Acta

Herpetologica

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Can neutral genetic differentiation explain geographical variation in body size of the natterjack toad, *Epidalea calamita*?

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Abstract. Population genetic studies are crucial for evolutionary biologists because the population is the basic substrate on which evolution is forged. However little empirical evidence has been able to demonstrate the role that isolation and gene flow play in maintaining differentiation in populations at short geographic scales. *Epidalea calamita* exhibits a steep variation in body size and reproductive traits in southwestern Spain, associated with changes in the geological substrate. This implies a decrease of 70.9% of body mass and 28.5% in snout-vent length, on a microgeographic scale of only 60 km. Previous results from both metamorphic and juvenile common garden experiments showed that genetic differentiation may be a causal determinant of geographic variation in adult. This study tested whether neutral genetic differentiation can explain the geographical variation in the body size observed in *E. calamita*. It was addressed analyzing the level of genetic structuring and gene flow among populations along the cline, comparing the genetic diversity between and within populations, as well as between ecological environments. The study showed that the geographic variation in body size observed in *E. calamita* has evolved in absence of geographic isolation, with moderate gene flow connecting the populations. Thus, neutral genetic differentiation cannot explain the geographical variation observed. Future studies are needed on the interaction between the genetic component with the environmental factors and will be necessary to analyze the contribution of the maternal effects in the origin and evolution of the geographical variation in the body size observed in *E. calamita* from southern Spain.

Keywords. *Epidalea*, F_{ST}, microsatellite loci, population differentiation, body size.

INTRODUCTION

Geographic variation in phenotypic and genetic characteristics among species' populations is a phenomenon that has been very well documented since the 1950s (Stebbins, 1950; Mayr, 1963; Harper, 1977). Adaptive explanations for the evolution and maintenance of geographic variation in body size have been put forward, particularly considering macrogeographical patterns as a response to environmental gradients (Bergmann, 1847; Ray, 1960; Lindsey, 1966; Adams and Church, 2008;

Ashton, 2002; Cvetkovic et al., 2009; Sinsch et al., 2010). Nevertheless, few studies have evaluated it at smaller geographical scales with, in many cases, lack of genetic isolation between populations (e.g., Skelly, 2004; Gomez-Mestre and Tejedo, 2004; Lee et al., 2020; Albert and García-Navas, 2022), and thus, what causes and maintains those patterns is still not well understood. Therefore, to infer on the processes causing these patterns, we need to know the mechanisms underlying the observed phenotypic variation, how they are connected to genetic variation, and how they interact with other traits and the environ-

ment (Stearns, 1989). This will also help us to understand the evolutionary significance of the geographic variation, which ultimately can lead to the formation of new species (Endler, 1977; Foster and Endler, 1999).

The agents that change the gene frequencies of populations, that is, the factors of evolution, are mutation, genetic drift, gene flow, and natural selection (Slatkin, 1987). While drift and selection tend to increase population differentiation, gene flow promotes homogenization among connected populations, either increasing or decreasing the genetic diversity of the system (Lenormand, 2002). Thus, gene flow is a major component of population structure because it determines the extent to which each local population of a species is an independent evolutionary unit. If there is a high gene flow between local populations, then all the populations evolve together; whereas in the presence of low gene flow, each population evolves almost independently (Slatkin, 1985).

The natterjack toad (*Epidalea calamita*) populations from southwestern Spain exhibit a steep variation in body size and reproductive traits associated with changes in the geological substrate (Marangoni et al., 2008). This implies a decrease of 70.9% of body mass and 28.5% in snout-vent length, on a micro-geographic scale of only 60 km (Fig. 1). Previous studies suggested that considerable genetic differentiation may be the mechanism underlying the observed geographic variation in metamorphic traits in *E. calamita* (Marangoni, 2006) and *Pelobates cultripes* (Marangoni and Tejedo, 2008), which exhibit the same geographic pattern of adult body size variation (Marangoni et al., 2008; Lee et al., 2020). Moreover, the study of age structure and growth pattern across populations of *E. calamita* suggests that both environmental variations in resources availability associated with the sandy substrate (Marangoni, 2023), but also different growth and maturity pathways, may happen in response to contrasting selective pressures (Marangoni et al., 2021). Two hypotheses could be suggested to explain the evolution and maintenance of the observed cline in body size and reproductive parameters in *E. calamita* (Marangoni et al., 2008). In the first place, it could be expected that in the presence of gene flow between populations, the alleles favored by a selection pressure of intensity s do not decrease their high frequency, because the rate of immigration m of other alleles is lower than the intensity of selection s, that is, $m < s$ (Slatkin, 1985). The second hypothesis would be that the differentiation between the populations along the cline had occurred in a context of relative population isolation or in the presence of scarce gene flow. Little empirical evidence has been able to demonstrate the role that gene flow plays in maintaining differentiation in populations at short geographical scales. It

has been suggested that when gene flow is not homogeneous, evolutionary differentiation can be rapid and can occur on small spatial scales (Kennington et al., 2003; Garant et al., 2005; Postma and van Noordwijk, 2005).

In previous studies on *E. calamita* that exhibited local adaptation to osmotically stressful environments, microsatellite markers revealed little population differentiation, lack of an isolation-by-distance pattern, and moderate gene flow connecting the populations (Gomez-Mestre 2001; Gomez-Mestre and Tejedo, 2004). Present study assess whether neutral genetic differentiation can explain the geographical variation in the body size, age and reproductive parameters observed in natterjack toad, from southern Spain (Marangoni et al., 2008; 2021). This comparison between patterns of population genetic differentiation in neutral markers and quantitative traits can provide valuable insights into the mechanisms driving variation within populations (Leinonen et al., 2007). Thus, this comparison can help us understand evolutionary forces by discerning the relative influences of genetic drift and natural selection on the evolution of quantitative traits (Gomez-Mestre and Tejedo, 2004; Knopp et al., 2007; Páez-Vacas et al., 2001). Secondly, it aids in identifying adaptive traits, for example those traits which might are related to adaptation to different environmental conditions (Gomez-Mestre and Tejedo, 2003; Luque et al., 20015). In addition, it also allows us to understand the distribution of genetic effects within the architecture of quantitative traits (see Leinonen et al. 2007 and references therein).

The main goals in the present study were: i) analyze the level of genetic structuring and gene flow in *E. calamita* populations along the geographic variation in body size observed (Marangoni et al., 2008), ii) compare genetic diversity between and within populations, as well as between ecological environments and iii) test for the existence of an isolation-by-distance pattern of populations differentiation. We expect that the observed geographical variation in the body size of natterjack toad has been evolved in absence of isolation-by-distance, and with a gene flow connecting the populations.

MATERIAL AND METHODS

Populations

Seven populations of *Epidalea calamita* were selected, representing the cline in body size observed in a previous study (Marangoni et al., 2008), which encompass three areas with different geological substrates. These populations included: two Large-bodied populations, Pedroso (L1) and Navas (L2), from Sierra Morena (old hercinic granites schist soils): four Small-bodied popula-
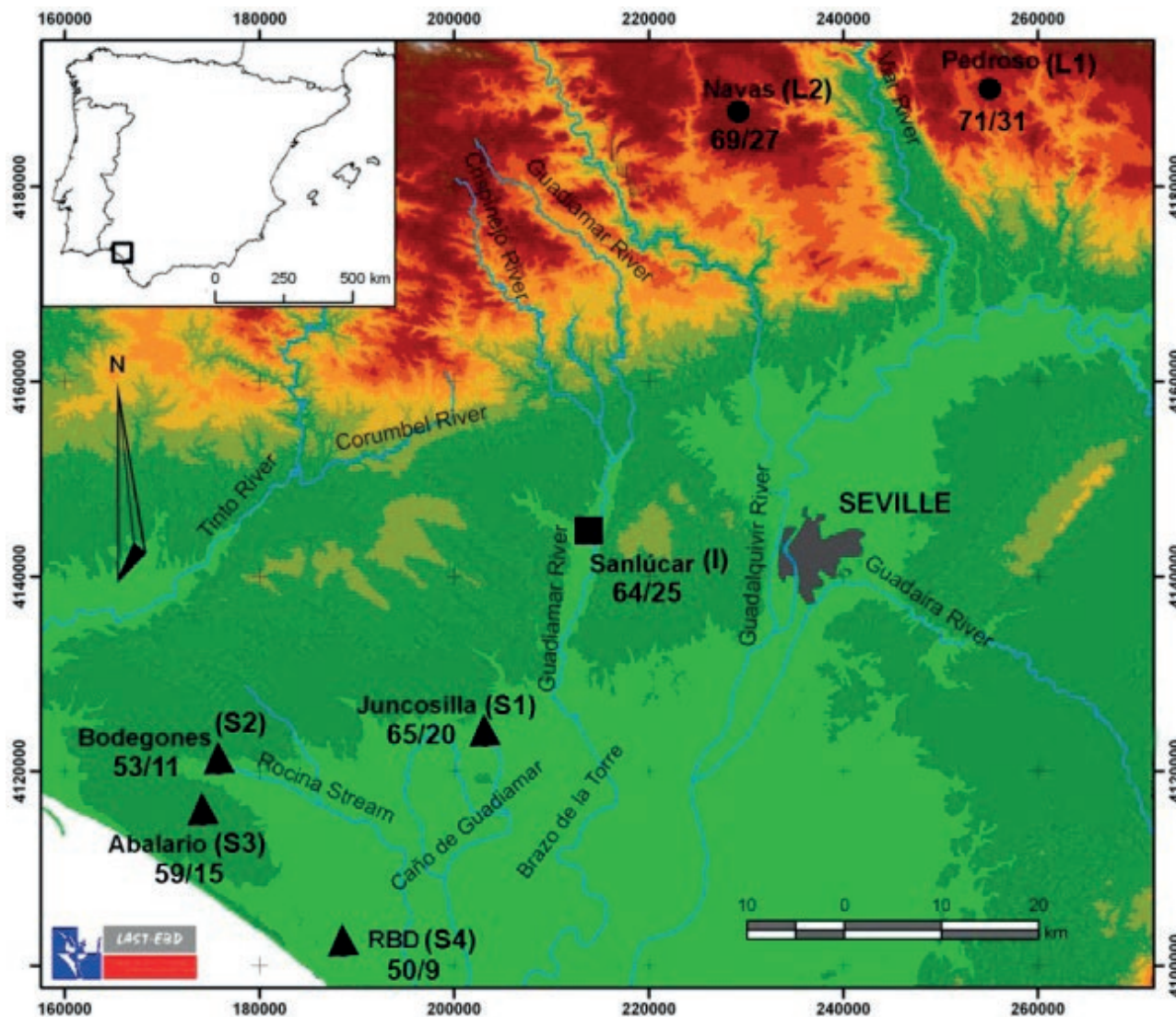
Fig. 1. Location and geological substrate of the studied *Epidalea calamita* populations. Abbreviated names of sampling localities, geographic coordinates (Coordinates UTM x/y in meters, Datum European 1950, Spain and Portugal, Zone: 30), elevation, and sample size are as follows: L1, 255170/4190574, 395 m, n = (39); L2, 229255/4187617, 420 m, n = (43); I, 213349/4144548, 34 m, n (22); S1, 203208/4124509, 23 m, n = (44); S2, 175577/4120711, 32 m, n = (45); S3, 174267/4115417, 63 m, n = (35); S4, 188450/4102197, 24 m, n = (42). Circles: Sierra (Paleozoic, Granite + Schist rocks), square: Intermediate (Miocene-Pliocene, Clay + Sandy), triangle: Doñana (Holocene, Sandy soil). Below each abbreviated names of sampling localities are indicated the mean body size (snout-vent length in mm/weight in g) from Marangoni et al. (2008).

tion, Juncosilla (S1), Bodegones (S2), Abalario (S3) and Reserva Biológica de Doñana (S4), from the Doñana area (quaternary sandy eolian deposits): and one population with intermediate body size, Sanlúcar (I), hereafter), geographically located between Sierra and Doñana (mixture of clays and sand) (Fig. 1). A more detailed description of the environments and the life history traits (body size, age, growth patterns and reproductive output) of the populations selected, is available in Marangoni and Tejedo (2008) and in Marangoni et al. (2008, 2021).

Sampling and molecular genetic analysis

Twenty recently laid clutches of similar larval stage (Gosner stage 10, Gosner, 1960) from each of the seven population of *Epidalea calamita* were sampled during the breeding season (January 2003). Each clutch (full-sib families) were brought to the laboratory and kept separately in plastic trays filled with dechlorinated tap water until tadpoles reached Gosner's stage 25 (Gosner, 1960), to be included in a common garden experi-

ments (Marangoni, 2006). Tissue samples for the present genetic study were obtained by cutting the tip of the tail of 15-20 tadpoles from each population. They were randomly taken from a sample in which the twenty clutches from each population were previously mixed, to maximize the chances of sampling unrelated tadpoles. In present study were used the same eight microsatellite loci that previously were analyzed by Gomez-Mestre (2001) and Gomez-Mestre and Tejedo (2004), which included *Bcal 1*, *Bcal 2*, *Bcal 3*, *Bcal 4*, *Bcal 5*, *Bcal 7* (Rowe et al. 1997), *Bcal 10*, and *Bcal 11* (Rowe et al., 2000). The 5' to 3'-primers were labeled with a color fluorophore, either HEX, TET, or FAM (Gomez-Mestre, 2001). DNA was obtained using an DNA Dneasy Tissue Extraction Kit (QIAGEN). The DNA was extracted by digesting each sample, approximately 25 mg of tissue minced into small pieces, to which 180 µl of ATL buffer and 20 µl of proteinase K were added. The samples incubated for 12 hours at 55 °C. Afterwards, if the tissue was not completely digested, an additional 20 µl of proteinase K was added and incubated for another 3 hours. Once the samples were fully digested, they were shaken for 15 seconds, 200 µl of buffer AL was added and incubated at 70 °C for 10 minutes. Subsequently, after adding 200 µl of ethanol, the mixture was centrifuged for 1 minute at 8000 rpm using the columns provided by the kit. Transferring the columns to other 2 ml tubes, 500 µl of buffer AW1 was added and centrifuged once more. This last step was repeated one more time, but adding 500 µl of buffer AW2, and centrifuged for 3 min at 15,000 rpm. Finally, once the columns were transferred to 1.5 ml Eppendorf tubes, 200 µl of AE buffer was added, incubated at room temperature for 1 min and centrifuged at 8000 rpm. This step was repeated twice, obtaining a final volume of 400 µl of buffer and the DNA extracted from the sample, which was stored at –20 °C until further procedures. Loci amplification was conducted using polymerase chain reactions (PCRs) of 1.5 ml total volume with 5 ml of DNA. The PCR amplifications followed a 66-50 °C touchdown procedure. PCR products of each of the eight loci for each individual sampled were aliquoted and mixed according to their abundance. An aliquot of 1.5 ml of the resulting mix was added to 13 ml of formamide plus 0.3 ml of Tamra 500 (Applied Biosystems, Foster City, CA) standard. Samples were analyzed in a fluorescence-based automatic fragment analyzer (ABI-PRISM 310 Genetic Analyzer, Applied Biosystems). Allele sizes for each locus were resolved by comparison of the peaks obtained to those yielded by the Tamra 500 standard using the software GeneScan version 3.1.2 (Applied Biosystems). All procedures described were performed in the Laboratorio de Ecología Molecular, at the Estación Biológica de Doñana (EBD-CSIC), Seville, Spain. Permits for capture and sampling of *E. calamita*, including all ethical considerations, were acquired from the regional authorities.

Statistics

Arlequin software packages (Schneider et al., 2000) was used to perform a nested molecular analysis of variance (nested AMOVA) comparing genetic diversity between and within populations, as well as between ecological environments population groups (Large-bodied populations from Sierra and Small-bodied populations from Doñana). This analysis provides genetic structure parameters in the form of F statistics (Wright, 1951; Excoffier et al., 1992). The significance of these statistics is assessed with the null distributions of the variance components, since both are highly correlated (Excoffier et al., 1992). Using the Arlequin software packages, were analyzed the allele frequencies, mean number of alleles per locus, and observed and expected heterozygosity. The significance of each of the variance components in subsequent analysis was estimated using 10,000 permutations. Genepop (version 3.4 online; Raymond and Rousset, 1995) was used to: i) analyze the population differentiation computing F_{ST} estimators (Weir and Cockerham, 1984) and their confidence intervals, ii) tests for linkage disequilibrium between loci using a Fisher exact test using Markov chains, and iii) analyze the existence of isolation-by-distance through Mantel tests carried out between matrices of log-transformed geographic distances and odds-transformed genetic distances ($F_{ST}/[1-F_{ST}]$; Rousset, 1997). Mantel Tests were also performed using *Isolation By Distance Web Service* (IBDWS) Version 2.5 (Jensen et al., 2005). Departures of the allelic frequencies from Hardy-Weinberg expectations were performed with the GENETIX Version 4.04 program (Belkhir et al., 2000) and allelic richness was estimated using the FSTAT program (Goudet, 1995). Finally, was conducted the analysis at a significance level of α = 0.05, and applied the Dunn-Sidak sequential correction of the level of significance for multiple tests (Sokal and Rohlf, 1995) when necessary.

RESULTS

No large differences between populations were found regarding genetic diversity and allelic richness (Table 1). Moreover, no allele had a frequency greater than 95%, which indicates that all analyzed loci were polymorphic. The mean number of alleles per locus ranged between 8 and 25 (*Bcal 2* and *Bcal 4*, respectively), while in the remaining loci were: *Bcal 1* = 23, *Bcal 3* = 17, *Bcal 4* = 25,

Table 1. Molecular variation in populations of *Epidalea calamita* from southern Spain. Populations (mean number of alleles), number of alleles per loci (NA), expected (H_e) and observed (H_o) heterozygosity, genetic diversity (GD) and allelic richness (AR). Significant deviations from Hardy-Weinberg equilibrium are marked with an asterisk.

