0.845, 0-1.0 and 0-0.871, respectively. None of the loci was observed in linkage disequilibrium. One locus (FMA294) was deviated from Hardy-Winberg equilibrium in each population separately and combined. These informative microsatellite loci will be applicable for conservation genetic studies of *F. multistriata* across varying scales from inter-individual to inter-population.

Keywords. *Fejervarya multistriata*, genome, microsatellite, next-generation sequencing, polymorphism.

INTRODUCTION

Microsatellite DNA loci, also known as simple sequence repeats, occur at thousands of locations within the eukaryotic genome, and are highly variable and sufficient in nuclear genome (Ellegren, 2004; Wei et al., 2015; Shao et al., 2017). Therefore, microsatellite DNA loci are widely applied as molecular markers in population genetics, species identifying, genetic breeding and genetic map (Selkoe and Toonen, 2006; Abe et al., 2012; Wambulwa et al., 2016; Soulard et al., 2017). Thanks to the development of next-generation sequencing technology, both throughput and efficiency of developing microsatellite DNA has increased with a decreased cost of sequencing process. In recent years, microsatellite DNA markers have been quickly developed in many species at a low cost when using the next-generation sequencing technology on Illumina HiSeq and Ion Torrent PGM platforms (Yang et al., 2012; Lü et al., 2013; Zhang et al., 2013; Sultana et al., 2014; Igawa et al., 2015; Song et al., 2017).

Fejervarya multistriata (Anura: Dicroglossidae) is a species of frog, which is widely found in south of the Yellow River in China and some countries (regions) in Southeast Asia, such as northern India, Vietnam and Myanmar (AmphibiaChina, 2018). The conservation status of this species is listed as data deficient in IUCN (AmphibiaChina, 2018). This species prefers to inhabit paddy field and still water and its ovulation remains active from April to September every year (AmphibiaChina, 2018). As a dominant amphibian species, *F. multistriata* plays an important role in farmland ecosystem, and its population density in field has decreased due to urbanization (Li et al., 2016). Meanwhile, environmental degradation also threatens the survival of this species (Othman et al., 2009). In previous studies, mitochondrial D-loop sequences were used as molecular mark-

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ers to study phylogeography of *F. multistriata* populations (Zhong et al., 2008). Twenty-one microsatellite loci, mainly including dinucleotide repeats, had been isolated for the species, and only approximately 24% loci had AT repeats (Chen et al., 2018). Here, we sequentially developed 18 new microsatellite DNA loci containing AT-rich repeats for *F. multistriata* by Illumina HiSeq sequencing. These new AT-rich microsatellite loci definitely would be useful in examining genetic diversity and protecting genetic resources of *F. multistriata*.

MATERIAL AND METHODS

Forty-eight *F. multistriata* individuals (n = 16 for each population) used in this study were collected by hand and net from 3 localities in Lishui City, Zhejiang Province, China, which were Lanshantou (LST, 119.7607°E, 28.36366°N), Baimashan (BMS, 119.1337°E, 28.63823°N) and Jiulongshan (JLS, 118.8452°E, 28.39538°N), respectively. Our experimental procedures are compliant with current laws on animal welfare and research in China, which are also specifically approved by the Animal Research Ethics Committee of Lishui University (Permit No. AREC-LU 2017-04).

Genomic DNA was extracted from toe muscle tissue of one male *F. multistriata* from LST population using the DNeasy Tissue Kit (Qiagen). The concentration of DNA sample was measured by using a spectrophotometer at 260 and 280 nm and DNA sample was quantified on an agarose gel. A 200-400 bp sequencing library was constructed according to the manufacturer instructions (Illumina). This library was sequenced using an Illumina HiSeq 2500 Platform with RAD-Tag at Novogene Bioinformatics Technology Co., Ltd (Beijing, China, http://www.novogene.com/). The microsatellite primer pairs of *F. multistriata* were designed using Primer Premier 3.0 software, which was used to check against potential primer dimers, hairpin structures and the occurrence of mismatches. Parameters for designing the primers were set as follows: primer length ranged from 18 bp to 24 bp with 22 as the optimum; PCR product size ranged from100-280 bp; melting temperature ranged from 50 °C to 65 °C with 55 °C as the optimum annealing temperature; GC content ranged from 30% to 70% with 50% as the optimum. Finally, thirty newly designed primer pairs were selected to synthesize and initially screened for microsatellite loci using total genomic DNA isolated from 6 *F. multistriata* individuals collected from the LST population.

PCR amplification reactions were performed using a thermal cycler (T100, Bio-Rad, USA). The total volume of each PCR mixture was 20 μL, containing 1 μL genomic DNA (100 ng/μL), 10 μL Premix Taq (TaKaRa, Japan), 1 μL of each primer (10 μM) and 6 μL double distilled H_2O. The conditions of the PCR amplification were as follows: 95 °C for 5 min, then 35 cycles at 95 °C for 30 s, T_a (the optimal annealing temperatures, see Table 1) for 30 s, 72 °C for 30 s, and a further extension at 72 °C for 10 min. Twenty-six primer pairs were further selected due to successful amplification in the 6 individuals, and the forward primer was labeled with FAM or HEX fluorescent dye (Sangon Biotech Ltd. Co., Shanghai, China). The PCR products were genotyped on an ABI 3730 sequencer (Applied Biosystems) and following data were analyzed with GeneMarker v1.8 software.

Population genetic parameters for polymorphic loci such as number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphic information content (PIC), P values in Hardy-Weinberg equilibrium (HWE) tests and linkage disequilibrium were calculated by Genepop 4.0 (Rousset, 2008), Cervus 2.0 (Marshall et al., 1998) and Fstat 2.9.3.2 (Goudet, 1995), respectively.

RESULTS AND DISCUSSION

Sequence data from Illunima HiSeq

We obtained a total of 6970707900 bp and 23235693 reads in a single sequencing run on Illunima HiSeq™ using RAD-Tag. The distribution frequency of read length for this species had a single peaks at approximately 125 bp. In a total of 307793 reads with more than 125 bp, 2561 reads contained microsatellite loci (0.83%).

Compared to a traditional library-based approach such as magnetic beads enrichment (Guo et al. 2015; Chang et al. 2016), next-generation sequencing technology is a more powerful approach to develop microsatellite markers due to its efficiency and low cost. Sufficient microsatellite sequences can be constructed in a genomic DNA sequence library on Illunima HiSeq™ using RAD-Tag. This result agrees with recent studies on other anuran species (Wei et al., 2015; Shao et al., 2017). Furthermore, microsatellite obtained rate maybe higher on Illunima HiSeq platform (e.g., 0.83% in our study) than on Ion Torrent PGM platform (e.g., 0.32–0.57% in Igawa et al., 2015).

Frequency and distribution of microsatellite loci in the genome

The length of the microsatellite loci ranged from 12 to 33 bp (15.7 ± 5.2, mean ± SD). The microsatellite DNA loci included 5 motif types: dinucleotide repeats (36.20%), trinucleotide repeats (52.60%), tetranucleotide repeats (9.64%), pentanucleotide repeats (0.90%) and hexanucleotide repeats (0.66%) (Fig. 1A). The frequency distribution of the 5 motif types was different significantly (G-test, G = 3085.9, df = 4, $P < 0.001$). The motif repeat number of microsatellite loci ranged from 4 to 16, while 97.66% of the microsatellite loci had 4-12 motif repeats, and motifs with more than 12 repeats were only with a frequency of <1.0% (Fig. 1B). There were 4 dinucleotide motif types, and the main types were AC/GT (50.70%), AT (35.17%) and AG/CT (13.92%) (Fig. 1C),