Locus	L1 (11.1)			L2 (13)			I (9.7)			S1 (12)			S2 (12.4)			S3 (12.5)			S4 (15.2)		
	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o	NA	H_e	H_o
Bcal μ1	17	0.901	0.897	18	0.891	0.837	14	0.888	0.818	15	0.846	0.704*	14	0.884	0.777	13	0.863	0.885	14	0.855	0.785*
Bcal μ2	7	0.762	0.575*	5	0.500	0.138*	5	0.555	0.200*	5	0.651	0.232*	6	0.507	0.157*	6	0.527	0.416*	6	0.559	0.114*
Bcal μ3	11	0.871	0.281*	14	0.878	0.333*	10	0.879	0.272*	13	0.886	0.405*	15	0.902	0.342*	14	0.875	0.428*	14	0.898	0.323*
Bcal μ4	16	0.889	0.550*	17	0.880	0.555*	12	0.885	0.381*	16	0.862	0.780	15	0.894	0.522*	17	0.859	0.512*	18	0.801	0.642*
Bcal μ5	8	0.735	0.525*	12	0.752	0.767	10	0.792	0.736	12	0.818	0.697*	11	0.803	0.681	11	0.836	0.666*	14	0.860	0.795
Bcal μ7	11	0.807	0.717	13	0.792	0.613*	6	0.420	0.450	11	0.616	0.613*	14	0.752	0.600	13	0.717	0.717	13	0.773	0.744
Bcal μ10	9	0.854	0.906	11	0.840	0.795	9	0.807	0.727*	9	0.839	0.846*	11	0.832	0.750	10	0.810	0.815	8	0.817	0.825
Bcal μ11	10	0.756	0.240*	14	0.826	0.282*	12	0.883	0.181*	14	0.887	0.620*	13	0.851	0.410*	16	0.897	0.552*	15	0.920	0.700*
continuation:	GD	AR		GD	AR		GD	AR		GD	AR		GD	AR		GD	AR		GD	AR	
Bcal μ1	0.913	12.948		0.903	12.308		0.911	12.298		0.858	10.063		0.895	10.496		0.876	10.558		0.867	9.970	
Bcal μ2	0.777	5.825		0.513	4.019		0.588	5.000		0.664	4.831		0.519	5.255		0.537	4.709		0.574	5.641	
Bcal μ3	0.895	9.396		0.901	11.591		0.915	9.538		0.906	10.917		0.922	12.125		0.895	10.935		0.921	11.889	
Bcal μ4	0.905	11.969		0.897	11.400		0.920	10.959		0.874	11.265		0.910	12.094		0.875	11.283		0.813	11.268	
Bcal μ5	0.748	6.127		0.761	8.076		0.816	9.241		0.830	8.996		0.814	8.535		0.850	8.457		0.872	10.435	
Bcal μ7	0.820	8.420		0.804	9.089		0.430	5.236		0.623	6.924		0.763	8.291		0.727	8.643		0.783	8.468	
Bcal μ10	0.867	8.088		0.851	8.565		0.829	7.853		0.851	8.097		0.843	8.521		0.821	7.685		0.828	7.464	
Bcal μ11	0.783	8.652		0.845	9.845		0.921	11.274		0.908	12.174		0.868	10.248		0.914	11.952		0.935	12.912	

Bcal 5 = 19 and *Bcal 7* = 18. Additionally, the mean number of alleles per locus in each studied population ranged between 9.75 (I) and 12.75 (S4). No private alleles were found, indicating that each allele was found in two or more populations. However, allele frequencies significantly differed across populations for all loci. The analysis of linkage disequilibrium showed that only 19 of all possible comparisons between pairs of loci in each population (28 × 7 = 119) were significant for α = 0.05, which represented 9.6%. However, all of them lost their significance after applying the Dunn-Sidak significance level correction for multiple comparisons. Genepop estimates for pairs of loci taking all populations together did not yield any significant evidence of linkage disequilibrium (for α = 0.05), so the eight loci were treated as independent henceforth. Furthermore, were detected significant departures from Hardy-Weinberg expectations in different loci across all populations (Table 1). *Bcal 11* and *Bcal 2* were departed in all analyzed populations, while *Bcal 4* in six of them. The population with the highest number of loci (7) outside the H-W expectations was S1, while in S2 we found the lowest (4). The rest of the populations presented five loci outside the H-W balance. Observed heterozygosity in the studied loci was generally lower than expected under Hardy-Weinberg equilibrium, indicating a deficiency of heterozygotes.

Regarding the relation between geographic distance (km) and, estimated distance genetics (F_{st}) and gene flow (Nm) of the pairwise comparisons between populations are shown in Table 2. There were significant differences at 13 of the 21 pairwise comparison between populations, before Bonferroni sequential correction (shown in bold in the Table 2). Nevertheless, all of them lost statistical significance after the Bonferroni correction was applied (critical level of significance was P = 0.0023; Sokal and Rohlf, 1995). Neutral genetic distance among populations was not correlated with geographical distance (r = 0.140, P = 0.217), rejecting the hypothesis of isolation by distance (Fig. 2). Two groups were clearly differentiated, one containing the pairwise comparisons between nearby populations (SS or LL), and another group containing the pairwise comparisons of distant populations (SL or LS), however these two groups did not show significant differences in the mean values of genetic distances (Fig. 2). For example, the S4 population had similar genetic distances when compared with both its most distant population (L2, F_{st} = 0.056/110 km) and its closest one (S3, Fst = 0.042/19 km). I also did not find any significant covariation between geographic distance and gene flow (y = 2.027 - 0,003*x; r = -0.129; P = 0.578; r^2 = 0.017).

The two AMOVA performed showed that the 95.4% of overall variation was held within populations, where-

Table 2. Geographical distance (km), distance genetic (*Fst*) and gene flow (*Nm)* between populations of *E. calamita*. Significant values before Bonferroni sequential correction are shown in bold.

Pairwise comparison	Geographic distance	Genetic distance	Gene flow
S2-S3	5.5	**0.006**	41.7
S3-S4	19.4	**0.042**	5.86
S2-4	22.5	**0.039**	6.35
S1-I	22.5	0.094	2.65
L1-L2	26.1	**0.042**	5.88
S1-S4	26.8	0.06	4.14
S1-S2	27.9	**0.02**	12.13
S1-S3	30.3	**0.025**	9.66
S2-I	44.7	0.064	3.86
L2-I	45.9	**0.021**	11.39
S3-I	48.7	0.079	3.13
S4-I	49.1	**0.024**	10.18
L1-I	62.2	0.07	3.56
L2-S1	68.3	0.068	3.63
L1-S1	84.1	**0.032**	7.76
L2-S2	85.8	**0.046**	5.41
L2-S3	90.8	0.062	4.03
L2-S4	94.7	**0.025**	9.64
L1-S2	105.9	**0.015**	16.31
L1-S3	110.4	**0.038**	6.49
L1-S4	110.7	0.056	4.41

Table 3. Nested molecular analysis of variance, where df stands for degrees of freedom, SS for sum of squares, Varcomp for variance components, and %Var for proportion of total variance accounted for by each source. Environments are Hercinic (L1 and L2) and Sandy soils (S2-S4).

Source of variation	df	SS	Varcomp	% Var
Between environments	1	19.985	0.023	0.69
Among populations within environments	4	58.991	0.133	3.91
Within populations	508	1660.179	3.268	95.4
Total	513	1739.156	3.425	100

as the lowest variance components were associated to the differences between environments (Sierra Morena vs Doñana), which suggests a lack of population substructuring (Table 3).

DISCUSSION

This study showed the highest allelic diversity found in *Epidalea calamita* populations. This was greater than that previously found by Gomez-Mestre and Tejedo (2004) in Spanish populations, and even greater than that found in the northernmost populations of the specie distribution (Rowe et al., 1998; Beebee and Rowe, 2000) (Table 4). The differences in the allelic diversity between this study and that of Gómez-Mestre and Tejedo (2004), particularly in the S1 population (Fresh 1 in Gómez-Mestre and Tejedo, 2004), could be attributed to a significant increase in the sample size in this study. However, I found no difference in the L1 population (Fresh 3 in Gómez-Mestre and Tejedo, 2004) (Table 4). The lower allelic diversity found in the British populations also supports the hypothesis that the Iberian Peninsula constituted a Pleistocene refuge for *E. calamita* (as it was for other species in other Mediterranean peninsulas; Hewitt, 1996; Taberlet et al., 1998). From this refuge, the species would have expanded rapidly to north and east during the postglacial stage (Beebee and Rowe, 2000), resulting in a pattern of high levels of genetic diversity in populations derived from southern refuge and a progressive loss of diversity in recolonized areas to the north (Avise, 1994).

Despite the high variability, there were significant departures from Hardy-Weinberg expectations in different loci across all populations, due to deficiency of heterozygotes. It is possible that there is a certain degree of variability in the primer pairing regions, so that their sequence would not be completely homologous and would fail to amplify some alleles. In this case, by amplifying only one of the two alleles present, the proportion
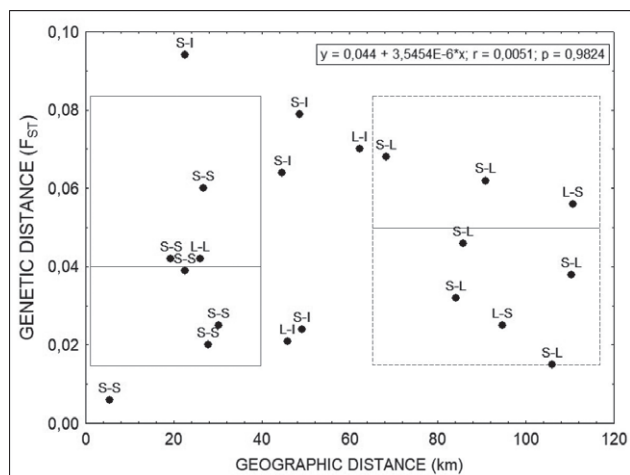

Figure 2. Pairwise comparisons between populations from the three environments studied, relating the geographic distance and estimated genetic distance. Two groups are clearly differentiated, one (inner square with continues lines) containing the pairwise comparisons between nearby populations (SS or LL), and another group (inner square with broken lines) containing the pairwise comparisons of distant populations (SL or LS). Nevertheless, mean values of genetic distances (middle lines) are not significantly different. L = large-bodied population (Sierra), I = intermediate and S = small-bodied population (Doñana).

Table 4. Genetic diversity. Mean number of alleles per locus (MAPL), percentage of polymorphic loci (P^{95}), and expected (He) and observed (Ho) heterozygosity of *E. calamita* throughout its distribution range. PS (in bold): present study, 1: Gómez-Mestre and Tejedo (2004), 2: Beebee and Rowe (2000). * and #, large and small-bodied population respectively, are showing the same population studied in PS and 1, using the same eight microsatellite loci.

Population	N	MAPL	P^{95}	He	Ho	Source
L1 (Spain)*	39	11.12	100	0.822	0.586	PS.
Fresh 3 (Spain)*	22	11.25	100	0.767	0.677	1
S4 (Spain)	**42**	**12.75**	**100**	**0.810**	**0.616**	**PS.**
S2 (Spain)	**45**	**12.37**	**100**	**0.803**	**0.530**	**PS.**
S3 (Spain)	**35**	**12.5**	**100**	**0.798**	**0.624**	**PS.**
S1 (Spain)#	**44**	**12**	**100**	**0.801**	**0.612**	**PS.**
Fresh 1 (Spain)#	22	10.5	100	0.698	0.581	1
L2 (Spain)	**43**	**13**	**100**	**0.795**	**0.540**	**PS.**
I (Spain)	**22**	**9.75**	**100**	**0.764**	**0.471**	**PS.**
Saline 1 (Spain)	23	9.38	100	0.664	0.626	1
Fresh 2 (Spain)	20	7.5	100	0.629	0.534	1
Velez (Spain)	11	4.88	100	0.689	0.607	2
Brittany (France)	32	4.38	87.5	0.491	0.355	2
Boulogne (France)	15	3.88	75	0.461	0.455	2
Ooy-Polder (The Netherlands)	40	5.13	87.5	0.520	0.466	2
Kerry (Ireland)	40	2.38	62.5	0.344	0.335	2
Merseyside (England)	40	2.63	62.5	0.294	0.295	2
Cumbria (England)	40	3.75	75	0.391	0.344	2
E/SE (England)	40	2.50	75	0.352	0.289	2
Texel (The Netherlands)	40	2.63	62.5	0.367	0.430	2
Sweden	40	1.63	25	0.119	0.144	2
Poland	40	2.00	62.5	0.245	0.283	2

of heterozygotes may have been underestimated, which would move the observed frequencies away from those expected for a Hardy-Weinberg equilibrium situation (Gómez-Mestre, 2001). However, we cannot rule out the homogenizing effect that gene flow (see below) may have in the departures from Hardy-Weinberg expectations observed. In addition, we could consider that the genes responsible for the expression of body size are potentially under selection and need to be studied.

Considering that some estimates of gene flow between populations were quite high, the lack of a relation between geographical distance and the degree of genetic differentiation (Fig. 2) is well exemplified by the L1 population. This population is genetically more similar to the S2 population, located 105 km apart, than to the L2 population, located only 26 km apart. As most of the observed variability corresponds to within-population differences (95.4%), this suggests that the populations are not structured. In addi-

tion neutral genetic differentiation cannot explain the geographical variation in body size observed, since only 0.6% of the total variance could be attributed to differences between the two environments.

The evolution and geographic variation of the Mediterranean herpetofauna has been influenced by a succession of geographic barriers to faunistic exchange over the last 23 myr (López-Martínez, 1989). The Guadalquivir River basin in the present study area has been suggested as major factor in the speciation processes in amphibians and as a barrier to dispersal of *Salamandra salamandra* (García-París et al., 1998). This intracontinental barrier has also been suggested as responsible of the geographic variation pattern in water salinity tolerance among *Epidalea calamita* populations in southern Spain (Gomez-Mestre, 2001). Nevertheless, since all our studied populations are all geographically located on the west bank of the Guadalquivir River, without population in the east, we cannot evaluate the hypothesis that considers the river as a barrier to gene flow.

A possible explanation for the lack of isolation-by-distance could be associated with the high dispersive capability that Bufonidae species can potentially present (e.g., 1.3 km/night, in *Rhinella marina*, Leblois et al., 2000), which could result in high gene flow despite populations being geographically distant. However, although this hypothesis is probable, it does not explain the high difference between closer populations (e.g., L1 and L2, or S3 and S4, Table 2). Alternatively, other less conspicuous physical barriers could exist between closer populations and need to be evaluated in futures studies. So, given the pattern of the gene flow observed in our study, there are some possible explanations for the maintenance of the geographical variation in the body size of *Epidalea calamita*. Populations subject to selection in two or more environmental patches, completely connected by gene flow, may develop phenotypic plasticity or adaptive reaction norms (Schmalhausen, 1949; Bradshaw, 1965), such that genetically similar individuals express different phenotypes in each environment. Then, it could be expected that the alleles that cause different phenotypes in *E. calamita* between Sierra and Doñana environments can evolve by natural selection, if the plastic response of the phenotype produces an increase in biological fitness (Via and Lande, 1985), as was demonstrated in *Pelobates cultripes* using reciprocal transplant experiments (Marangoni, 2006). This and other studies made in *E. calamita* and newts have shown that the dwarfism could be involved in response to some common environmental factor in Doñana (Díaz-Paniagua et al., 1996; Díaz-Paniagua and Mateo, 1999). It is clear that sandy soil substrates, directly or indirectly impose a strong effect (e.g., by imposing

high energetic costs of maintaining water balance or limiting availability of food resources; Marangoni, 2023), on adult body size, age and growth pattern (Marangoni et al. 2008, 2021). In addition, it could also be possible that gene flow would have prevented differentiation of these *E. calamita* populations for neutral loci, while intense selection would have maintained differences in adaptive traits of size and reproduction as has been suggested for other processes of adaptive divergence (Bensch et al., 1999; Gómez-Mestre and Tejedo, 2004). Recently, in the first study reporting genetic diversity estimates in the Iberian endemic pygmy newt (*Triturus pygmaeus*) from Doñana environment, Albert and García-Navas (2022) showed differences in genetic variability between temporary and permanent ponds. The authors suggested that the pond connectivity may constitute a more important factor than hydroperiod length in determining the genetic diversity and viability of pygmy newt populations. Moreover, given the geologically recent formation of the Guadalquivir basin, which may have occurred in mid-Holocene (about 5,000 years ago), it is possible that current genetic diversity patterns still reflect the historical distribution and gene flow among populations and that the effect of current gene flow (or lack thereof) is still not visible.

In conclusion, the neutral genetic differentiation cannot explain the geographical variation in the body size of natterjack toad, in accordance with the previous study by Gomez-Mestre and Tejedo (2004). Therefore, it is suggested that future studies are needed on the interaction between the genetic component with the environmental factors, and life history traits (e.g., age and growth pattern, Marangoni et al., 2021; food resources, Marangoni, 2023) at both larval and juvenile stages.

Finally, it is considered necessary to investigate other potential source of both within- and between-population components of variance, in addition to purely additive genetic variance, since that preliminary analyses showed that maternal effects may potentially contribute to the origin and evolution of the geographical variation in body size observed in *E. calamita* (Marangoni, unpublished data), and others amphibians (Bernardo, 1996; Mousseau and Fox, 1998; Räsänen et al., 2003, 2005).

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Interpopulation variation in prebreeding energy reserves of plateau brown frog (*Rana kukunoris*)

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Abstract. The energy storage plays a prominent part in the life histories of many animals inhabiting cold harsh environment, thus anuran amphibians rely on larger residual energy stores from post-hibernation to cope with unfavourable weather conditions before breeding. As the habitat conditions often change in different locations, selective pressure for energy storage could also vary. However, we still have limited knowledge on whether anurans living under different environmental backgrounds would adopt suitable strategies to cope with energy demands during reproduction. Here, we examined patterns of energy storage in spring (after hibernation and before reproduction) in seven plateau brown frog (*Rana kukunoris*) populations with different climatic conditions in the eastern Tibetan Plateau. After removing the effects of age and body size, liver weights of both sexes and fat body weights in males were negatively correlated with temperature, suggesting that these reserves were critical for this anuran to survive through harsh and extended dormancy period under uncertain environmental conditions. Additionally, reproductive investment in both sexes could partly explain the increase in post-hibernation energy stores with decreasing temperature. Males had relatively heavier liver and fat body weights than females, indicating that males store more energy reserves for breeding activity compared with females. Therefore, we suggest that both the variation of environmental conditions and sexual differentiation of reproductive roles play a prominent role in shaping the energy allocation and reserve patterns in the dormancy and breeding of amphibian species.

Keywords. Energy storage, post-hibernation, plateau brown frog, Tibetan plateau.

INTRODUCTION

Acquisition and expenditure of energy are fundamental factors influencing life history traits intimately linked with fitness and population viability (Jönsson et al., 2009). Populations from environments with different constraints often evolve different patterns of energy demands and acquisition to adapt to local environmental conditions. Since the active period to breed and forage between dormancies are limited (15-30 days) for amphibians living at temperate zones or higher altitudes (> 2000m asl), energy storage strategies were expected to evolve. Therefore, energy storage plays a prominent part in the life histories of many animals inhabiting cold environments (e.g., high-altitude or high-latitude, Wells, 2007).

The organisms are usually classified as either income breeders (e.g., feeding after emergence and before breeding) or capital breeders (e.g., refrain from feeding until after breeding) based on the origin of the energy (Stearns, 1992; Jönsson, 1997; Drent and Daan, 1980). Capital breeding was widely found among ectothermic vertebrates living in cold environments that they reproduce immediately after a long hibernation (Wells, 1977; Chen et al., 2018). The energy stores before the dormancy were critical for their survival and reproductive success through the harsh winter and early spring. Generally, amphibians, such

as burrowing amphibians or species with hibernating in water can store energy in the form of lipids, proteins and carbohydrates, but lipids should be the most important energy form because the carcass, fat bodies of abdomen and liver deposit high amounts of fat (Fitzpatrick, 1976).

Populations within species living in different environments face the environmental constraints and challenges, which may be highly variable (Gasc et al., 1997; Peng et al., 2020). In this case, high energy reserves represent a general tactic to cope with stochastic energy supply or predicted energy shortages (McNamara and Houston 1990; Jönsson, 1997). The energy demands and acquisition can determine overwintering survival rate and breeding success (Bodie and Semlitsch, 2000; Wells, 2007), compared with warm environments, anurans living in cold environments require more accumulated energy to survive the harsh and extended winter and subsequently breed in spring. Also, more residual energy stores for reproduction at post-hibernation emergence might be expected at areas with lower annual average temperature because there is a higher probability of occurrence of harsh weather conditions during early springs.

Furthermore, differences in the time of energy allocation towards reproduction between the sexes are expected to explain for sexual differences in energy reserve before breeding (Jönsson et al., 2009; Chen et al., 2011a). For example, female amphibians allocate their main energy into egg growth prior to hibernation (Lu et al., 2008; Jönsson et al., 2009), most females enter the pond unamplexed, and usually leave the pond immediately after depositing eggs. Conversely, males invest energy into breeding activities after hibernation, arriving the breeding ponds is earlier than females, usually search actively or engage in physical struggles for females until the end of the breeding season. Thus, males need larger residual energy stores and allocate more energy for demanding activities during the breeding season than females do (Wells, 1977). So far, there are limited studies exploring the patterns of energy storage based on comparisons of different populations with varied environmental conditions (see Elmberg, 1991; Elmberg and Lundberg, 1991; Chen et al., 2011a), thus we have a very poor understanding of how energy stores in the life histories varies among populations of amphibian species.