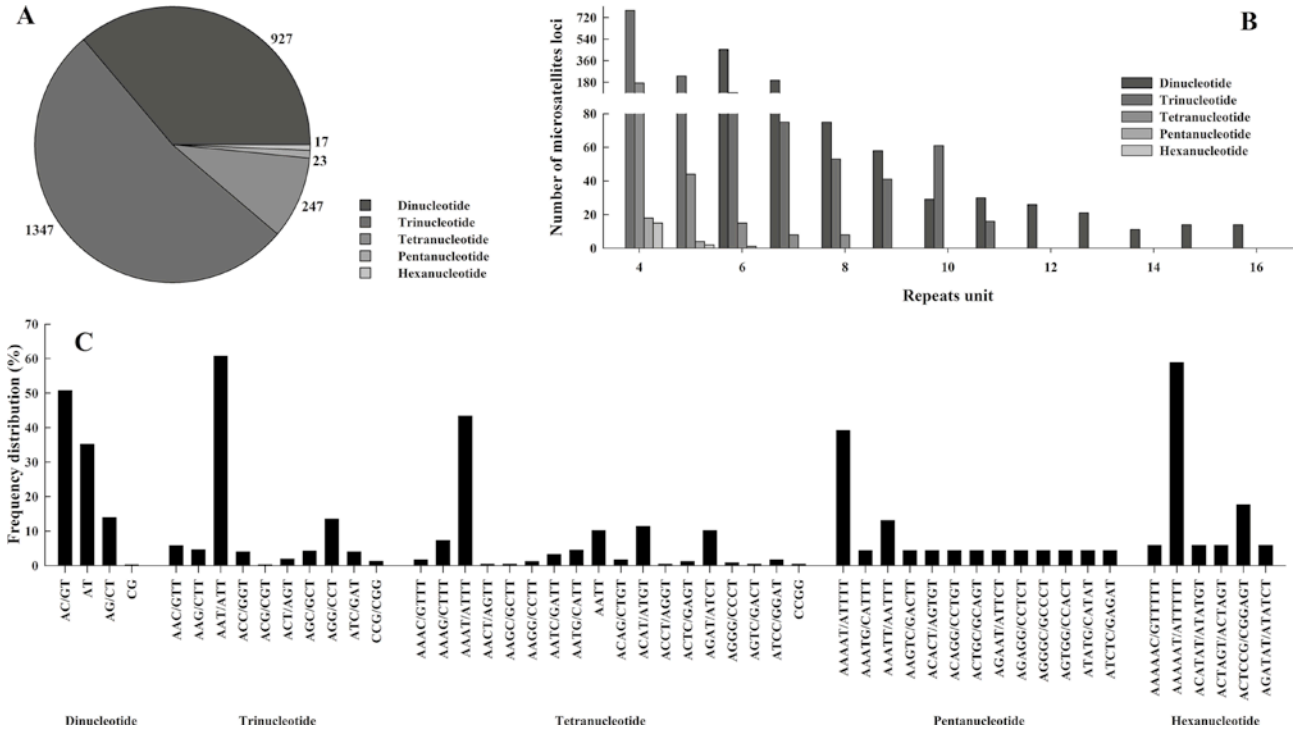

Fig. 1. Characterization of microsatellite loci in *Fejervarya multistriata* genome. (A) distribution of different repeat motif types of microsatellite loci; (B) number of different repeat motifs; (C) frequency distribution of 5 different repeat types based on different motif types. Number represent number of sequence.

respectively. There were 10 trinucleotide motif types, and the main types were AAT/ATT (60.65%) and AGG/CCT (13.51%) (Fig. 1C), respectively. There were 18 tetranucleotide motif types, and the main types were AAAT/ATTT (43.32%), ACAT/ATGT (11.34%), AATT (10.12%) and AGAT/ATCT (10.12%) (Fig. 1C), respectively. There were 13 pentanucleotide motif types, and the main types were AAAAT/ATTTT (39.13%) and AAATT/AATTT (13.04%) (Fig. 1C), respectively. There were 6 hexanucleotide motif types, and the main types were AAAAAT/ATTTTT (58.82%) and ACTCCG/CGGAGT (17.65%) (Fig. 1C), respectively.

The frequency of different repeat types of microsatellites in *F. multistriata* was different from *Xenopus tropicalis* (Xu et al., 2008), *Odorrana narina*, *Hoplobatrachus tigerinus*, and *Buergeria japonica* (Igawa et al., 2015). The dominant repeat type was trinucleotide in *F. multistriata*, but dinucleotide in the last 4 anuran species (Xu et al., 2008; Igawa et al., 2015). Such results may be related to the different next-generation sequencing platforms used in constructing sequence library. Since the library of microsatellite sequences was constructed by Illunima HiSeq platform for *F. multistriata*, however, by Ion Torrent PGM platform for *O. narina*, *H. tigerinus* and *B. japonica* (Igawa et al., 2015). In addition, the results suggested that the frequency of the repeat type changed randomly for each species and was species-specific. The frequencies decreased when the repeat unit length in each repeat type motif of *F. multistriata* increased (Fig. 1B), indicating that a relatively short repeat unit of microsatellites might be a main component in the genome of *F. multistriata.*

Our finding suggested that the ratio of the repeat motifs with an AT content of approximately 56% in our *F. multistriata* was similar to other reported anuran species (e.g., *X. tropicalis*, Xu et al., 2008; *O. narina*, *H. tigerinus* and *B. japonica*, Igawa et al., 2015), suggesting that the AT content could be an important repeat unit in anurans. The dominant repeat motif in the trinucleotide type of *F. multistriata*, (AAT/ATT repeat) was similar to the other four reported anuran species (Xu et al., 2008; Igawa et al., 2015). However, other types of repeat motif were different among these species. For example, *F. multistriata* had a higher frequency in AC/GT and AAAT/ATTT repeats, but *O. narina*, *H. tigerinus*, *B. japonica* and *X. tropicalis* in AT and AGAT/ATCT repeats (Xu et al., 2008; Igawa et al., 2015). These results implied that the accumulation rates of repeat motifs were maintained in modern anurans, but skewed in a common ancestor (Igawa et al., 2015).