The aim of this study was to analyze the patterns of energy stores in breeding the plateau brown frog (*Rana kukunoris*) populations along an altitudinal gradient in the eastern Tibetan Plateau. This species is endemic to the eastern Tibetan plateau, inhabiting many different habitats, and their habitats are widely distributed from 2200 to 4400 m in altitude (Chen et al., 2023; Chen et al., 2023a), and thus remarkably different environmen-

tal stresses on energy stores and investments might be expected. This species is also a typical explosive breeder with shorter active period in colder regions (Wells, 2007: Chen et al., 2011b). Further, males stay in the spawning site during breeding season and do not feed, while they are forming a chorus and actively search and competing for females (Yu et al., 2013). Most of unamplexed females enter the spawning site, and usually mate with male on the same day and leave the spawning site immediately after oviposit. In this study, *R. kukunoris* enter another dormancy period after breeding because the environmental condition (e.g., cold temperature) is not suitable for individuals to feed and survive. Thus, the more energy storage before hibernation or residual energy stores for dormancy is very important for their survival and reproductive success. Here, we explored differences in organ size of liver and fat bodies in *R. kukunoris* across seven populations along an altitudinal gradient. We predicted that 1) energy stores should decrease with temperature rather than altitude since the two factors might not be strongly correlated in our studies, and 2) males should have greater energy storage before reproduction compared with females due to differences in allocation of breeding energy investment during varied periods.

MATERIALS AND METHODS

Study site and sample collection

We collected *Rana kukunoris* individuals from seven populations (elevations ranging from 2506 to 3478 m, Fig. 1; Table 1, 2) along the eastern Tibetan Plateau, China. The beginning of the breeding period for *R. kukunoris* tends to be later at higher altitudes (2297 m site: mid-March, 9 days; 2594 m site: late-March, 10 days; 2789 m site: mid-April, 15 days; 3049 m site: late-March, 11 days; 3060 m site: late-March, 10 days; 3233 m site: early-April, 11 days; 3441 m site: mid-April, 15 days, Table 1). Following the protocol of Chen et al. (2013), the frogs begin to be active when the mean daily ambient temperature is close to 6 °C. Thus, six degrees Celsius could be a threshold which is similar across study sites (unpublished data). In our study site, the length of activity period was calculated by estimating number of days with a mean daily temperature above 6 °C (Table 1). In fact, we found that *R. kukunoris* has a short dormancy period after reproduction because low temperature is not suitable for feeding activities and prey is few. Here, the length of dormancy period was calculated by estimating number of days from after breeding until mean daily temperature above 6 °C, when the length of hibernation period was calculated by estimating number of days from mean daily tempera-
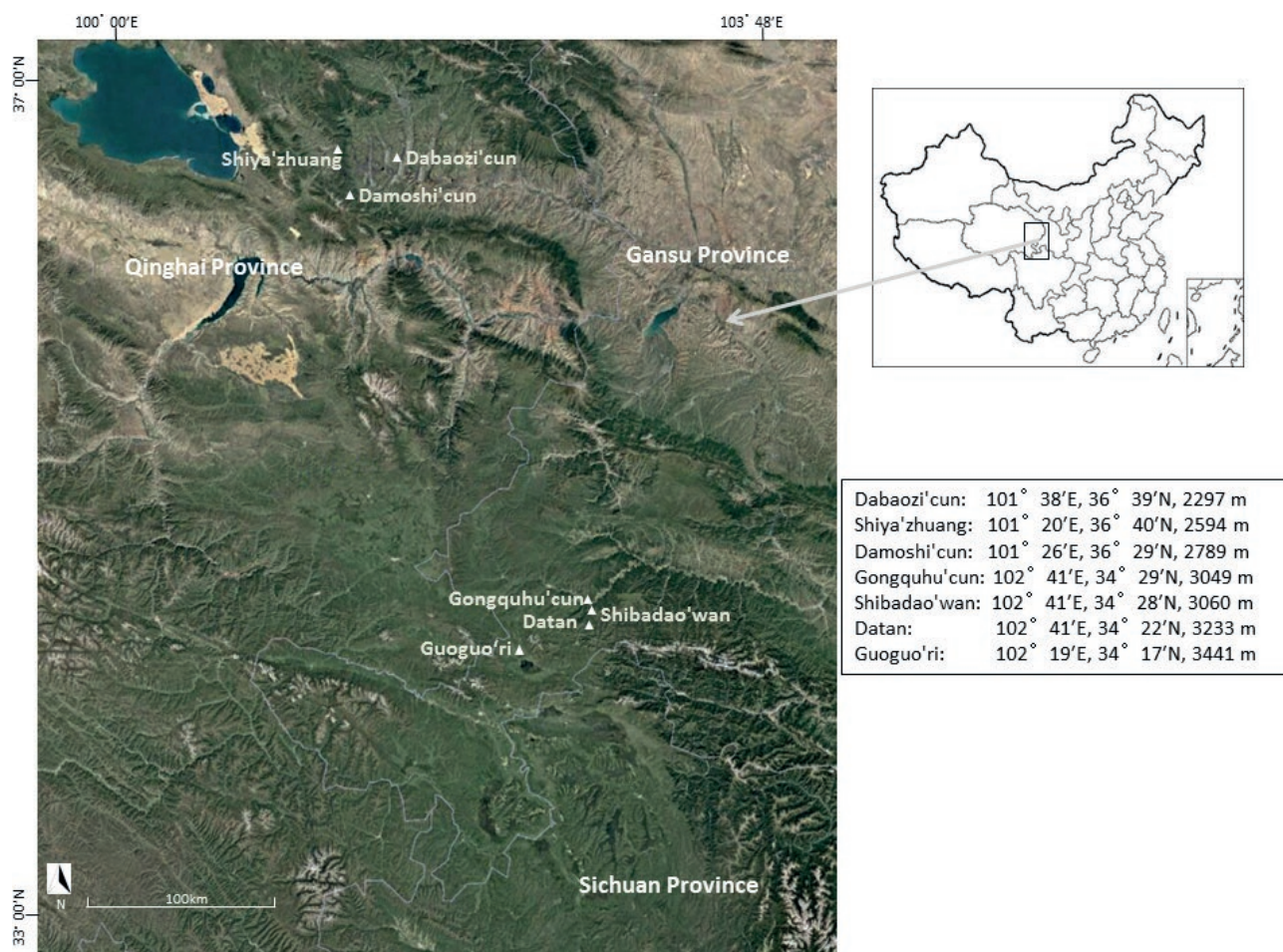
Fig. 1. Topographic map showing the location of the seven *Rana kukunoris* study populations in the eastern Tibetan plateau.

ture below 6 °C until before breeding. We randomly collected 10–97 individuals in the ponds or swamps by hand before spawning period (males haven't form a chorus and females are unamplexed), just after their emergence from hibernation at each site. Then, the frogs were identified preliminarily as adult males when the fore digits were discovered bulging brown nuptial pads (SVL > 35mm), others as females (which had large abdomen, indicative of well-developed oocytes; SVL > 40mm). All captured frogs were brought to our field lab near spawning sites. However, at the highest altitude (Guoguo'ri), all the females ran away because the plastic bucket containing the female was broken on the way back to the lab. At room temperature, they were put into individual plastic opaque containers (diameter = 16.75 cm), filled 2 cm deep with fresh water. Then, the snout-vent length (SVL, to the nearest 0.1 mm) was measured with a vernier caliper, and body mass (to the nearest 0.01 g) was weighed with an electric balance. Subsequently, frogs were maintained in fresh water for

48 hours to allow full hydration before being euthanised with an overdose of TMS (Tricaine methane sulfonate, CAS: 886-86-2, Purity: > 97.0%, Sigma-Aldrich). The liver and fat bodies were divided and weighed to the nearest 0.1 mg with an electronic balance after placement on water-absorbing paper for around five minutes (Lu, 2004). Finally, the determination of sex and sexual maturity was confirmed by the condition of the gonads at autopsy.

Age determination

We removed the longest phalange of the left hindfoot of adults from each population and preserved in 10% aqueous solution of formaldehyde. Following the protocol of Ma et al. (2009), we produced histological sections of the frog phalanges and counted the number of lines of arrested growth (LAG) in the sections to determine age. Numerous studies have confirmed that improved method of Ehrlich's haematoxylin stain and paraffin section dis-

Table 1. Characteristics of the sample-collecting sites in the east Tibet Plateau, China.

Population	Altitude (m)	Latitude (degrees)	Annual mean temperature (°C)	Hibernation period (days)	Breeding period (days)	Dormancy period (days)	Activity period (days)
Dabaozi'cun	2297	36.65	5.30	152	9	14	190
Shiya'zhuang	2594	36.68	3.50	168	10	17	170
Damoshi'cun	2789	36.49	0.20	196	15	24	130
Gongquhu'cun	3049	34.49	1.50	187	11	17	150
Shibadao'wan	3060	34.47	1.40	191	10	19	145
Datan	3233	34.37	1.00	193	11	21	140
Guoguo'ri	3441	34.29	0.80	193	15	22	135

Table 2. Comparisons of SVL, body mass, age, liver, and fat body of *Rana kukunoris* from seven altitudes in the east Tibet Plateau, China. Values represent mean ± SE for each measure. n = number of individuals.

Population	Sex	n	Collected frogs/ the total frogs (%)	SVL (mm)	Body mass (g)	Age (years)	Liver (mg)	Fat body (mg)
Dabaozi'cun	Female	7	7.29	61.71 ± 1.96	32.07 ± 3.64	4.00 ± 0.31	412.50 ± 91.92	85.66 ± 17.72
	Male	17	17.71	54.70 ± 0.90	17.38 ± 0.86	2.82 ± 0.13	492.86 ± 38.14	90.05 ± 25.48
Shiya'zhuang	Female	13	9.29	58.20 ± 1.35	28.93 ± 2.50	4.00 ± 0.25	201.49 ± 40.84	5.57 ± 2.04
	Male	39	27.86	51.24 ± 0.58	18.35 ± 0.62	3.05 ± 0.10	511.41 ± 23.15	65.91 ± 24.00
Damoshi'cun	Female	44	23.16	56.12 ± 0.61	25.58 ± 0.92	3.48 ± 0.12	376.10 ± 18.75	42.63 ± 6.35
	Male	53	27.89	50.25 ± 0.58	15.39 ± 0.69	2.77 ± 0.09	526.45 ± 30.29	83.28 ± 8.23
Gongquhu'cun	Female	18	26.47	59.82 ± 0.62	32.53 ± 1.26	4.06 ± 0.13	379.56 ± 18.37	8.33 ± 3.25
	Male	19	27.94	54.57 ± 0.59	18.23 ± 0.79	3.32 ± 0.13	586.21 ± 33.28	81.81 ± 10.78
Shibadao'wan	Female	9	21.43	60.42 ± 1.43	31.72 ± 2.10	4.11 ± 0.26	155.60 ± 12.51	3.28 ± 0.74
	Male	11	26.19	51.23 ± 0.90	14.74 ± 0.72	3.18 ± 0.12	421.87 ± 38.01	39.04 ± 7.15
Datan	Female	30	24.59	56.44 ± 0.52	26.82 ± 0.95	3.97 ± 0.12	190.39 ± 13.68	16.02 ± 3.34
	Male	24	19.67	47.40 ± 0.70	12.46 ± 0.41	2.92 ± 0.12	359.83 ± 16.93	44.17 ± 6.25
Guoguo'ri	Male	10	8.26	51.74 ± 098	17.56 ± 0.94	3.20 ± 0.13	515.00 ± 30.10	51.37 ± 7.33

play seasonal growth of amphibian species (e.g., Yu and Lu, 2013; Yu et al., 2019; Yu et al., 2021).

Environmental factor collection

The annual mean temperature did not decreased significantly with elevation (Spearman's correlation: r_s = -0.643, P = 0.119), and latitude (r_s = 0.607, P = 0.148). Thus, we used annual mean temperature as environmental factor in this study. Temperature was obtained from WorldClim (the period of 1950–2000; http://www.worldclim.org) at a resolution database of 0.167° × 0.167° grid cells (Hijmans et al., 2005).

Statistical analyses

Prior to analyses, the organ weights, body mass, SVL and age were log transformed to approximately conform to normality. Then, we performed a General Linear Model (GLM) to test whether absolute weights of both liver and fat body differed across populations and between sexes, where population and sex as fixed factors, including an interaction between sex and population. If the overall GLM results were significant, we did pairwise multiple comparisons (post hoc LSD tests) to evaluate differences among populations. To further investigate the differences in relative organ weights across populations and between sexes, we performed a GLM treating weights of liver and fat bodies as dependent variable, population and sex as fixed factors, body mass and age as a covariate. To test the effects of variation in temperature on relative organ weights, we also used GLM treating the organ weights as dependent variables, variation in temperature, body mass and age as a covariate. SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

RESULTS

Absolute weights of both liver and fat body variation

We detected significant differences in absolute weights of both liver and fat body among populations and between sexes (Table 2, 3). Post hoc LSD tests exhibited those frogs from population Datan, Shibadao'wan and Shiya'zhuang, had respectively lesser absolute weights of both liver and fat body, compared with population of Gongquhu'cun, Dabaozi'cun and Damoshi'cun, respectively (Fig. 2). Males had heavier livers (t_{292} = 10.824, P < 0.001) and fat bodies (t_{292} = 11.297, P < 0.001) than females. Moreover, larger individuals had heavier livers ($F_{1,284}$ =33.380, P < 0.001), but not heavier fat bodies ($F_{1,284}$ = 3.021, P = 0.083).

Relative weights of both liver and fat body variation

The significant differences in relative weights of both liver and fat body were observed among populations and between the sexes (Table 4). For female, the population at Shibadao'wan, Shiya'zhuang and Dabaozi'cun, had respectively the smallest relative weights of both liver and fat body whereas frogs from population Gongquhu'cun, Dabaozi'cun and Datan had respectively the heaviest weights of liver and fat body (Fig. 2). For male, relative weights of liver and fat body were the smallest in population Dabaozi'cun, Shiya'zhuang and Shibadao'wan, whereas population Gongquhu'cun, Damoshi'cun and Shiya'zhuang were characterized by the largest weights of liver and fat body, respectively (Fig. 2). Generally, males had relatively heavier livers (t_{292} = 12.861, P < 0.001) and fat bodies than females (t_{292} = 8.406, P < 0.001). Moreover, the relative weights of liver in each sex and relative weights of fat body in male were negatively correlated with temperature (Fig. 3; Table 5).
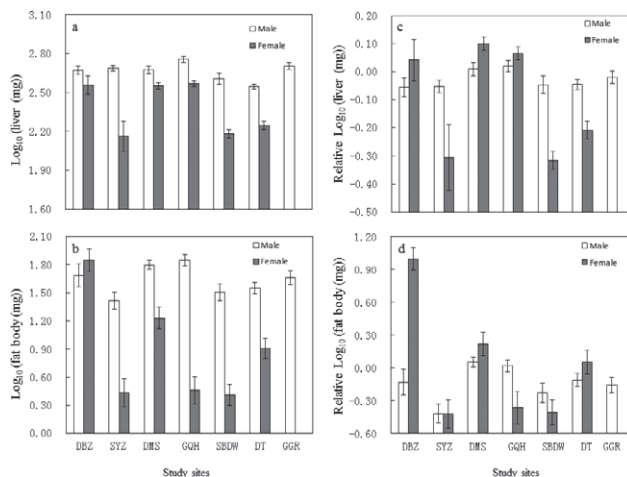

Fig. 2. Absolute liver mass (a), fat body mass (b), relative liver mass (c), and relative fat body mass (d) among seven populations in *Rana kukunoris* (DBZ: Dabaozi'cun, SYZ: Shiya'zhuang, DMS: Damoshi'cun, GQH: Gongquhu'cun, SBDW: Shibadao'wan, DT: Datan, GGR: Guoguo'ri). Relative organ mass represents the residual mean value for a given population corrected for the effect of body size.

DISCUSSION

Anurans living in colder regions usually store more energy after hibernation and before breeding (Jönsson et al., 2009). Our results supported this prediction: when removing the effects of age and body size, liver weights in both sexes and fat body weights in male were negatively correlated with temperature.

Here, larger energy stores in cold environments could be that anurans need chronically plenty of energy

Table 3 The generalized linear model for liver and fat body among the seven high-altitude populations of *Rana kukunoris* along the eastern Tibetan Plateau.

Response variable	Source of variation	df	MS	F	P
Liver	Intercept	1	1112.437	31237.714	< 0.001
	Sex	1	4.172	117.160	< 0.001
	Population	6	0.448	12.569	< 0.001
	Error	286	0.036		
Fat body	Intercept	1	270.373	943.468	< 0.001
	Sex	1	36.575	127.628	< 0.001
	Population	6	2.358	8.227	< 0.001
	Error	286	0.287		

Table 4 The generalized linear model for liver and fat body (with age and body mass as a covariate) among the seven high-altitude populations of *Rana kukunoris* along the eastern Tibetan Plateau.

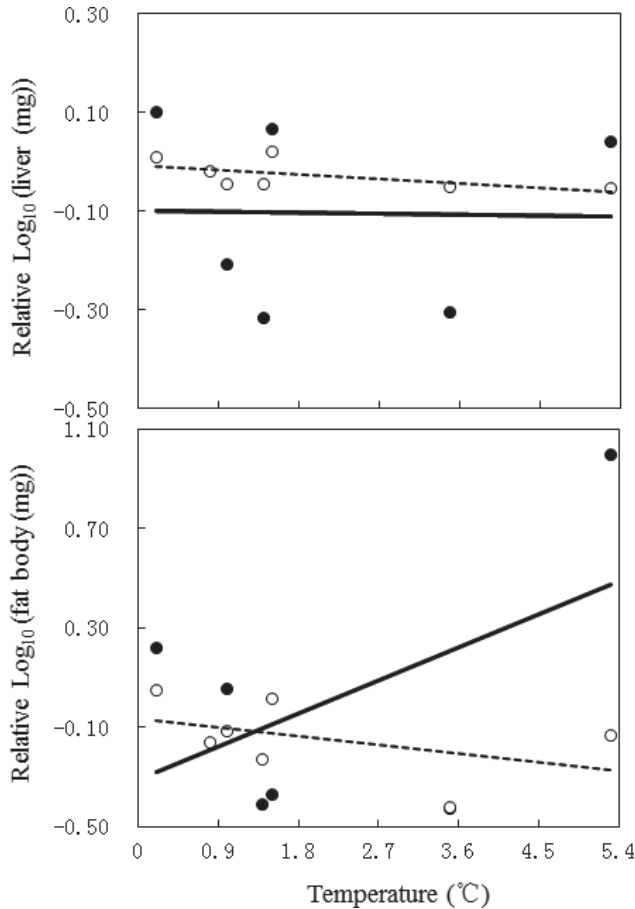| Response variable	Source of variation	df	MS	F	P
Liver	Intercept	1	5.090	163.409	< 0.001
	Sex	1	5.077	162.986	< 0.001
	Population	6	0.387	12.414	< 0.001
	Age	1	0.004	0.133	0.716
	Body mass	1	1.040	33.380	< 0.001
	Error	284	0.031		
Fat body	Intercept	1	1.022	3.580	0.059
	Sex	1	20.168	70.665	< 0.001
	Population	6	2.209	7.741	< 0.001
	Age	1	0.421	1.473	0.226
	Body mass	1	0.862	3.021	0.083
	Error	284	0.285		

Fig. 3. The effect of annual mean temperature on mean organ mass in males and females from seven populations of *Rana kukunoris* along the eastern Tibetan plateau. Solid and dashed lines are fitted lines for females and males, respectively.

Table 5 The generalized linear model for the effects of temperature on relative organ size of energy storage among the seven high-altitude populations of *Rana kukunoris* along the eastern Tibetan Plateau.