Table 1. Characterization of 18 microsatellite DNA markers developed for *F. multistriata*. Size range: size range of fragment; bp: base pair; T_a,: annealing temperature of primer pairs; N_a: number of alleles; H_o: observed heterozygosity; H_E: expected heterozygosity; HWE: Hardy-Weinberg equilibrium; PIC: polymorphic information content; bold: significant deviation from HWE after Bonferroni correction ($P < 0.05$).

Locus (GenBank #)	Primer sequences (5'-3')	Repeat motif	T_a (°C)	Size range (bp)	N_A	H_o	H_E	P_{HWE}	PIC
FMA102 MG744293	F: GCACTGTAGAGCACTGGATTC R: GAGCGTCATAGGGGTCAAATAG	(TA)16	53	129-219	13	0.4792	0.7592	1.000	0.72
FMA349 MG744294	F: CACTCATGTTATCACTCTACTCTC R: CCTCCTACCTCTTGTACTAAATTG	(TAT)11	53	156-237	11	0.3333	0.8145	1.000	0.782
FMA117 MG744295	F: ACTTGAGTCTATTCTATTCTGCTG R: ACTGCTGCTCTGATCTCTATG	(ATA)7	53	149-158	4	0.4167	0.5893	0.996	0.495
FMA402 MG744296	F: AGACATTACCTTAAAGCCATAGTG R: CTTCTGACATGACCTGTTCTTC	(AGAT)6	53	189-201	4	0.4583	0.6090	0.975	0.538
FMA041 MG744297	F: CCAGGAGGATTCTAGTGACAG R: ATGAAGGCAAGAGCAATGTAC	(AT)12	53	152-192	12	0.3958	0.8169	1.000	0.789
FMA466 MG744298	F: GGTGCCACTGTCTTAACTATCC R: AGTCCAATCAAGTCCAATTCAAAC	(TTTTTA)4	53	199-205	2	0.3125	0.4086	0.975	0.323
FMA294 MG744299	F: GTCCTCCTACCTCTTGTACTG R: CGAATGAGAACCTTCACAGAC	(ATA)10	53	187-238	6	0.9375	0.6515	**< 0.01**	0.586
FMA302 MG744300	F: TCCGACCTCTTGAAACTGTATTG R: AGGATCACCACTAGGAGCATC	(ATA)10	53	205-259	13	0.6042	0.8680	1.000	0.845
FMA355 MG744301	F: TATGACCACAGTCTAGCATCC R: CTCCAGTAGTTATCACCTTCTTG	(AAAT)5	58	158	1	–	–	na	0
FMA188 MG744302	F: CCTCTTGTGTTGGTGTATTTCTG R: TTATGCTTGTGTTCTGGTCATTC	(AAT)8	63	209-215	3	0.6042	0.5340	0.170	0.434
FMA231 MG744303	F: GCTGCTGCATGATAGTGTCTC R: TGATGTCTGATGGTCGTCCTG	(TAT)8	53	155-185	10	0.7917	0.8353	0.992	0.805
FMA072 MG744304	F: TGCAGTAGACATCGGAGTTG R: GCCTCTCTCATCTTATTAAGTGG	(TA)13	53	209-237	10	0.6875	0.7934	0.998	0.757
FMA140 MG744305	F: TTCATTGTGCCAAGTGTAACG R: TAACAAAGAGGTCATCACTAATCC	(ATT)7	63	153-165	3	0.1875	0.2254	0.927	0.206
FMA403 MG744306	F: GCGTGGATCGTTATTGAAGTG R: GGTGACCTAATGTGAAATTCCTG	(ATTT)6	63	193-197	2	0.2708	0.2945	0.858	0.249
FMA116 MG744307	F: CTCCCTAACTATTGTAAAGCACTG R: ATTATAGATGGAAGCAACAGGAAC	(ATA)7	55	165-195	8	0.5208	0.7825	1.000	0.743
FMA399 MG744308	F: TTCAGGCTACAGGCATTACAG R: ATAAGGGTGTTCTGCTAAATCAAG	(AATA)6	55	168-228	4	0.2708	0.4476	1.000	0.416
FMA269 MG744309	F: AATGCTTGCAGAACTATTCACAC R: TACGGCGGTCCTAAGATGG	(TAT)9	55	177-192	6	0.2500	0.6732	1.000	0.616
FMA139 MG744310	F: GATTGATGGATTGATGATGGACTG R: AATGTTCAAGATGGACGAATTACC	(ATG)7	55	177-189	5	0.6042	0.6535	0.813	0.584