Sex	Response variable	Source of variation	Slope	SE	t	P
Female	Liver	Intercept	1.691	0.293	5.767	< 0.001
		Temperature	-0.042	0.017	-2.467	0.015
		Age	-0.120	0.327	-0.367	0.714
		Body mass	0.588	0.253	2.327	0.022
	Fat body	Intercept	2.332	0.823	2.834	0.005
		Temperature	0.013	0.048	0.276	0.783
		Age	-2.649	0.917	-2.889	0.005
		Body mass	0.065	0.709	0.092	0.927
Male	Liver	Intercept	1.634	0.106	15.363	< 0.001
		Temperature	-0.013	0.006	-2.028	0.044
		Age	-0.165	0.126	-1.310	0.192
		Body mass	0.947	0.103	9.221	< 0.001
	Fat body	Intercept	0.568	0.318	1.790	0.075
		Temperature	-0.072	0.019	-3.767	< 0.001
		Age	-0.127	0.377	-0.338	0.736
		Body mass	1.061	0.307	3.458	0.001

Sceloporus undulates between higher latitude and altitude population. Thus, Chen et al. (2013) suggested that liver weights of *R. kukunoris* in increased high altitudes where indicate low temperature might be an adaptation to the hypoxic aquatic environment in cold regions.

Another possible explanation is allocating more energy for reproduction in cold environments. Due to limited food sources, intense interspecific competition and greater annual mortality risk, females living in cold environments were more likely to allocate more energy to current breeding efforts (Chen et al., 2013). For example, females of *R. kukunoris* living in high-altitude environments favours allocating more energy for maternal investment independent of body size, which cope with the unfavorable and unpredictable weather conditions (Chen et al., 2013). Furthermore, female frogs must utilize stored energy to support early egg development due to lacking of a resting period, indicated that females rely on a single tactic of capital breeding, thus the energy storage should be larger in cold populations. Instead, testis mass of *R. kukunoris* tend to increase with temperature, suggesting that reproductive investment in males is not high in cold environments (Yu et al., 2022). However, we found that the length of breeding period is negatively correlated with temperature (r_s = −0.909, n = 7, P = 0.005, Table 1), indication of longer breeding period in cold populations. We speculated that relatively stable warm temperatures in low altitudes promote synchro-

storages to survive the harsh and extended hibernation period. However, *R. kukunoris* enter another short dormancy period after breeding because the environmental condition (e.g., cold temperature) is not suitable for individuals to feed, which could be one possible explanation for more residual energy stores. Further, length of dormancy period decreased with increasing temperature (r_s = −0.991, P < 0.001, Table 1), indication of longer dormancy period in cold populations. Hence, they must deposit sufficient energy to survive the harsh and extended dormancy period. Additionally, larger energy reserves are necessary for amphibians living with less predictable environmental conditions because more extreme weathers (e.g., temperature plummet) would occur right after their last dormancy (McNamara and Houston, 1990; Jönsson, 1997; Jönsson et al., 2009; Chen et al., 2011a). In accordance with this, Niewiarowski (2001) reported also detected a similar pattern for the eastern fence lizard

nous breeding of most toads, resulting in shorter breeding period. In this case, males living at cold environments need more energy reserves to calling and breeding activities in early spring. Therefore, maternal investment in both sexes could partly explain pro-hibernation energy stores increased with decreasing temperature.

Considering the difference between sexes in the timing of the main energy allocation for reproduction, females allocate most of their energy storage in egg development before the mating period, while males put more of their energy investment into their mating efforts during the mating period (Jørgensen, 1981; Jönsson et al., 2009; Chen et al., 2023a,b). Thus, sexual differences in energy stores might be expected after hibernation. Our results showed that males had relatively heavier livers and fat bodies males than females. For capital breeding anurans, in adult females primarily exploit fat bodies to provide energy for the developing gonads (Jørgensen et al., 1979; Jönsson et al., 2009). Here, female *R. kukunoris* finish their main follicular growth before hibernation, and their gonad weight increases before overwintering (Chen et al., 2013), whereas adult males utilize the fat bodies to conduct breeding activities in spring (Yu et al., 2013; Yu et al., 2016; Yu et al., 2018). Thus, males may have more energy reserves prior to breeding than females.

In general, we found that *R. kukunoris* from cold environments had more energy storages after hibernation prior to breeding than individuals from warm environments. Parental investment in both sexes could partly explain pro-hibernation energy stores increased with decreasing temperature. Additionally, these reserves were also used for surviving the harsh and extended dormancy period, indicating of buffering against uncertain environmental conditions. Males had more energy reserves than females due to differences in energy allocation in varied reproductive periods. Therefore, we suggest that a combination of environmental conditions and reproductive roles is the important to analyses energy allocation and reserve patterns in breeding amphibian species.

ACKNOWLEDGMENTS

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Intra- and inter-drainage variation in population structure, body condition, shape morphology and sexual dimorphism in *Mauremys leprosa saharica* from southern Morocco

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Abstract. The Mediterranean pond turtle *Mauremys leprosa* is widely distributed in Morocco. Significant variations could be observed in this species due to the fact that it occupies a vast and environmentally diverse area. Thus, the analysis of population parameters is crucial in elucidating the differences between populations. Differences between individuals may be linked to environmental factors, including many habitat characteristics. In the present study, variation in population structure, body condition, morphology and sexual dimorphism, were examined for the Sahara Desert blue-eyed turtle *M. l. saharica* in southern Morocco from seven distinct localities distributed in four different drainages in southern Morocco. A total of 224 turtles were captured, sexed, weighed and then measured for their carapace and tail dimensions before being released. Among the studied populations, four were dominated by adult individuals (Taakilt: 96%, Oued Guir: 87%, Oued Ziz: 75%, Sidi ElMehdaoui: 70%), two comprised 100% of adults (Oued Noun and Tarmigt) and one population consisted of 50% adults (Lower Draa). The sex ratio was significantly different between populations, being balanced in two populations (Tarmigt and Sidi El Mehdaoui), male-biased in three populations (Oued Zizi, Oued Noun, Lower Draa) and female-biased in the two remaining populations (Oued Guir and Taakilt). Body Condition Index (BCI, g/cm³) was significantly different among populations but not between sexes, or for the Sex × Locality interaction. The Sexual Size Dimorphism (SSD), females being larger than males, varies among population with the greatest degree observed in Oued Guir population (SSD = 0.41) and the lowest in Sidi El Mehdaoui population (SSD = -0.14). Length-Weight Relation (LWR) shows that *M. l. saharica* has isometric growth for both male and female of Oued Guir and Tarmigt, for female of Oued Ziz, Taakilt, Oued Noun and lower Draa and hypo-allometric for male of Oued Ziz, Taakilt, Oued Noun, Lower Draa and for both male and female of Sidi El Mehdaoui. Morphological variation of carapace is shown in this species associated with population variation among basins. Increased understanding of population characteristics and their fitness status must be considered in conservation and management action plans for the species and its habitat.

Keywords. Structure, morphology, sexual dimorphism, *M. l. saharica*, southern Morocco.

INTRODUCTION

Freshwater turtle species are still poorly understood, and many species are in decline due to unsustainable trade as well as human alteration of freshwater ecosystems (Palacios et al., 2015). These species are strongly linked to their habitats and consequently greatly affected by their deterioration: fragmentation and degradation. In the arid regions of North Africa, turtles are faced with extreme environmental conditions of arid climate and anthropogenic and climate change-mediated water and land salinization.

The Mediterranean pond turtle, *Mauremys leprosa*, Geoemydidae family, is a freshwater turtle species that mainly inhabits streams and ponds with riparian vegetation (Da Silva, 2002). This turtle species is found in southwestern Europe and northwestern Africa (Da Silva, 2002). In Europe, its distribution is limited almost exclusively to the Iberian Peninsula and includes a small area in southern France in the Eastern Pyrenees. In the northern limit, *M. leprosa* apparently presents a wide distribution although it appears in small and fragmented populations (Llorente et al., 1995; Da Silva and Blasco, 1997; Rivera et al., 2011). Populations in Northwest Africa are often isolated from one another by intervening arid terrain, and a complex pattern of local variation in shell markings has occurred (Bertolero and Busack, 2017). *Mauremys leprosa* tolerates brackish to saline water, and has a high tolerance for polluted freshwater habitats, reduced water levels, and elevated ambient temperatures; carnivorous by preference, it can also feed freely upon vegetation and has been reported ingesting nitrogenous animals (freshwater fish, amphibian larvae…) and human wastes (Bertolero and Busack, 2017). Two subspecies are currently recognized: *M. l. leprosa* (Mediterranean Pond Turtle) (distribution: from northwestern Morocco through the Iberian Peninsula to southern France) and *M. l. saharica* (Saharan Pond Turtle) (distribution: from southern and eastern Morocco through Algeria to northwestern Libya, with scattered populations in the northern Saharan margin).

In Morocco, *M. leprosa* is characterized by a great ecological valence occupying all available aquatic environments (streams, rivers, ponds…) and can tolerate clear, eutrophic, brackish or excessively polluted waters. Across most of its range, *M. leprosa* is currently considered threatened by habitat fragmentation and/or destruction, alien species, pollution, aquifer water extraction, with less significant threats from harvesting for the pet trade (Pleguezuelos and Feriche, 2003; Polo-Cavia et al., 2011) and pathogens (Hidalgo-Vila et al., 2008; Verneau et al., 2011). For these reasons, *M. leprosa* is listed as vulnerable in Europe on the IUCN Red List (version 13.2) (Van Dijk et al., 2004) and is listed in Appendix II of the Berne Convention and in Appendix II and IV of the Habitat Directive (92/43/CEE) (Cox and Temple, 2009). The different threats that the Mediterranean pond turtle faces across its distribution area could lead to an important population stress due to forced adaptations to changing environment contributing thus to marked reductions in the size of populations (Glynn, 1988; Hoffmann and Pearsons, 1991).

The study of population structure is an important parameter of the life history patterns of animals often related to ecological and ethological aspects of the individuals (Peters, 1986; Schmidt-Nielsen, 1984; Roff, 1992; Stearns, 1992). Differences between males and females in body size and morphology (sexual dimorphism) are common among reptiles (Dunham et al., 1988; Shine, 1989; Randriahamazo, 2000). Particularly, body size and sexual dimorphism have been extensively described in turtles (e.g., Berry and Shine, 1980; Iverson, 1985; Gibbons and Lovich, 1990; Lambert, 1995; Zuffi and Gariboldi, 1995; Yasukawa et al., 1996; Ernst et al., 1998; Graham and Cobb, 1998; Willemsen and Hailey, 1999; Zuffi et al., 1999; Ayres and Cordero, 2001; Bonnet et al., 2001; Boone and Holt, 2001). Body size of turtles can vary significantly at the intraspecific level between geographic locations, which is usually explained by phenotypic plasticity, or local adaptation (Gibbons and Lovich, 1990; Rowe, 1997).

Body condition (mass per unit of volume) is an important determinant of an individual animal's fitness. Many authors have addressed the relationship between body condition and ecological parameters such as survivorship, reproductive investment, parasite load or investment in characters used in sexual display in a wide range of studies of amphibians (Reading and Clarke, 1995), reptiles (Bradshaw and Dèath, 1991; Cuadrado, 1998), birds (Carranza and Hidalgo De Trucios, 1993; Dufva, 1996) and mammals (Dobson and Michener, 1995). The variation in morphometric parameters is generally induced by genetic and environmental factors that may have an effect on growth process and then on the species survival (Barlow, 1961; Somers, 1986). Thus, the morphological analysis of intra-population variation is crucial in elucidating the characteristics of a population. The morphological differences among individuals may be linked to environmental factors, including air and water temperatures and other habitat characteristics (Litzgus and Smith, 2010), or to historical factors limiting gene flow among the basins (Clavijo-Baquet et al., 2010). However, turtles and tortoises are known with the existence of morphological differences (Germano, 1993; Packard et al., 1999). Turtles are a model clade for which to study sexual size dimorphism (SSD), as this attribute varies dramatically across species. Sexual Size Dimorphism is a widespread phenomenon

among plants and animals that often results from differential selection operating on different body sizes between males and females (Fairbairn, 1997). Biologists generally have explained these differences in terms of sexual selection (Darwin, 1874; Trivers, 1972; Ghiselin, 1974). Body size is among the most frequently used variables used to quantify the sexual dimorphism because it is a fundamental property of organisms in anatomy, ecology, physiology… (Peters, 1986; Calder III, 1996; Cardillo et al., 2005; Lynch, 2007). The standard body size measurement in turtles is the carapace length (CL), a linear measurement of the dorsal shell. Carapace length is often considered as a stable measurement of size across turtles, with little or no apparent seasonal or daily variation (Regis and Meik, 2017). Most turtle species are female-biased in carapace length. Despite this trend, the SSD varies within most families even within genera and species (Lovich et al., 2010). In *M. leprosa*, females are the largest sex. Berry and Shine (1980) suggested that the smaller body size of males in most species turtles could be attributed to the low importance of intrasexual selection processes in turtles. It has been suggested for other species of turtles and tortoises that the small size of one of the sexes can also be related to age at maturity (Gibbons and Lovich, 1990). In *M. leprosa*, males attain sexual maturity at smaller sizes than females (Keller, 1997), and this could be one of the causes of the SSD in this species. Apart from two studies (Meek, 1987; Lovich et al., 2010), there were no investigations on the Saharan and sub-Saharan populations. In this study, we reported on the characteristics of seven Saharan populations (including population structure along with shape morphology, body condition and sexual dimorphism) of *M. leprosa* from southeast to southwest Morocco.

MATERIALS AND METHODS

Study site

The Mediterranean Stripe-necked Terrapins, *M. leprosa*, were collected during spring period from seven sites in southern Morocco (six sites located at different oueds [rivers] belonging to four different drainages: noun, Guir, Ziz, Draa (Lower draa, Taakilt, Tarmigt, and an isolated brackish pond at Sidi El Mehdaoui oasis). The locality of Oued Noun (28°58'20.3"N, 10°13'6.71"W, 198 m a.s.l, about 70 km southwest of Guelmim city (28°59'17"N, 10°03'27"W) on southwestern Morocco. The average of annual rainfall varies between 90 and 120 mm. Maximum and minimum temperatures reach respectively 45 °C and 0.1 °C. The annual average temperature is around 20.5 °C. (The National Meteorological Department, Morocco, 2018). The substrate is dominated by rocks characterized by the

presence of shales. The vegetal cover is made of tree species: *Acacia raddiana*, *Tamarix aphylla*, *Phoenix dactylifera* (Palm date) and other plant species such as *Haloxylon scoparium*, *Aizoon canariense*, *Launaea arborescens*, and *Euphorbia officinarum*. The sampling site of Oued Ziz (31°55'42.56"N, 04°18'41.75"W, 1027 m a.s.l) at 7 km north to Erfoud city (31°26'20"N, 04°14'37"W) Southeast Morocco. The average annual rainfall is very low and decrease from north to south ranging from 270 mm at the high atlas ending on the north to 66 mm. Mean annual temperatures are very high and are characterized by strong daily amplitudes (20-50 °C) (The National Meteorological Department, Morocco, 2018). The substrate is rocky with limestone and sandstone. The vegetation is mainly composed of *Acacia*, *Atriplex* and palm date. The Oued Guir site (32°13'59.9"N, 03°56'30.9"W, 1124 m a.s.l) at 22 km south to Gourrama village southeastern Morocco. The rainfall is low, generally poorly distributed over time and space. The monthly temperature represents a striking seasonal variation with an extremely cold winter (January). The summer (July-August) is very hot with mean maximal temperatures up to 41 °C. The National Meteorological Department, Morocco, 2018). The substrate is characterized by rocks occupying stony arid flat areas. The vegetation is in the form of a steppe characterized by the presence of *Acacia* and *Tamarix* and a vegetation consisting mainly of *Stipa tenacissima* tussocks and palm dates. The four last sites are related to the Draa river (Taakilt: 30°37'17.77"N, 06°09'44.62"W, 845 m a.s.l; Tarmigt: 30°52'13.7"N, 06°50'54.0"W, 1152 m a.s.l; Sidi El Mehdaoui (pond): 29°29'03.66"N, 07°59'08.29"W, 453 m a.s.l and Lower Draa: 28°31'6.62"N, 10°56'8.78"W, 58 m a.s.l). The temperatures are high especially between June and September (43 to 50 °C) (The National Meteorological Department, Morocco, 2018), the evapotranspiration is strong especially in summer with the scarcity and the very strong annual and inter-annual variability in precipitations. The substrate is isohumic with brown colour and the texture is sandy-silty on the surface and clay-silty on depth. The vegetation is characterized by an association of *Artemisia herba alba* and *Stipa parviflora* and *Poa bulbosa* with *Festuca spp*. We can also distinguish *Haloxylon scoparium* with *Stipa parviflora* and *Aristida obtusa* (Fig. 1).

Sampling methods

Turtles were collected using hoop traps baited with canned sardines in spring 2017. Upon capture, each individual was weighed (to the nearest 0.1 g) then measured using a Vernier caliper (to the nearest 0.1 mm) for the following shell dimensions (Fig. 2; Muñoz and Nicolau, 2006): Carapace Length (CL), Anterior Width of
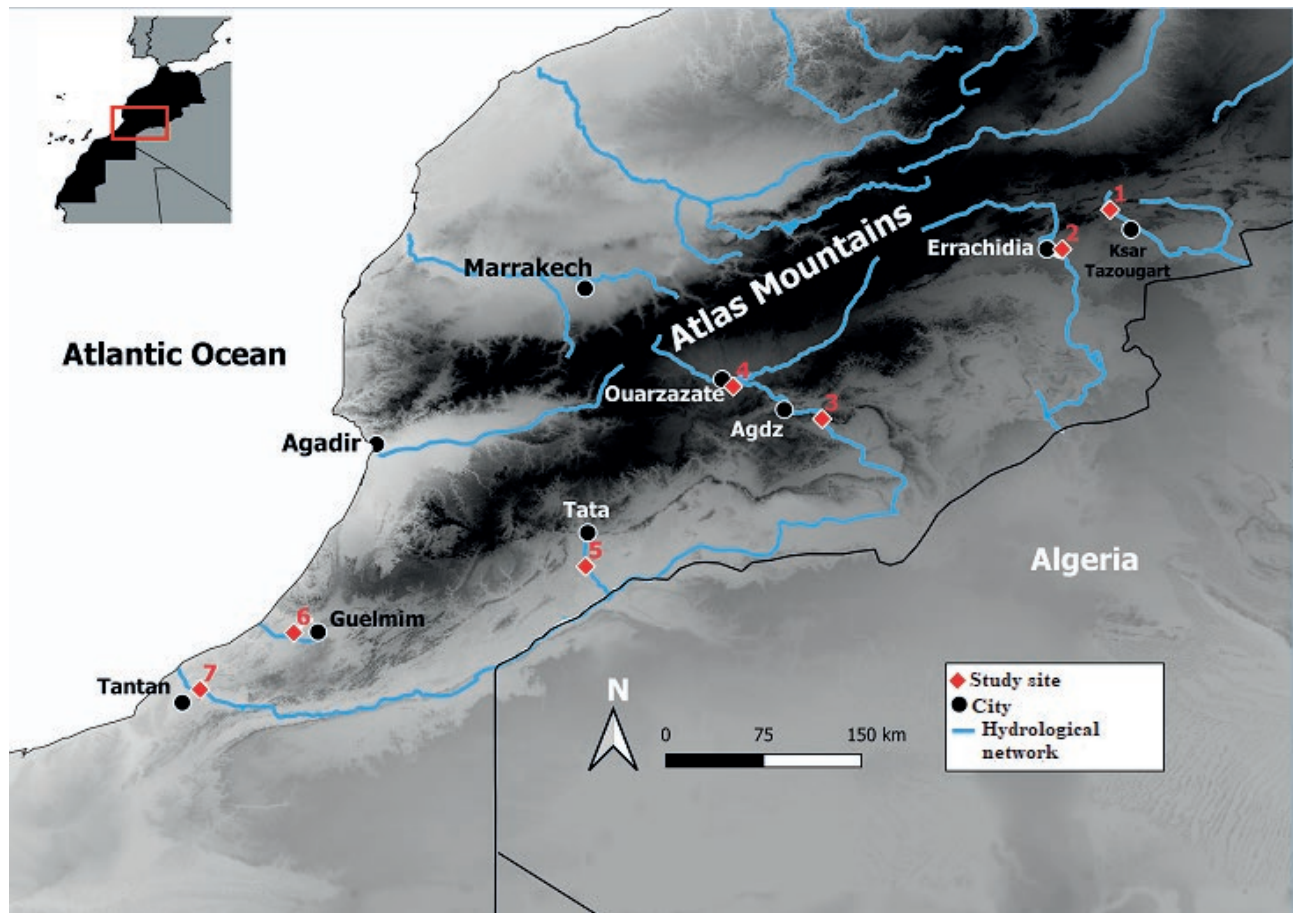
Fig. 1. Map showing the geographic location of the seven study sites in Southeast and Southern Morocco.