Characterization of microsatellite loci

Twenty-six primer pairs were used to successfully amplify genomic DNA of *F. multistriata* from LST population. Of the 26 pairs, 18 pairs generated target bands, and the other 8 pairs generated non-target bands. Finally, a total of 18 primer pairs were characterized, of which 17 were polymorphic. The genomic sequences containing a microsatellite locus, which were used to design these primers, were deposited in GenBank (accession number: MG744293–MG744310). The information of primer sequences, repeat motifs, T_a, N_A, PIC and heterozygosity for each locus were shown in Table 1. The N_a, PIC and heterozygosities (H_O and H_E) ranged from 1 to 11 (4.815 ± 2. 699, mean ± SD), 0 to 0.845 (0.549 ± 0.240, mean ± SD), 0 to 1.0 (0.452 ± 0.246, mean ± SD), 0 to 0.871 (0.571 ± 0.237, mean ± SD) within each population, respectively. No significant linkage disequilibrium was observed after Bonferroni correction for multiple tests (all $P > 0.05$). Of the 18 loci, one (FMA294) deviated significantly from HWE testing each population separately and combined (all $P < 0.05$; Table 1), which indicated that null alleles may be present in this locus (Song et al., 2017). The locus was assessed to contain moderately high

polymorphism degree when the value of PIC was larger than 0.5 (Song et al., 2017). Overall, 11 loci had high polymorphism degree (PIC > 0.5), 6 loci had low polymorphism degree (PIC < 0.5). These informative microsatellite loci will be applicable for conservation genetic studies of *F. multistriata* across varying scales from inter-individual to inter-population.

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REFERENCES

Abe, H., Hayano, A., Inoue-Murayama, M. (2012): Forensic species identification of large macaws using DNA barcodes and microsatellite profiles. Mol. Biol. Rep. **39**: 693-699.

AmphibiaChina (2019): The database of Chinese amphibians. Kunming Institute of Zoology (CAS), Kunming, Yunnan, China. Available from: http://www.amphibiachina.org/ [*accessed on 1 March 2019*]

Chang, Y.C., Li, S.H., Lin, H.Y., Chen, S.L., Chang, M.H. (2016): Development of 22 polymorphic microsatellite markers for Taipei grass frogs (*Hylarana taipehensis*). Amphibia-Reptilia **37**: 117-120.

Chen, Y.D., Li R., Wei, L., Lin, Z.H., Li, Y.D., Ding, G.H. (2018): Development and characterization of 21 microsatellite loci for *Fejervarya multistriata* (Anura: Dicroglossidae) using genome-wide data. Microsatellite records for volume 10, issue 1. Conserv. Genet. Resour. **10**: 127-140.

Ellegren, H. (2004): Microsatellites: simple sequences with complex evolution. Nat. Rev. Genet. **5**: 435-445.

Goudet, J. (1995): FSTAT (Version 1.2): a computer program to calculate F-statistics. J. Hered. **86**: 485-486.

Guo, K., Ding, G.H., Sun, Y.Y., Lu, H.L. (2015): Isolation and characterization of microsatellite loci in the northern grass lizard, *Takydromus septentrionalis* (Squamata: Lacertidae). Conserv. Genet. Resour. **7**: 583-584.

Igawa, T., Nozawa, M., Nagaoka, M., Komaki, S., Oumi, S., Fujii, T., Sumida, M. (2015): Microsatellite marker development by multiplex Ion Torrent PGM sequencing: a case study of the endangered *Odorrana narina* complex of frogs. J. Hered. **106**: 131-137.

Li, B., Zhang, W., Shu, X.X., Pei, E.L., Yuan, X., Sun, Y.J., Wang, T.H., Wang, Z.H. (2016): The impacts of urbanization on the distribution and body condition of the rice-paddy frog (*Fejervarya multistriata*) and gold-striped pond frog (*Pelophylax plancyi*) in Shanghai, China. Asian Herpetol. Res. **7**: 200-209.