Carapace (ACW), Posterior Width of Carapace (PCW), Height of the carapace (H), Plastron Length (PL), Tail Length (TL), Pre-Anal Length (PAL).

Turtle's sex was determined on the basis of the concavity of the plastron (concave in males and flat in females) and confirmed by evaluating the position of the cloaca (located well outside and just at the margin of the carapace, respectively in males and females). For each population, individuals that lacked male secondary sex characteristics and were smaller than the minimum-sized males were considered as juveniles and those that were larger than the minimum-sized males were considered as females. The minimum carapace length of females known to be reproductive (Rowe, 1994) was used as the lower limit for adult female carapace length in each population.

Body Condition Index

We calculated the volumetric Body Condition Index (BCI), which allowed estimating body density as the ratio of the live body mass of the animal to its estimated volume in cm³. This index expresses the weight status of the individual in relation to its size in terms of body mass loss (wasting), overweight (accumulation of energy reserves) or normal weight (no loss or gain of weight). It was calculated using the following formula (Nagy et al., 2002):

$$BCI = \frac{mass(g)}{Volume(cm^3)}$$

Where V= volume calculated by equating the shape of the turtle to an ellipsoid using the formula:

$$V = \pi \times H \times \frac{(ACW+PCW)}{2} \times CL$$

Sexual Dimorphism Index

The Sexual Dimorphism Index (SDI) defined by Lovich and Gibbons (1992) was used to evaluate the degree of sexual dimorphism in turtles. The Index is sim-
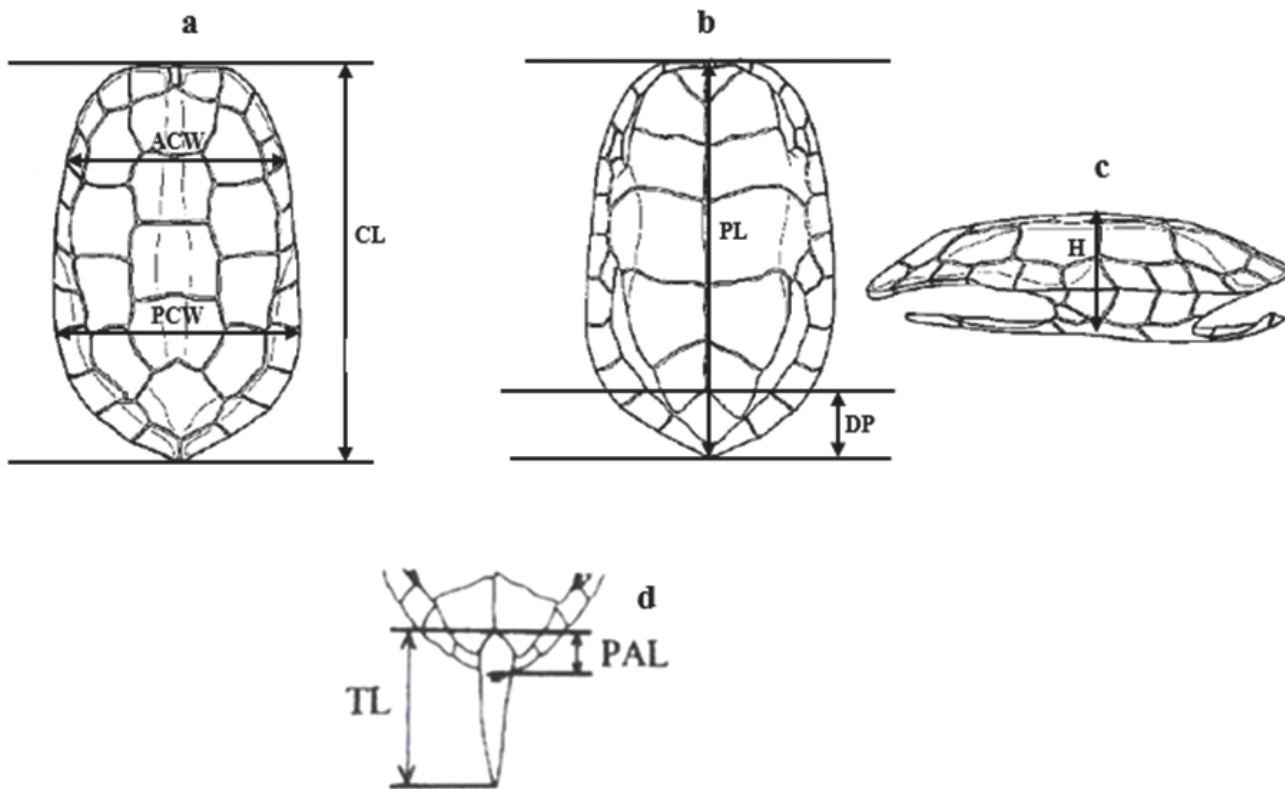
Fig. 2. Main measurements on the carapace. Dorsal (a), ventral (b and d) and lateral (c) views.

ply the ratio between the mean size of the largest sex and the mean size of the smallest sex subtracted by 1 in cases where mean adult female size is larger than mean adult male size. This index is positive when females are larger than males and negative in the opposite case. The SSDI formula is as follows:

$$SDI = \left(\frac{A}{B}\right) - 1$$

Where A is the average size of females and B the average size of males, in the case where females are larger than males (*M. leprosa* case).

Sphericity and Flatness Indices

Sphericity and flatness indices were estimated using Krumbein's Sphericity Index (SI) (Krumbein, 1941) and Cailleux's Flatness Index (FI) (Cailleux, 1947):

$$SI = \left(\frac{qr}{p^2}\right)^{1/3} \text{ and } FI = \frac{p+q}{2r}$$

Where p, q and r were given by CL, CW and CH, respectively. Higher values of sphericity index and lower values of flatness index mean a more domed shell, which entails lower energy barriers between stable and unstable equilibriums.

Statistical analysis

The mean values are given along with their standard deviations and the range of extreme values is eventually indicated. The sex ratio was compared among populations using Pearson Chi-Square (χ^2) test.

Initially, all data were evaluated for normality requirements using the Kolmorov-Smirnov test, in order to determine the application of parametric or non-parametric analyses. A two-way ANOVA was used to compare BCI, SI, and FI among populations. One-factor ANOVAs was used to determine differences in BCI, SI and FI between sexes for each population and among populations. Significant differences among the different populations were determined using a Tukey post hoc test.

One-factor ANOVA was used to compare CL and weight of males and females among populations and Student t-tests were used for comparison of CL and weight between pairs of study populations. Length-weight relations (LWR) were calculated for all localities using the equation $W = aL^b$ (Bagenal and Tesch, 1978) and the

growth type is determined: isometric (b = 3), positive allometric (b > 3) or negative allometric (b < 3).

To analyze shape changes filtering allometric effects, we conducted linear regressions between body mass and standard length. Linear variables are usually transformed with a method that removes size and allometric effects. Hence, we transformed linear measurements into size-free new variables with a transformation that considers allometric effects (Lleonart et al., 2000) with the following equation:

$$Z = Y \times (X0 / X)^b$$

Where X0 is the mean standard length. In this case, we chose straight carapace length, but any measure related with size can be used. The X represents the standard length of each individual; Y is the variable to being transformed; b is the allometric coefficient of the variable Y with standard length obtained from a linear regression between the logarithms of Y and X; and Z is the new variable that we used in our statistical analyses.

All statistical analyses were carried out using the STATISTICA software (version 10.0). The statistical significance level was set at 5%.

RESULTS

Distribution by size and body mass

A total of 224 individuals of *M l. saharica* were captured in the seven populations (Table 1). Among the studied populations, and according to the juveniles-to-adults ratios, four populations were dominated by adult individuals (Table 1): Taakilt, Oued Guir, Oued Ziz, Sidi El Mehdaoui and two out of them were made up of 100% adults (Oued Noun and Tarmigt). The other populations consisted of 50%, 29%; 25%, 12.5% and 4.8% of juveniles, respectively in Lower Draa, Sidi El Mehdaoui, Ziz, Guir and Taakilt. Size frequencies based on carapace length are

shown in Fig. 3. About 37.5% of the Lower Draa population is composed of individuals smaller than 80 mm *vs.* 12.5%, 0%, 29%, 7.14%, 0% and 16%, respectively in Oued Guir, Oued Noun, Sidi El Mehdaoui, Taakilt, Tarmigt and Oued Ziz. Carapce length and body mass of the visibly mature individuals are summerized for each population in Table 2.

The distribution by size for juveniles was significantly different among population for the following size class: 61-80 (χ^2 = 22.00, df = 6, P = 0.001), 101-120 (χ^2 = 18.00, df = 6, P = 0.006), 161-180 (χ^2 = 18.00, df = 6, P = 0.006); but not different for: 20-40 (χ^2 = 6.00, df = 6, P = 0.423), 41-60 (χ^2 = 8.66, df = 6, P = 0.193), 81-100 (χ^2 = 10.40, df = 6, P = 0.109), 141-160 (χ^2 =12.00, df = 6, P = 0.062). The distribution by size for males differed significantly among the studied population for the size class 80-100 (χ^2 =23.25, df = 6, P < 0.001) and for 181-200 (χ^2 = 35, df = 6, P < 0.001); but it does not differ for: 101-120 (χ^2 =11.58, df = 6, P = 0.072), 121-140 (χ^2 = 9.07; df = 6, P = 0.169), 141-160 (χ^2 = 10.15, df = 6, P = 0.118), 161-180 (χ^2 = 8.00, df = 6, P = 0.238), >200 (χ^2 = 6.00, df = 6, P = 0.423). The distribution by size for females was significantly different among population for: 81-100 (χ^2 =26.33, df = 6, P = 0.002), 101-120 (χ^2 = 18.00, df = 6, P = 0.043), 161-180 (χ^2 =22.80, df = 6, P < 0.001); but not different for: 60-80 (χ^2 =12.00, df = 6, P = 0.062), 121-140 (χ^2 = 6.00, df = 6, P = 0.423), 141-160 (χ^2 = 6.933, df = 6, P = 0.327), 181-200 (χ^2 = 8.20, df = 6, P = 0.223), >200 (χ^2 = 6.00, df = 6, P = 0.423).

Among the studied populations, five populations comprised juveniles, which have the following sizes and body masses (Min-max/Mean ± SD): Lower Draa (57.0-89.5/73.73±10.07, 29.2-97/59.04±22.07), Oued Guir (67.2-79.4/72.8±6.2, 45.1-72.3/57.4±13.8), Sidi EL Mehdaoui (34.9-77.6/66.2±16.05, 9.3-70.0/51.98±23.49), Taakilt (75.9-81.2/78.55±2.65, 66.0-77.0/71.6±5.5) and Oued Ziz (61.8-85.5/77.85±5.96,41.3-89.9/71.71±13.22) (Fig. 3 and 4).

Table 1. Sex/age distribution of turtles *Mauremys leprosa saharica* from seven distant populations in southern Morocco. Sex-ratio: males to females; J-A ratio = Juveniles to Adult ratio.

	Oued Guir	Oued Ziz	Taakilt	Tarmigt	Sidi El Mehdaoui	Oued Noun	Lower Draa
Juveniles	3 (12.5%)	14 (25%)	2 (4.8%)	0	9 (29%)	0	12 (50%)
Males	8 (33.3%)	23 (41.1%)	11 (26.2%)	11 (50.0%)	11 (35.5%)	18 (72%)	7 (29.2%)
Females	13 (54.2%)	19 (33.9%)	29 (69%)	11 (50.0%)	11 (35.5%)	7 (28%)	5 (20.8%)
Total	24	56	42	22	31	25	24
Sex-ratio	1:1.63	1:0.83	1:2.64	1:1	1:1	1:0.39	1:0.71
J-A ratio	1:7	1:3	1:20	0:22	1:2.44	0:25	1:1

Table 2. Carapace length and body mass descriptive statistics for adult males and females from the seven study localities. For each case, values are given as: mean ± SD [sample size] and (min-max).

Locality	Carapace length (mm)		Body mass (g)	
	Males	Females	Males	Females
Oued Guir	114.01 ± 9.78 [8] (105.5 - 128.1)	145.29 ± 28.62 [13] (105.1 - 187.0)	218.04 ± 56.48[8] (175.2 - 303.1)	490.3 ± 279.7 [13] (173.4 - 1010.0)
Oued Ziz	114.45 ± 27.54 [23] (83.6 - 168.4)	131.71 ± 36.12 [19] (90.5 - 196.3)	228.38 ± 170.1 [23] (80.5 - 588.4)	400.17 ± 322.71 [19] (117.5 - 1036.0)
Sidi El Mehdaoui	108.67 ± 16.9 [11] (83.2 - 132.0)	93.19 ± 12.08 [11] (80.6 - 107.8)	193.39 ± 84.6 [11] (89.0 - 323.9)	130.16 ± 50.67[11] (84.9 - 191.0)
Taakilt	118.78 ± 33.36 [11] (90.9 - 198.6)	140.72 ± 35.70 [29] (77.5 - 207.7)	270.81 ± 258.42 [11] (127.0 - 989.0)	479.79 ± 303.26 [29] (82.0 - 1037.0)
Tarmigt	114.1 ± 36.76 [11] (85.0 - 203.4)	151.73 ± 35.47 [11] (97.9 - 210.3)	260.00 ± 289.18 [11] (91.0 - 990.0)	614.54 ± 414.87 [11] (156.0 - 1468.0)
Oued Noun	161.48 ± 35.60 [18] (95.5 - 194.4)	174.45 ± 26.02 [7] (135.7 - 200.6)	591.56 ± 284.00 [18] (122.8 - 816.0)	806.58 ± 319.96 [7] (331.4 - 1014.5)
Lower Draa	149.42 ± 21.09 [7] (128.1 - 184.3)	153.76 ± 15.96 [5] (130.5 - 169.3)	436.35 ± 172.70 [7] (268.4 - 728.6)	548.16 ± 140.67 [5] (320.7 - 634.0)

Sex and juveniles-to-adults ratios

The sex ratio was significantly different among populations (Pearson χ^2 = 14.66, df = 6, P = 0.023). It is male-biased in Oued Guir and Taakilt populations. On the other hand, the sex ratio is female-biased in Ziz, Noun and Lower Draa populations while it is balanced (close to 1) in Tarmigt and Sidi El Mehdaoui (Table 1).

The juveniles-to-adults ratio differed significantly among the different populations (Pearson χ^2 = 37.09, df= 6, P < 0.001). There were no juveniles in Tarmigt (0/22) and Oued Noun (0/25), few in Taakilt (2/40), Oued Guir (3/21), Oued Ziz (14/42) and Sidi El Mehdaoui (9/22). The ratio is well balanced in Low draa: 1 (12/12) (Table 1).

Body Condition Index

BCI did not differ considerably from a normal distribution for all populations (Kolmogorov-Smirnov test: all P values > 0.05) and is significantly different among populations but non-significantly different between sexes (Table 3). The interaction Sex×Locality has no significant effect on BCI (Table 3).

BCI for each population was non-significantly different between sexes for all the studied populations, but significant for Sidi El Mehdaoui ($F_{1,20}$ = 11.24, P = 0.003). The BCI was significantly different among populations ($F_{6,170}$= 7.44, P < 0.001). Table 4 shows significant differences (in bold) for each population from Tukey Post-hoc test.

Sexual Dimorphism Index

The SDIs for the studied populations were as follows: Oued Guir = 0.41, Oued Ziz = 0.15, Taakilt = 0.19, Tarmigt = 0.32, Sidi El Mehdaoui = -0.14, Oued Noun = 0.080, Lower Draa = 0.03 (Fig. 5). Significant results of sexual dimorphism using t-test were observed in the following populations: Oued Guir (t-test = -3.492, df = 12; P = 0.003); Oued Ziz (t-test = -1.713, df = 27; P = 0.047); Taakilt (t-test = -1.821, df = 27, P = 0.043) and Tarmigt (t-test = -2.437, df = 19, P = 0.012). The differences were not significant for Sidi El Mehdaoui (t-test = 2.471, df = 14, P = 0.987); Oued Noun (t-test = -1.003, df = 20, P = 0.165) and lower Draa (t-test = -0.407, df = 9, P = 0.344).

Sphericity and Flatness Index

The SI and FI did not significantly depart from a normal distribution (Kolmogorov-Smirnov test, all P values > 0.05). The factorial ANOVA that compared SI and FI between sexes and among the seven populations demonstrated that both indices differed significantly, but the interaction Sex×Locality had no significant effect (Table 3).

The comparison of SI for each population shows that there are significant differences between sexes for Lower Draa ($F_{1,10}$ = 33.62, P < 0.001), Sidi El Mehdaoui ($F_{1,20}$ = 20.09, P < 0.001), Oued Ziz ($F_{1,40}$= 32.20, P<0.001), Oued Noun ($F_{1,23}$ = 6.30, P = 0.019), Taakilt ($F_{1,38}$ = 7.79, P = 0.008), and Tarmigt ($F_{1,20}$ = 7.91, P = 0.010), but non-significant differences for Oued Guir ($F_{1,19}$ = 0.01, P = 0.923). FI differs significantly between sexes in Lower
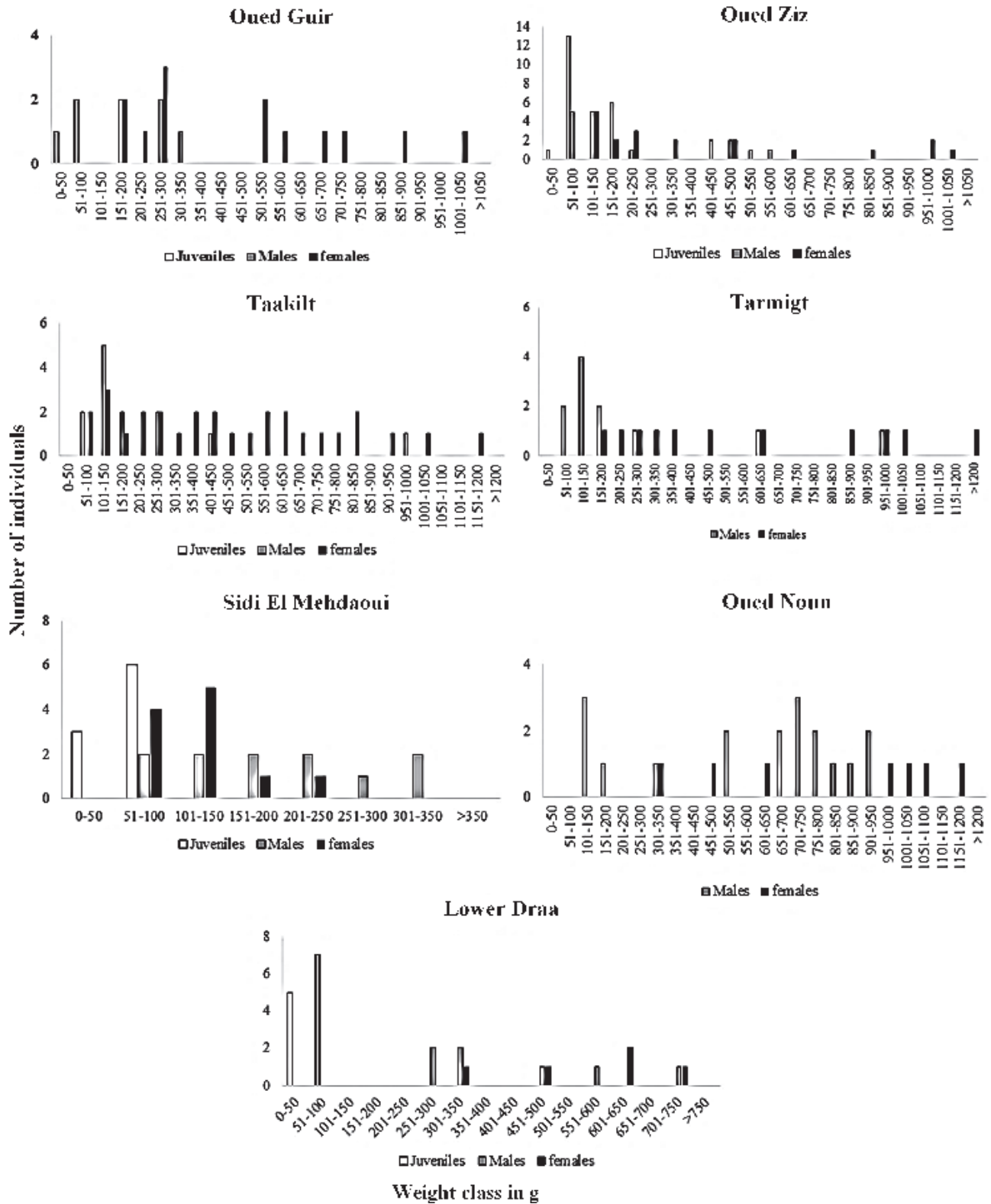
Fig. 3. Distribution by size and sex of individuals from the studied populations of *Mauremys leprosa saharica*.
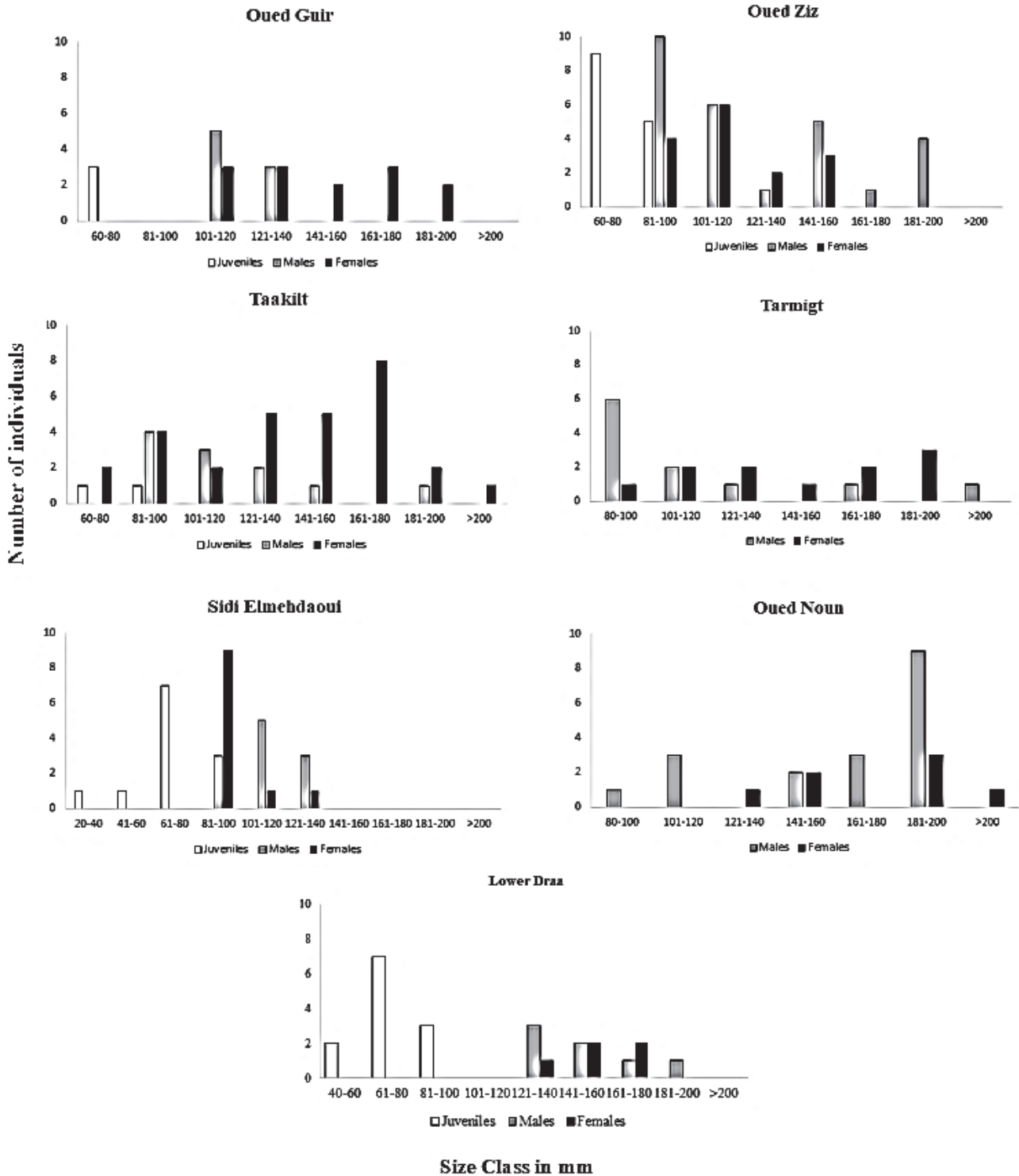
Fig. 4. Distribution by body mass and sex of individuals from the studied populations of *Mauremys leprosa saharica*.

Draa ($F_{1,10}$ = 45.46, P < 0.001), Sidi El Mehdaoui ($F_{1,20}$ = 16.18, P < 0.001), Taakilt ($F_{1,38}$=14.78, P < 0.001), Tarmigt ($F_{1,20}$ = 23.16, P < 0.001), Oued Ziz ($F_{1,40}$ = 22.48, P < 0.001) and Oued Noun ($F_{1,23}$= 5.69, P = 0.025) but non-significant differences in Oued Guir ($F_{1,19}$= 3.72, P = 0.068). Differences among populations in SI and FI were

Table 3. ANOVA for comparison of Body condition Index (BCI), Sfericity Index (SI) and Flatness Index (FI) between sexes, localities and interaction Sex × Locality. Significant effects are bolded; df_{num} and df_{den} represents degrees of freedom for the numerator and denominator of the F stastitic, respectively.

Variable	Effect	df_{num}	df_{den}	F	P
	Sex	1	20	0.232	0.630
BCI	**Locality**	6	170	5.034	**<0.001**
	Sex × Locality	6	170	0.377	0.893
	Sex	1	20	31.77	**<0.001**
SI	**Locality**	6	170	3.64	**<0.001**
	Sex × Locality	6	170	1.00	0.425
	Sex	1	20	36.28	**<0.001**
FI	**Locality**	6	170	20.79	**<0.001**
	Sex × Locality	6	170	0.35	0.908

Fig. 5. Means and standard deviations for carapace length of *Mauremys leprosa saharica* from 7 localities in southern Morocco.

significant for both SI ($F_{6,170} = 9$, P < 0.001) and FI ($F_{6,170} = 81.91$, P < 0.001). Significant differences among each population are shown from Tukey post-hoc test in Tables 5 and 6.

Comparison of Carapce Length and body mass

There are significant differences in CL and body mass of males among populations (CL: $F_{6,82} = 9.993$, P < 0.05; body mass: $F_{6,82} = 11.452$, P < 0.05) and females (CL: $F_{6,88} = 11.28$, P < 0.05; body mass: $F_{6,88} = 9.35$, P < 0.05). Significant differences were observed in CL between males of the following pairs: Oued Guir-Oued Noun, Oued Ziz-Oued Noun, Taakilt-Sidi El Mehdaoui, Taakilt-Oued Noun, Tarmigt-Sidi El Mehdaoui, Tarmigt-Oued Noun, Sidi El Mehdaoui-Oued Noun and Oued Noun-Lower Draa. For body mass in males, significant differences were observed between Tarmigt-Oued Guir, Tarmigt-Oued Ziz, Tarmigt-Taakilt, Sidi El Mehdaoui-Tarmigt, Oued Noun-Oued Guir, Oued Noun-Oued Ziz, Oued Noun Taakilt, Oued Noun-Sidi El Mehdaoui, Lower Draa-Tarmigt and Lower Draa-Oued Noun (Table 7).

Comparison of CL among populations showed significant differences for females of: Oued Guir-Sidi El Mehdaoui, Oued Guir-Oued Noun, Oued Guir-Lower Draa, Oued Ziz-Taakilt, Oued Ziz-Tarmigt, Oued Ziz-Sidi El Mehdaoui, Oued Ziz-Oued Noun, Taakilt-Sidi El Mehdaoui, Taakilt-Oued Noun; Taakilt-Lower Draa, Tarmigt-Sidi El Mehdaoui, Tarmigt-Oued Noun, Tarmigt-Lower Draa, Sidi El Mehdaoui-Oued Noun, Sidi El Mehdaoui-Lower Draa and oued Noun-Lower Draa. For Body mass in females, significant differences were observed between Taakilt-Oued Ziz, Tarmigt-Oued Ziz, Sidi El Mehdaoui-Oued Guir, Sidi El Mehdaoui-Oued Ziz, Sidi El Mehdaoui-Taakilt, Sidi El Mehdaoui-Tarmigt, Oued Noun-Oued Guir, Oued Noun-Oued Ziz, Oued Noun-Taakilt, Oued Noun-Sidi El Mehdaoui, Lower Draa-Oued Guir, Lower Draa-Taakilt, Lower Draa-Tarmigt, Lower Draa-Sidi El Mehdaoui and Lower Draa-Oued Noun (Table 8).

Length-mass relationships

LMR were calculated for all localities (Table 9). The value of "b" of LMR was found to be significantly differ-

Table 4. Comparison of Body Condition Index (BCI) among populations of *Mauremys leprosa saharica*. P-Values are from Tukey post-hoc test with df = 177. Significant values are bolded.

	Oued Ziz	Taakilt	Tarmigt	Sidi El Mehdaoui	Oued Noun	Lower Draa
Oued Guir	**0.039**	1.000	0.999	0.275	1.000	**0.030**
Oued Ziz		**0.005**	0.056	0.999	**0.015**	0.960
Taakilt			0.999	0.144	0.999	**0.011**
Tarmigt				0.343	0.999	**0.041**
Sidi El Mehdaoui					0.185	0.879
Oued Noun						0.017

Table 5. Comparison of Sfericity Index (SI) among populations of *Mauremys leprosa saharica* from Tukey post-hoc test. P-values are from Tukey post-hoc test with df = 177. Significance is indicated in bold.

	Oued Ziz	Taakilt	Tarmigt	Sidi El Mehdaoui	Oued Noun	Lower Draa
Oued Guir	0.998	1.000	0.999	0.221	0.225	0.990
Oued Ziz		0.997	0.979	0.297	**0.017**	0.999
Taakilt z			0.999	0.105	0.087	0.986
Tarmigt				0.108	0.367	0.958
Sidi El Mehdaoui					**<0.001**	0.871
Oued Noun						0.091

Table 6. Comparison of Flatness Index (FI) among populations of *Mauremys leprosa saharica* from Tukey post-hoc test. P-values are from Tukey post-hoc test with df = 177. Significance is indicated in bold.

	Oued Ziz	Taakilt	Tarmigt	Sidi El Mehdaoui	Oued Noun	Lower Draa
Oued Guir	0.999	**<0.001**	0.542	0.999	**0.001**	1.000
Oued Ziz		**<0.001**	0.547	0.996	**<0.001**	0.999
Taakilt z			**<0.001**	**<0.001**	**<0.001**	**<0.001**
Tarmigt				0.367	0.360	0.733
Sidi El Mehdaoui					**<0.001**	0.999
Oued Noun						**0.017**

Table 7. Pairwise comparison of Carapce length (CL; below matrix diagonal) and body mass (above matrix diagonal) between males of the studied populations, using Student t-tests. Significant differences (P < 0.05) are in bold; degrees of freedom are reported as subscript.

	Males	Body mass (g)						
		Oued Guir	Oued Ziz	Taakilt	Tarmigt	Sidi El Mehdaoui	Oued Noun	Lower Draa
	Oued Guir	—	0.105_{46}	-0.836_{22}	$\mathbf{-3.441_{20}}$	1.266_{29}	$\mathbf{-6.300_{16}}$	-0.352_{28}
	Oued Ziz	0.235_{46}	—	-1.211_{48}	$\mathbf{-5.486_{46}}$	1.0372_{55}	$\mathbf{-8.287_{42}}$	-0.584_{54}
	Taakilt	-0.830_{22}	-1.249_{48}	—	$\mathbf{-2.735_{22}}$	1.808_{31}	$\mathbf{-4.419_{18}}$	0.514_{30}
CL (mm)	Tarmigt	-0.886_{20}	-1.296_{46}	-0.104_{22}	—	$\mathbf{5.050_{29}}$	-1.040_{16}	$\mathbf{3.654_{28}}$
	Sidi El Mehdaoui	1.401_{29}	1.429_{55}	$\mathbf{2.161_{31}}$	$\mathbf{2.128_{29}}$	—	$\mathbf{-8.695_{25}}$	-1.304_{37}
	Oued Noun	$\mathbf{-4.944_{16}}$	$\mathbf{-6.873_{53}}$	$\mathbf{-3.843_{29}}$	$\mathbf{-3.436_{27}}$	$\mathbf{-7.060_{36}}$	—	$\mathbf{5.650_{24}}$
	Lower Draa	0.087_{28}	-0.110_{54}	0.805_{30}	0.644_{28}	-1.105_{37}	$\mathbf{4.784_{35}}$	—

Table 8. Pairwise comparison of Carapce length (CL; below matrix diagonal) and body mass (above matrix diagonal) between females of the studied populations, using Student t-tests. Significant differences (P < 0.05) are in bold; degrees of freedom are reported as subscript.

	Females	Body mass (g)						
		Oued Guir	Oued Ziz	Taakilt	Tarmigt	Sidi El Mehdaoui	Oued Noun	Lower Draa
	Oued Guir	—	1.6397_{47}	-0.467_{45}	-1.485_{25}	$\mathbf{4.526_{34}}$	$\mathbf{-2.284_{21}}$	$\mathbf{2.153_{31}}$
	Oued Ziz	1.930_{47}	—	$\mathbf{-2.555_{62}}$	$\mathbf{-3.115_{42}}$	$\mathbf{2.495_{51}}$	$\mathbf{-4.412_{38}}$	0.699_{48}
	Taakilt	-0.427_{45}	$\mathbf{-2.918_{62}}$	—	-1.352_{40}	$\mathbf{5.0956_{49}}$	$\mathbf{-2.707_{36}}$	$\mathbf{2.874_{46}}$
LC (mm)	Tarmigt	-1.346_{25}	$\mathbf{-3.259_{42}}$	-7.149_{40}	—	$\mathbf{5.584_{29}}$	-1.040_{16}	$\mathbf{3.319_{26}}$
	Sidi El Mehdaoui	$\mathbf{5.029_{34}}$	$\mathbf{3.003_{51}}$	$\mathbf{6.071_{49}}$	$\mathbf{7.222_{29}}$	—	$\mathbf{-9.892_{25}}$	-1.926_{35}
	Oued Noun	$\mathbf{-2.602_{21}}$	$\mathbf{-4.286_{38}}$	$\mathbf{-2.496_{36}}$	-1.457_{16}	-10.05_{25}	—	$\mathbf{5.049_{22}}$
	Lower Draa	$\mathbf{2.504_{31}}$	1.002_{48}	$\mathbf{3.407_{46}}$	$\mathbf{3.715_{26}}$	-1.630_{35}	4.749_{22}	—

Table 9. Carapace Length-Body mass relation and growth types for *M. l. saharica* according to localities.

Locality		a	b	r²	95%CI	Growth type
Oued Guir	M	0.0002	2.953	0.999	2.899-3.006	Isometric
	F	0.0002	2.951	0.997	2.871-3.031	Isometric
Oued Ziz	M	0.0004	2.767	0.986	2.652-2.881	(-) Allometry
	F	0.0002	2.921	0.993	2.855-2.965	Isometric
Taakilt	M	0.0005	2.731	0.994	2.585-2.877	(-) Allometry
	F	0.0004	2.824	0.978	2.664-2.984	Isometric
Tarmigt	M	0.0002	2.899	0.986	2.634-3.164	Isometric
	F	0.0002	2.964	0.987	2.702-3.226	Isometric
Sidi El Mehdaoui	M	0.0007	2.658	0.995	2.561-2.745	(-) Allometry
	F	0.0007	2.672	0.996	2.584-2.759	(-) Allometry
Oued Noun	M	0.0003	2.843	0.988	2.677-3.008	(-) Allometry
	F	0.0002	2.971	0.954	2.223-3.718	Isometric
Lower Draa	M	0.0003	2.822	0.999	2.779-2.865	(-) Allometry
	F	0.0001	3.020	0.996	2.937-3.102	Isometric

ent from 3.0 in *M. l. saharica for* some localities. According to the results, the type of growth for *M. l. saharica* is isometric for both male and female of Oued Guir and Tarmigt, for female of Oued Ziz, Taakilt, Oued Noun and lower Draa and hypo-allometric for male of Oued Ziz, Taakilt, Oued Noun, and Lower Draa and for both males and females in Sidi El Mehdaoui. The analysis of covariance of Log BW with locality as categorical predictor and log CL as continuous predictor revealed a difference among populations for both Males ($F_{6,82}$ = 2863.71, P < 0.05) and females ($F_{6,88}$ = 2658.8, P < 0.05).

DISCUSSION

Several studies have been conducted on the Iberian Population of *M. leprosa* (e.g., Perez et al., 1979; Andreu and Villamor, 1989; Da Silva, 1995). Data on the ecology and biology of this species in Morocco are limited, indeed, with the exclusion of population ecology (Meek, 1987) and on the geographical variation of sexual dimorphism (Lovich et al., 2010). Studies on the population structure of *M. leprosa* are rather rare and only few studies have been done in Spain (Keller, 1997; Alarcos et al., 2008). The only work on the population structure of *M. leprosa* in North Africa is that of Meek (1987), carried out in May-September in the region of Tiznit, Souss valley, Morocco. The population studied by this author in September 1981, was large (sample of 67 individuals) dominated by young animals (60%) with carapace lengths not exceeding 80 mm. However, adults and sub-adults were 4.5 times more abundant than hatchlings. Moreover, in this population, the sex ratio in adults was biased

in favour of females (1: 2.12 = 0.47). The total length of the carapace in females is on average higher than that of males (97.1 vs 82 mm) with maximum values of 186 and 149 mm respectively. Two populations in the present study are approximately large as the population of Tiznit (Meek, 1987) (Oued Ziz = 56 and Taakilt = 42) followed by Sidi El Mehdaoui, which was 31 comparatively to the following population that were rather small Tarmigt = 22, Oued Guir = 24, Lower Draa = 24 and Oued Noun = 25 that were rather small.

The sex ratio is variable among the seven populations: it is balanced for Sidi El Mehdaoui and Tarmigt with a value of 1, biased towards males for Oued Ziz (1.21), Oued Noun (2.57) and Lower Draa (1.4) biased towards females for Oued Guir (0.62) and Taakilt (0.39) and the same for the population studied by Meek with a value of 0.47. The differences in sex ratio in the studied populations may be due to either a low impact rate or a difference in the mortality rate between males and females. The sex ratio information is, however, not reliable in explaining the fluctuation in the proportion of males and females and seems to be dependent on the locality or month of capture, reflecting rather different behaviours between the two sexes. Another cause of this variation could be related, as in various other turtle species of the family Geoemydidae, to the phenomenon of sex determinism by temperature (during the incubation of eggs). As in Japan, *M. japonica* (Okada et al., 2010), a species closely related to *M. leprosa*, with incubation temperatures above 29-30 °C, would produce almost exclusively females that could affect sex ratio within natural populations. According to the scenario of a climate change with a tendency to the increase of the

temperature, a predominance of females could contribute strongly to the functional disappearance then to the total extinction of some populations.