Lü, Z., Li, H., Liu, L., Cui, W., Hu, X., Wang, C. (2013): Rapid development of microsatellite markers from the large yellow croaker (*Pseudosciaena crocea*) using next generation DNA sequencing technology. Biochem. Syst. Ecol. **51**: 314-319.

Marshall, T.C., Slate, J., Kruuk, L.E.B., Pemberton J.M. (1998): Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* **7**: 639-655.

Othman, M.S., Khonsue, W., Kitana, J., Thirakhupt, K., Robson, M. G., Kitana N. (2009): Cadmium accumulation in two populations of rice frogs (*Fejervarya limnocharis*) naturally exposed to different environmental cadmium levels. B. Environ. Contam. Tox. **83**: 703-707.

Rousset, F. (2008): Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Resour. **8**: 103-106.

Selkoe, K.A., Toonen, R.J. (2006): Microsatellite for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol. Lett. **9**: 615-629.

Shao, W.W., Lin, Z.H., Fan, X.L., Ding, G.H., Wei L. (2017): Characterization of 25 microsatellite loci for the Guenther's frog *Hylarana guentheri*. Conserv. Genet. Resour. **10**: 31-33.

Song, W., Zhu, D.M., Lv, Y.F., Wang, W.M. (2017): Isolation and characterization of 37 polymorphic microsatellite loci of *Megalobrama hoffmanni* by next-generation sequencing technology and cross-species amplification in related species. J. Genet. **96**: 39-45.

Soulard, L., Mournet, P., Guitton, B., Chaïr, H. (2017): Construction of two genetic linkage maps of taro using single nucleotide polymorphism and microsatellite markers. Mol. Breeding. **37**: 37.

Sultana, N., Igawa, T., Nozawa, M., Islam, M.M., Hasan, M., Alam, M.S., Khan, M.M., Sumida, M. (2014): Development and characterization of 27 new microsatellite markers for the Indian bullfrog *Hoplobatrachus tigerinus* and its congeneric species. Genes Genet. Syst. **89**: 137-141.

Wambulwa, M.C., Meegahakumbura, M.K., Chalo, R., Kamunya, S., Muchugi, A., Xu, J.C., Liu, J., Li, D.Z., Gao, L. M. (2016): Nuclear microsatellites reveal the genetic architecture and breeding history of tea germplasm of East Africa. Tree Genet. Genome. **12**: 11.

Wei, L., Li, L.M., Chen, J.P., Shao, W.W., Lin, Z.H. (2015): Characterization of 14 microsatellite loci for the pan-oriental narrow-mouthed toad, *Microhyla ornata* (Amphibia: Microhylidae). Conserv. Genet. Resour. **7**: 33-35.

Xu, Z.K., Gutierrez, L., Hitchens, M., Scherer, S., Sater, A. K.,Wells, D.E. (2008): Distribution of Polymorphic and Non-Polymorphic Microsatellite Repeats in *Xenopus tropicalis*. Bioinform. Biol. In. **2**: 157-169.

Yang, T., Bao, S.Y., Ford, R., Jia, T.J., Guan, J.P., He, Y.H., Sun, X.L., Jiang, J.Y., Hao, J.J., Zhang, X.Y., Zong, X.X. (2012): High-throughput novel microsatellite marker of faba bean via next generation sequencing. BMC Genomics **13**: 602.

Zhang, L.N., Peng, J., Li, X. J., Liu, Y.L., Cui, C. J., Wu, H., Wu, R.N., Tian, P.P., Li, Y. (2013): Development of 27 trinucleotide microsatellite markers for *Saccharina japonica* using next generation sequencing technology. Conserv. Genet. Resour. **6**: 341-344.

Zhong, J., Liu, Z.Q.,Wang, Y.Q. (2008): Phylogeography of the rice frog, *Fejervarya multistriata* (Anura: Ranidae), from China based on mtDNA D-loop sequences. Zool. Sci. 25: 811-882.

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Top left: *P. latastei* from Ponza Island, male with spotted color pattern . Photo by Jean-Michel Delaugerre.
Top right: *P. latastei* from Ponza Island, female with "quasi" concolor pattern. Photo by Jean-Michel Delaugerre.
Bottom left: *P. latastei* from Ponza Island, female with "quasi" concolor pattern. Photo by Jean-Michel Delaugerre.
Bottom right: *P. latastei* from Ponza Island, male from with reticulate color pattern. Photo by Jean-Michel Delaugerre.

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