In Oued Noun, Tarmigt, Oued Guir, Sidi El Mehdaoui and Oued Ziz the juvenile rate varies between 0% and 30%. The value reported by Meek (1987) is approximately similar to those reported for the populations of this study with 25.4%. One population of the seven studied populations which is that of Lower Draa has a rate of juveniles (50%) equal to that of adults (50%). This could indicate a low turnover rate, which may be caused by a high mortality of juveniles due to predation, habitat destruction or drought or to the differences in the minimum size at sexual maturity.

The largest sizes (carapace length) of males and females captured are respectively observed in Tarmigt (203.4 mm) and in Oued Noun (209.6 mm). Busack and Ernst (1980) reported a value for the largest size of CL in Tunisia of 174.5 mm, in Spain it is 114 mm in males and 182 mm in females (Bertolero and Busack, 2017), while in the population studied by Meek (1987), the CL was 146 mm in males and 186 mm in females. The variations observed in the different studied populations could be explained by the variability of habitat and the availability of conditions that allow the animal to grow well, they may also be due to sampling problems. The wide geographic distribution of *M. leprosa* showed that this species is able to be adapted to different habitat conditions ranging from cold/temperate to extremely arid climate and from fresh to brackish waters (Bertolero and Busack, 2017). Studying the condition factors such as the Body Condition Index is very important. The Body Condition Index is the most appropriate parameter to monitor the vitality of populations in different habitat. It was considered as an indicator of past foraging success instead of the ability to cope with environmental pressures that may ultimately impact the population viability (Jakob et al., 1996). In the present study, we found that the BCI was significantly different among populations but not significantly different between sexes among populations, for each studied population and for the interaction sex×population. The observed difference would be mainly attributed to the environmental conditions of turtle habitat (dryness, salinity…) and availability of food resources.

The main differences in shell dimensions between males and females are evident in most turtle species. Thus, it is clear that sexual dimorphism exists, with females being generally larger than males. Berry and Shine (1980) suggested that the smaller body size of males in most freshwater turtles could be the result of a low degree of intrasexual selection processes. In some turtle species, males are larger than females when forced

insemination is occurred. The small size of male could facilitate the mobility reducing the costs of their daily/seasonal movements (Bonnet et al., 2011) and hence increased ability to detect females with the most of their available energy devoted to searching for females more than growth (Berry and Shine, 1980). In addition, sexual size dimorphism (SSD) correlates with habitat types, which could affect male mating strategy. It has been suggested for other species of turtles and tortoises that the small size of one of the sexes can also be related to age at maturity (Gibbons and Lovich, 1990). In *M. leprosa*, males attain sexual maturity at smaller sizes than females (Keller, 1997), and this could be one of the causes of the sexual size dimorphism in this species. Lovich et al (2010) noted that SSD was biphasic very unusual phenomenon in turtles, with males and females in one population exhibiting similar body sizes. Intraspecific changes in SSD was also observed for other turtle species by Lovich and Lamb (1995), Iverson (1985), and Yasukawa et al. (1996). The direction of size (large/small or equal) varied according to the sampled population, the growth patterns, mortality at a specific size and possibly to the food availability (Lovich and Gibbons, 1992). In the present study, the SSD varies among populations. The greatest SSD was observed in Oued Guir population (SSD = 0.41) and the lowest in Sidi El Mehdaoui population (SSD = -0.14). The negative SSD values indicate male size bias, whereas positive values indicate female size bias (Lovich and Gibbons, 1992). In the population of Sidi El Mehdaoui, males are even slightly larger than females (males: 108.67 ± 16.9 mm; females; 93.19 ± 12.08 mm) but the opposite case is onserved in in the other studied populations.This latter findings are similar to those for other population in Morocco (Meek, 1987; Lovich et al., 2010) and for those reported by Muñoz (2004) and Muñoz and Nicolau (2006) from the center of the Iberian Peninsula. Lovich et al. (2010) have indeed found, on the basis of the sexual dimorphism index (Lovich and Gibbons, 1992), a geographic variation in the size sexual dimorphism in *M. leprosa* along an environmental gradient in Morocco between the Atlantic coast and the upper Draa to the southeast. The extreme value of the SSD (0.92) assigned by Lovich et al. (2010) to the population of upper Draa is due to the very small sample of the latter. However, the very low value of SSD in the population of Sidi El Mehdaoui may be partly influenced by the possible inclusion of immature specimens as suggested by Gibbons and Lovich (1990).

Estimations of sexual size dimorphism can vary under the influence of several factors: 1) biased samples, 2) inappropriate dimorphism measures, 3) incorrect estimation of size at sexual maturity, 4) geographic variation

in growth or body size. These various factors have been cited and discussed in detail by Lovich et al. (2010). These authors deduced, the existence of a positive correlation between the degree of sexual size dimorphism and the productivity of the environment, with the smallest females in oligotrophic environments and the largest in the most productive environments. This suggests that the availability of trophic resources may limit growth in females of *M. leprosa* to such an extent that they acquire sexual maturity at a smaller size and begin earlier to allocate energy resources for egg production. This finding was also reported by Iverson (1985) who suggests that the limitation of food resources was responsible for the geographical variation of the size sexual dimorphism in the red-legged mud turtle, *Kinosternon hirtipes*, of North America.

Previous work has suggested that factors affecting the direction and magnitude of SSD in turtles may include fecundity (Cox et al., 2003). Other works, noted that sexual dimorphism is influenced by the characteristics of habitats as variables differ between localities such as food availability and partitioning and differences in hormone levels (Cox and John Alder, 2005 ; Shine, 1989, Vincent and Herrel, 2007).

Our geometric models of turtles among the studied population based on sphericity and flatness indices of the shell have yielded morphological shape differences among individuals of *M. l. saharica* in the different localities for both sexes, but not for the interaction locality×sex. In freshwater turtles, morphology can vary among populations of the species inhabiting different environmental conditions (Rowe, 1997; Zuffi et al., 2007). Many studies examining intraspecific morphological divergence have focused on the effects of biotic components of the environment, such as resource competition (Adams and Rohlf, 2000; Grant and Grant, 2006; Pfennig et al., 2006; Adams and Collyer, 2007) and the effects of predator–prey interactions (Brönmark and Miner, 1992; Milano et al., 2002; Langerhans and Dewitt, 2004; Eklov and Svanback, 2006; Brookes and Rochette, 2007). However, abiotic or physical features of the environment can also drive phenotypic divergence among intraspecific populations. Despite the potential constraints of a rigid shell, semi-aquatic freshwater turtles have adapted to life in a diverse array of aquatic flow regimes, ranging from ponds and lakes to fast flowing rivers (Ernst et al., 1994). Compared with terrestrial turtles, aquatic turtles possess flatter and more symmetrical shells, both of these characteristics are believed to create a difference between populations of the same species.

Two studies examining intraspecific variation in morphology across different flow regimes have suggested that the shells of freshwater turtles are suited to the hydrodynamic environments in which they are found (Aresco and Dobie, 2000; Lubcke and Wilson, 2007). Aresco and Dobie (2000) presented the first quantitative data, by showing that the shells of river cooters (*Pseudemys concinna*) from lotic sites were flatter than those from lentic sites. Lubcke and Wilson (2007) found that western pond turtles (*Actinemys marmorata*) from lotic habitats were flatter and narrower than those from lentic habitats.

The morphological data variation on *M. leprosa* are not well documented for all the known distribution area of the species. However, morphology of the species is not well investigated and information looking particularly at the variations between populations is almost unknown. Growth of turtles is a process that can changes considerably in response to genetic and environmental factors (Barlow, 1961; Somers, 1986). The results presented in this study shows that LWR has an isometric growth for both male and female of *M. l. saharica* in Oued Guir and Tarmigt, for female of Oued Ziz, Taakilt, Oued Noun and lower Draa but a negative allometric growth for male in Oued Ziz, Taakilt, Oued Noun, Lower Draa and in both male and female of Sidi El Mehdaoui. Few studies have reported *M. leprosa* growth patterns in Morocco (Meek, 1987) and Spain (Keller, 1997; Muñoz and Nicolau, 2006). The differences in growth between and among the different studied populations could be explained by local ecological parameters. Environmental conditions have an important influence on ecology of turtles and are considered to be the principal factors in intra- and interspecific growth differences.

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Temporal variation in diet and helminth abundance in the spiny-tailed lizard, *Strobilurus torquatus* Wiegmann, 1834 (Squamata: Tropiduridae) from the Brazilian Atlantic Forest

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Abstract. Spatial and temporal variations can influence the abundance and biology of parasite species and even the transmission process, as well as the diet, reproduction and distribution of hosts. We studied the influence of seasonality on the diet and endoparasites of the lizard *Strobilurus torquatus*. The lizards were collected during a period of one year, in the Engenho Gargaú Private Reserve of Natural Heritage, located in the municipality of Santa Rita, in the state of Paraíba, Northeast Brazil. Thirty-three lizards were analysed, of which 30 were parasitised (overall prevalence of 90%) with the helminths *Physaloptera lutzi* (prevalence of 69.6%), *Spauligodon loboi* (51.5%), *Strongyluris oscari* (15.1%) and *Haplometroides odhneri* (3.0%). Our analysis showed that endoparasite abundance was influenced by host body size. Host sex also influenced endoparasite abundance, where males were parasitised more than females. When comparing the two seasons (dry and rainy), we observed that the greatest endoparasites abundance occurred during the rainy season. Regarding the lizards' diet, we observed a high niche overlap (overlap index of 0.999), which indicates that males and females of *S. torquatus* consumed practically the same prey during the analysed period. The life cycle of the endoparasites found in *S. torquatus* was linked to seasonal variation, and the behavioural and physiological differences between sexes may have influenced the variations in endoparasite abundance.

Keywords. Brazil, endoparasites, Nematode, seasonality, Trematoda.

INTRODUCTION

Lizards are known to act as hosts to several parasites, such as nematodes (e.g., Brito et al., 2014a, b; Araújo-Filho et al., 2016; Teixeira et al., 2017), cestodes (e.g., Brito et al., 2014a, b; Araújo-Filho et al., 2016), trematodes (e.g., Teixeira et al., 2018; Amorim and Ávila, 2019; Araújo-Filho et al., 2020; Teixeira et al., 2021) and pentastomids (Almeida et al., 2008; Brito et al., 2014a, b; Araújo-

Filho et al., 2016). These animals are excellent models for studying how ecological and evolutionary relationships determine the distribution and parasite abundance, since they inhabit different environments, utilize various foraging strategies and present different trophic interactions, body sizes, and reproductive strategies (Aho, 1990).

Several factors can affect the survival and distribution of parasites in host populations (Laferty and Kuris, 2005), such as variations in environmental conditions

(i.e., temperature, humidity, and precipitation), changes in the availability of intermediate hosts, and physiological changes in final hosts (Noble, 1966; Lafferty and Kuris, 2005; Vasconcellos et al., 2010; Brito et al., 2014b; Araújo-Filho et al., 2016), which are commonly seen in environments with well-defined seasonal regimes (Da Silva et al., 2019; Amorim and Ávila, 2019).

Seasonal changes can influence parasite abundance, richness and composition in several ways (Miranda and Andrade, 2003; Fontes et al., 2003; Lafferty and Kuris, 2005). For example, an increase in temperature favours some parasite species, which may accelerate their reproduction rates, resulting in an increase in population sizes (Lafferty and Kuris, 2005). Temperature is also very important for host species that are ectothermic and require external heat sources for internal temperature regulation. Another climatic factor that can affect parasite species is precipitation, which may favour parasites that lay their eggs in the environment, as well as those that have an aquatic life stage (Lafferty and Kuris, 2005).

In recent years, many studies have sought to analyse the influence of seasonal variations on lizard populations and their endoparasites (Ribeiro and Freire, 2011; Gambhir et al., 2012; Brito et al., 2014a, b; Araújo-Filho et al., 2016; Oliveira et al., 2017; Amorim and Ávila, 2019). These studies demonstrated that seasonal variations can influence behaviour, reproduction, diet (Miranda and Andrade, 2003; Ribeiro and Freire, 2011) and endoparasite infection rates in lizards (Fontes et al., 2003; Salkeld et al., 2008; Gambhir et al., 2012; Brito et al., 2014a, b; Araújo-Filho et al., 2016; Amorim and Ávila, 2019). Additionally, historical factors (Brito et al., 2014a, b), coupled with ecological traits, can also influence the compositions of host parasite communities.

Tropiduridae is one of the most diverse lizard families in the Neotropical region (Torres-Carvajal, 2004; Rodrigues et al., 2013; Lima-Silva et al., 2021), comprising 138 species that are distributed across eight genera. One of these genera, *Strobilurus* Wiegmann 1834, is composed of only one species, *Strobilurus torquatus* Wiegmann, 1834 (Uetz et al., 2021), the spiny-tailed lizard, for which there is little available information on its ecology (Rodrigues et al., 1989), distribution (Rodrigues et al., 2013) and associated endoparasite fauna (Teixeira et al., 2020; Lima-Silva et al., 2021).

Strobilurus torquatus (Fig. 1) is diurnal, arboreal and insectivorous (Rodrigues et al., 1989). Males are usually larger-bodied than females, and have longer tibias, which may be related to their territorial behaviour (Rodrigues et al., 1989; Frost et al., 2001; Rodrigues et al., 2013; Lima-Silva et al., 2021). This species is distributed along the coastal Atlantic Forest, extending from the state of Rio de Janeiro

Fig. 1. Specimen of the rare lizard *Strobilurus torquatus* from the Atlantic Forest, north-eastern Brazil. (Photo: Silva, W.P)

in the extreme south, through the state of Pernambuco in the north, to the isolated enclaves of tropical forest within the Caatinga in the state of Ceará, in west. (Salles-Silveira et al., 2010; Rodrigues et al., 2013; Lima-Silva et al., 2021). Despite its wide distribution range, *S. torquatus* is considered a rare species due to its low local abundance, resulting in a lack of basic information about its biology, ecology and parasitic fauna (Lima-Silva et al., 2021). Therefore, this study aims to verify the influence of seasonality on the diet and endoparasites of *Strobilurus torquatus*.

MATERIAL AND METHODS

Study area and collections

The lizards were collected in the Engenho Gargaú Private Reserve of Natural Heritage (hereafter PRNHEG; 06°59'52"S, 34°57'30"W), located in the municipality of Santa Rita, in the state of Paraíba, Northeast Brazil (Fig. 2). PRNHEG covers an area of 1,058.62 ha, preserving one of the most significant remaining fragments of the Atlantic Forest in north-eastern Brazil (ICMBio, 1994). The climate is tropical, with an average temperature of 25.7° C. The collections took place over 8 days for each sampled month, where sampling during the months of lower rainfall occurred between September 2016 and January 2017 (average of 34 mm for these months) and sampling during the months of greater rainfall occurred from March 2017 to July 2017 (average of 135 mm for these months) (Climate-Data, 2017).

The primary sampling method was manual collection during active-search surveys. All campaigns were carried out with a team of researchers made up of three members, and searches were conducted between 09:00 am and
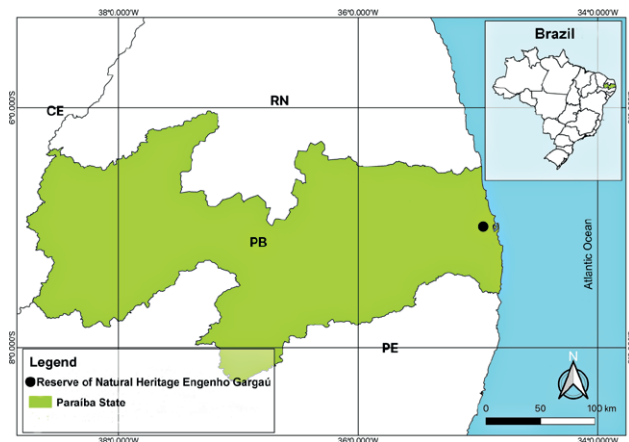
Fig. 2. Study area, remnant Atlantic Forest fragments, located in the municipality of Santa Rita, in the state of Paraíba - PB. Modified from Teixeira et al. (2021).

05:00 pm (after this period, a severe decrease in sunlight within the forest hindered lizard visualization), totalling 192 hrs of sample effort by campaign.

We complemented active-search sampling with pitfall traps. A total of 25 sets of traps were installed. Each set consisted of four buckets (volume of 20 litres) arranged in a "Y" shape, totalling 100 buckets distributed throughout the study area (see Oliveira et al., 2017).

The lizards were fixed in 10% formaldehyde, preserved in 70% alcohol, and deposited in the Herpetological Collection of the Universidade Federal da Paraíba (UFPB) sequential voucher number CHUFPB-29020 to CHUFPB-29058.

Parasitological identification

In the laboratory, the lizards were sacrificed following the current ethical guidelines, using doses of Lidocaine 2%. Subsequently, the snout-vent lengths (SVL) of the samples were recorded using a Mitutoyo digital calliper 150mm, accurate to 0.001mm. Finally, the body mass of each specimen was measured using a precision scale (accuracy: +/- 0.3%). During the necropsies, gonad inspections and animal sexing were performed. Afterwards, their lungs and gastrointestinal tracts were analysed in search of endoparasites.

All endoparasites found were collected, counted, and later mounted on temporary slides for identification. To identify the nematodes, the specimens were bleached with Lactophenol. In the case of trematodes, the specimens were stained with Carmin, before being mounted on slides with Eugenol solution. Taxonomic identification was achieved using the following relevant keys and

descriptions: Ramallo et al. (2002), Anderson et al. (2009), Pereira et al., (2017) and Alves et al., (2022) for nematode identification and Silva et al. (2007) for trematode identification. The endoparasites were deposited in the Paulo Young Invertebrate Collection (UFPB) - CHUFBP (UFPB-NEM: 03, 05, 06; UFPB-DIG: 03, 04, 05).

Data analysis

Mean intensity (MII) and prevalence (P%) of infection were calculated following Bush et al. (1997), who described the former as "the total number of parasites of a given species divided by the number of hosts infected with that species of parasite" and the latter as "the number of hosts infected with a species of parasite divided by the number of examined hosts".

We used generalised linear mixed models (GLMM) to verify if endoparasite abundance was influenced by the physical characteristics of the hosts (Mass and SVL) and by environment seasonality (dry and rainy seasons). Linear models allow for the exploration of one variable (the dependent variable) as a function of another (the independent variable), in addition to having special terms for inserting random effects (Bates et al., 2014).

In the first model, using a Poisson distribution, we verified whether host body mass influenced endoparasite abundance. Endoparasite abundance corresponded to the response variable, while body mass corresponded to the independent variable, and host sex was allocated as a random effect. In the second model, also using a Poisson distribution, we verified whether host SVL influenced endoparasite abundance. Endoparasite abundance corresponded to the response variable and SVL corresponded to the independent variable, while host sex was allocated as a random effect, to prevent the sexual dimorphism of the species from influencing the results. For the third model, we attempted to verify whether endoparasite abundance was influenced by seasonality, using the Poisson distribution and the log function. For this, we performed an interaction between parasite abundance, host sex and seasonality. All analyses were performed using R Software and the lme4 package (Bates et al., 2014; R Core Team, 2018).

We analysed all the stomach contents of the collected lizards and the prey found were identified to the lowest possible taxonomic level. Subsequently, we calculated niche width which was only based on the number of prey items. This index was calculated through the inverse of Simpson's diversity index (Simpson, 1949), which is calculated by the following equation:

$$B = \frac{1}{\sum_{i=1}^{n} P_i^2}$$

where i is the prey category, P is the proportion of prey category i and n is the number of categories. Niche width was based on the number of prey items from pooled stomachs.

We calculated niche overlap using the number of prey items from pooled stomachs to verify whether there was a difference in prey consumed by males and females and between seasons. Niche overlap was calculated using Pianka's index (Pianka, 1986), expressed by the following equation:

$$\emptyset_{jk} = \frac{\sum_{i=1}^{n} p_{ij} p_{ik}}{\sqrt{\sum_{i=1}^{n} p_{ij}^2 \sum_{i=1}^{n} p_{ik}^2}}$$

where p_{ij} and p_{ik} are the consumption rates of prey category i, and j and k represent the sexes and seasons compared. This index ranges from zero (no overlap) to one (total overlap).

Descriptively, we individually calculated the Volume (V) of consumed prey, using the width and length of each prey item. From the volume, we were able to calculate the importance index value for each prey category.

The volume (V) of individual prey was calculated using the following formula:

$$V = \frac{4}{3} \pi \left(\frac{w}{2}\right)^2 \left(\frac{l}{2}\right)$$

where w is prey width and l is prey length.

To determine the importance of each prey category in the diet, we calculated the importance index based on pooled stomachs, using the following formula:

$$I = \frac{F\% + N\% + V\%}{3}$$

where $F\%$ is the percentage of occurrence, $N\%$ is the numerical percentage and $V\%$ is the volumetric percentage of the prey.

RESULTS

We analysed 33 lizards, of which 19 were males (mean SVL = 65.67 ± 14.06 mm), 12 were females (mean SVL = 58.53 ± 10.20 mm) and two were juveniles (removed from the statistical analysis of sex and seasonality interaction). We collected 23 lizards during the dry season, and ten during the rainy season. Because this lizard species is arboreal, only two individuals were captured by pitfall traps, both during the dry season. The difference in sample sizes was probably due to the fact

that this species is more reclusive during rainy days, and the fact that the sampling effort was strictly the same may have caused a decrease in the likelihood of lizards being found in the environment during the rainy season.

Of the specimens examined, 30 were parasitised, resulting in an overall prevalence of 90%. Four endoparasite species were found, three of which were nematodes (*Physaloptera lutzi* Cristofaro, Guimarães and Rodrigues, 1976; *Spauligodon loboi* Ramallo, Bursey and Goldberg, 2002 and *Strongyluris oscari* Travassos, 1923) and one was a digenean trematode (*Haplometroides odhneri* Ruiz and Perez, 1959) (Table 1).

The models indicated that endoparasite abundance was positively influenced by host body mass (R^2m: 0.95; R^2c: 0.95; Z-value: -7.485; P < 0.001) and SVL (R^2m: 0.95; R^2c: 0.95; Z-value: 11.521; P < 0.001). Regarding host sex, males were more parasitised than females (Z-value: 6.260; P < 0.001). When comparing the two seasons (dry and rainy), we observed that the greatest endoparasite abundance occurred during the rainy season (Z-value: -2.422; P < 0.05). Furthermore, our analysis of the interaction between sex and season indicated that during the rainy season male lizards exhibited greater endoparasite abundance (Z-value: -5.019; P < 0.001) (Figure 3).

Regarding diet, we did not observe differences between the proportions of prey consumed by males and females. Both sexes mainly preyed on ants, which represented 95% of the entire dietary composition of *S. torquatus*. During the rainy season, Formicidae represented 98% of the species' food composition, with an importance index of 86.92. Similar to the results observed for the rainy season, in the dry season, Formicidae corresponded to 96% of the food items consumed by *S. torquatus* with an importance index of 64.61 (Table 2).

Based on the number of prey items in the pooled stomachs, niche width was similar between sexes and seasons (Table 2). Finally, we observed a high niche overlap between sexes and seasons, with an overlap index of 0.999 in both analysed pairs, thus showing that the individuals consumed basically the same prey regardless of sex and period of the year.

DISCUSSION

The endoparasite species identified in *S. torquatus* are frequently found to parasitize other lizard species (Bursey et al., 2005; Ávila et al., 2012; Brito et al., 2014b; Lima et al., 2017; Araújo-Filho et al., 2020), with the exception of the parasite *Haplometroides odhneri*, parasites of the genus *Haplometroides* Odhner, 1910 were previously only described as endoparasites of snakes and amphisbaenians

Table 1. Endoparasites infecting the lizard *Strobilurus torquatus* from the Atlantic Forest, north-eastern Brazil. Infection Site (I.S): Stomach (S), Small intestine (S.I), Large intestine (L.I), Body cavity (B.C); Prevalence (P%) and Mean infection intensity (I).

Phylum	Family	Species	I.S	P%	I
Nematoda	Physalopteridae	*Physaloptera lutzi*	S/S.I/L.I/B.C	69.6	18.6
	Pharyngodonidae	*Spauligodon loboi*	S.I/L.I	51.5	9.5
	Heterakidae	*Strongyluris oscari*	S.I/L.I	15.1	6.8
Platyhelminthes	Plagiorchiidae	*Haplometroides odhneri*	S.I	3.0	4

Table 2. Diet composition, number of prey (NP) and Importance Value Index (IVI) of the prey consumed by *Strobilurus torquatus* in relation to host sex and season (dry and rainy). Niche width based on the number of prey items in pooled stomachs (Niche # A).

Prey category	NP / IVI			
	Dry season	Rainy season	Males	Females
Araneae	1 / 1.65	-	1 / 2.59	-
Coleoptera	19 / 13.71	3 / 7.49	17 / 20.16	5 / 6.83
Diptera	4 / 1.70	-	4 / 2.15	-
Formicidae	652 / 64.61	283 / 86.92	497 / 63.10	418 / 71.19
Hemiptera	3 / 4.27	1 / 2.49	2 / 1.07	1 / 6.85
Hymenoptera(non-Formicidae)	3 / 4.13	-	-	3 / 8.30
Acari (Ixodida)	1 / 0.83	-	1 / 1.07	-
Insect larvae	5 / 6.63	-	3 / 7.78	2 / 3.42
Scolopendromorpha	-	1 / 3.08	-	1 / 1.71
Scorpiones	3 / 2.41	-	2 / 2.03	1 / 1.66
Niche # A	1.12	1.03	1.12	1.06

(Silva and Barrella, 2002; Santos et al., 2008), however, Teixeira et al. (2018) the species *H. odhneri* parasitizing the lizard *Dryadosaura nordestina* for the first time. Recent studies have expanded the host records of this parasite, which has been found in different species of lizards from the families Sphaerodactylidae, Dactyloidae, Mabuyidae and Tropiduridae (Teixeira et al., 2020; 2021).

Our GLMM analysis showed that host body size directly influenced the endoparasite abundance found in *S. torquatus*. This same pattern has been observed for other lizard species of the family Tropiduridae in different biomes of the Neotropical region (Fontes et al., 2003; Anjos et al., 2012; Pereira et al., 2012; Araújo-Filho et al., 2016) and in studies conducted with fishes (Isaac et al., 2000) and amphibians (Hamann et al., 2012). Thus, these studies indicate that the positive correlation between host body size and parasitism rate does not exclusively occur in lizards.

Fontes et al. (2003), studying the tropidurid *Eurolophosaurus nanuzae* in an area of rocky outcrops in the Cerrado, observed that an increase in the infection intensity of three endoparasite species was correlated with the hosts' body size. Anjos et al. (2012), who studied another tropidurid species, *Tropidurus hispidus*, in the Caatinga area, and Pereira et al. (2012), who worked with *Tropidurus torquatus* in an area of Atlantic Forest, also obtained similar results, where host body size influenced the infection intensity of the endoparasites found.
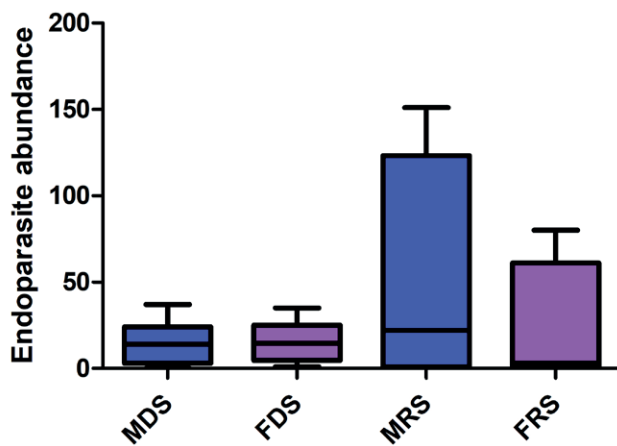
Fig. 3. Interaction between endoparasites abundance, host sex and seasonality. (FDS: Female dry season, MRS: Male rainy season, FDS: Female dry season).

A host's body can be considered a parasite's habitat (Poulin et al., 2011). Thus, larger hosts have a greater adhesion area and more available nutrients to parasitic species, in addition to having greater longevity, making these individuals the best sources of resources for endoparasites (Korallo et al., 2007; Pereira et al., 2012).

However, hosts may have other ecological characteristics, in addition to body size, which can influence parasite infection rate, for example population density, social behaviour and diet composition (Morand, 2000; Poulin, 2004). All these factors affect, not only the probability of an individual acquiring endoparasites, but also the parasitic species to which the hosts may be exposed to throughout their lives.

Host sex influenced the endoparasite abundance found in *S. torquatus*, where males were more parasitised than females. Similar to our study, Brito et al. (2014b), in their research involving lizards of the families Tropiduridae and Teiidae in areas of Caatinga in Northeast Brazil, also found that sex influenced endoparasite abundance, where males were also the most parasitised. Several studies on species of the family Tropiduridae performed in different areas of the Neotropical region, have demonstrated the influence of host sex on endoparasite infection rates (Fontes et al., 2003; Pereira et al., 2012; Galdino et al., 2014; Araújo-Filho et al., 2016). In studies carried out with lizards of the families Leiosauridae (Sousa et al., 2007), Gymnophtalmidae (Oliveira et al., 2017) and Dactyloidae (Campos et al., 2021), differences in infection rates were also observed between male and female hosts.

According to Poulin (1996), the biological differences (physiological, morphological and behavioural) between hosts of different sexes can make one sex more susceptible to parasitic infections than the other. Differences in endoparasite abundance between sexes may be related to host diet, habitat use and reproductive period (Goldberg and Bursey, 1989; Pereira et al., 2012; Brito et al., 2014b; Araújo-Filho et al., 2016). The males of *S. torquatus* present more territorial behaviour than females (Rodrigues et al., 1989), which may have influenced the fact that these individuals were more parasitized than females. Lizards that present this type of behaviour spend more time patrolling or participating in territorial disputes, raising stress levels that can compromise an individual's immune system. Additionally, during the reproductive period, this type of behaviour tends to intensify due to an increase in testosterone levels, and very high levels of testosterone can cause immunosuppression in the animal, making these individuals more susceptible to infections (Folstad and Karter,1992). However, due to the lack of studies on the ecology of *S. torquatus* and information about the differences that may exist between sexes, and

since we found that the males and females did not differ in terms of diet, we cannot say for sure which factors contributed to the increase in the endoparasites abundance in male *S. torquatus* individuals.

We observed that the highest levels of infection occurred during the rainy season. The endoparasite species that showed the highest prevalence were *P. lutzi*, *S. loboi* and *S. oscari*. Despite presenting different life cycles, heteroxene and monoxenes, respectively, all of these species depend on increased humidity and precipitation in the environment in order for their eggs and larval stages to persist longer in the environment (Anderson, 2000).

In a study by Brito et al. (2014b) in the semi-arid Caatinga, the environmental conditions during the rainy season influenced the endoparasite abundance in *Tropidurus hispidus*. The authors emphasized that seasonal variation in parasite abundance may have occurred because the identified endoparasites have a monoxenous (direct) life cycle and may encounter the best environmental conditions during the rainy season. Araújo-Filho et al. (2016) analysed populations of *T. hispidus* in Caatinga, Restinga and Atlantic Forest habitats, and observed different seasonal variations. In the Caatinga, the highest infection rates occurred during the rainy season, while in the Restinga areas the highest endoparasite abundance was observed during the dry season, and in the Atlantic Forest region no variation was observed. These publications have shown the importance of performing studies on the same species in different environments, as they can generate relevant information about seasonal and geographic differences in the infection process and variation in the endoparasites abundance.

According to Gambhir et al. (2012), temperature and precipitation are the main climatic factors that influence both the distribution of host species and the survival of parasites. Temperature can influence the transmission, development and mortality rates of endoparasite larval stages, and therefore, acts as a regulatory agent of endoparasite populations in hosts (Esch et al., 1977; Altizer et al., 2006). Rainfall intensity, in particular, is a critical factor because excessive rainfall can sweep eggs and larvae deposited in the environment away during runoff, thus limiting the probability of transmission to potential hosts (Stromberg, 1997).

In the present study, the increase in precipitation associated with humidity during the rainy season may have mainly favoured nematodes, whose transmission occurs through the ingestion of eggs or free-living juvenile stages by *S. torquatus*, because during the rainy season the humidity and temperature conditions are optimal for parasites eggs to persist longer in the environment. (Lafferty and Kuris, 2005).

The differences in infection prevalence and intensity between male and female hosts are usually attributed to the different types of diets observed in these individuals (Goldberg and Bursey, 1989). However, in our analysis, male and female lizards did not differ in terms of diet, and the same was observed when comparing dietary data between seasons (Table 2). We observed a high niche overlap 0.999, indicating that male and female *S. torquatus* consumed practically the same prey during the study period. Despite this high niche overlap, endoparasite abundance varied in relation to sex and seasonality. According to Fontes et al. (2003) and Galdino et al. (2014) the differences in infection parameters observed between sexes may be due to other factors that are complementary to the diet.

The life cycles of the endoparasites found in *S. torquatus*, were associated with seasonal variation in the environment and the behavioural and physiological differences between male and female hosts may have influenced the endoparasite abundance observed here. The nematode *P. lutzi* uses insects as intermediate hosts (Anderson, 2000), which, in turn, are the main food source of *S. torquatus* (Rodrigues et al., 1989). Although *S. torquatus* is an arboreal species, these individuals tend to forage at various heights along tree trunks and occasionally on the ground (Rodrigues et al., 1989). During the collections, individuals were found in treetops, standing tree trunks, fallen trunks (at various elevations), and in pitfall traps at ground level. The presence of *S. torquatus* on the ground, and at the different types of elevations, may explain how these individuals may have come into contact and became infected with the eggs of *S. loboi* and *S. oscari*.

Due to the low prevalence of *H. odhneri* and the general lack of information regarding the parasitic fauna of *S. torquatus*, we believe that the occurrence of this species of Trematoda parasitizing a single specimen of *S. torquatus* was accidental. However, further studies on the parasitic fauna of *S. torquatus* would be needed in order to confirm whether this species also acts as a definite host for *H. odhneri*. This is the first study analysing seasonal influence on the diet and endoparasites of *S. torquatus*, in addition to providing the first record of the trematode *H. odhneri* parasitizing this lizard species. We emphasize that, in addition to seasonal variation, other factors can influence the parasitic infection processes, such as host sex and body size, as well as the behaviour and diet of individuals and should be considered in future studies.

ACKNOWLEDGMENTS

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Diversity, distribution, habitat preferences and community assemblages of Amphibians and Reptiles in the "Cilento, Vallo di Diano e Alburni" National Park (Campania, Southern Italy)

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Abstract. Amphibians and reptiles are important ecological indicators of ecosystem status and play significant ecological roles. However, their populations are declining globally due to factors such as climate and land-use change. To effectively manage these species, it is crucial to study their distribution in protected areas. In this study, we provide new information on the distribution and ecology of amphibians and reptiles in the "Cilento, Vallo di Diano e Alburni" (CVDA) National Park in southern Italy. We used a comprehensive dataset consisting of 2465 records obtained from fieldwork, citizen science, and literature to evaluate species' rarity, habitat preferences and community assemblage. We investigated whether differences in species richness across different habitat types and elevations resulted from species selection of different environmental conditions. We quantified species probability of observation in different habitat types and estimated the significance of species-habitat associations. Species rarity was assessed by considering geographic range, population abundance, and habitat breadth. Collected data substantially improved knowledge of the distribution of numerous species compared to published data. Our findings are generally consistent with the species' ecological information available in Italy, but we also found some species' peculiar ecological aspects that are little-known. The CVDA National Park emerges as an effective protected area that ensures good conservation status of the herpetofauna, also in the case of species showing population and range declines at the national scale.

Keywords. Herpetofauna, protected areas, community ecology, elevation range, species rarity.

INTRODUCTION

Amphibians and reptiles play important roles in nutrient cycling and connecting aquatic and terrestrial environments. They are also considered ecological indicators of ecosystems' status and stability (Pereira et al., 2012). Nonetheless, the global biodiversity crisis includes a decline in amphibian populations worldwide, with over 40% of amphibian species at risk of extinction (Stuart et al., 2004; IUCN, 2023). Significant population declines have also been found for reptile populations, with more than 21% of the world's reptiles threatened by extinction (Cox et al., 2022). Amphibians and reptiles face similar stressors, including agriculture intensification, overexploitation of natural resources, urbanization, introduction of alien species and climate change. In particular, panzootic chytridiomycosis is a major threat to amphibians and it is causing widespread population collapses and extinctions worldwide (Scheele et al., 2019). These factors mainly operate at small spatial scales, resulting in numerous cases of local extinctions. Local conservation action within protected areas can be the key to halting the decline of

amphibians and reptiles. To achieve this, it is crucial to have a comprehensive picture of species' ecology and distribution in these areas. Studying the distribution of herpetofauna in protected areas provides information to identify areas of high conservation value and prioritize conservation actions, such as habitat and connectivity restoration, to improve landscape-scale conservation (Beale et al., 2013). This information can also guide decisions about land-use planning to ensure sustainable development in complex landscapes where people and biodiversity coexist (Guillera-Arroita et al., 2015). This is the case of the Cilento (Campania Region), a highly diverse but yet under-investigated area of southern Italy protected by the "Cilento, Vallo di Diano e Alburni" (CVDA) National Park, which ensures a remarkable equilibrium between nature and society. The Park hosts many endemic species and subspecies of herpetofauna (Romano et al. 2010; Romano, 2014) whose distribution has been historically overlooked. Here we provide detailed information on terrestrial herpetofauna distribution and ecology in the CVDA National Park by presenting and using a dataset resulting from 14 years of fieldwork, citizen science and literature. Using the distribution dataset, we evaluated species rarity, the influence of elevation on species distribution and richness, the terrestrial and aquatic habitat preferences and, finally, the assemblage of species at terrestrial and water sites.

MATERIAL AND METHODS

Study area

The "Cilento, Vallo di Diano e Alburni" National Park (CVDA) National Park is located in the Province of Salerno, southern Italy, and has a total area of 181,048 hectares, making it the largest national park in Italy. The park extends between 40°00' and 40°30'N and 14°50' and 15°40'E, stretching from the Tyrrhenian coast to the foot of the Apennines in Campania and Basilicata regions. It includes the peaks of Alburni Mountains, the Mt. Cervati (the highest mountain of the park, 1897 m asl), the Mt. Gelbison and the coastal buttresses of Mt. Bulgheria and Mt. Stella (Fig. 1). The park's topography is mainly hilly and mountainous except for the tectonic depression of the Vallo di Diano, which is the northern portion of the park. This complex topography translates into high environmental and climatic heterogeneity (Guarino et al., 2012; Guglietta et al., 2015). The lowland and coastal zone is characterized by a typically Mediterranean climate, with dry winters and warm summers, while the highland zone is more temperate, with dry winters and cold summers (Peel et al., 2007). According to the Corine

Land Cover Level 2 (Copernicus, 2018), the study area is mainly covered by Forests (52.13%) and Heterogeneous agricultural areas (hereafter Heterogeneous agrosystems, 15.02%) and Shrub and/or herbaceous vegetation associations (hereafter Scrub/herb vegetation, 13.49%), followed by Permanent crops (8.78%), Open spaces with little or no vegetation (6.29%, hereafter Scarce Vegetation), Arable land (2.43%), Urban fabric (1.37%) and Pastures (0.47%) (Fig. 1). Coastal areas are mainly characterized by Mediterranean scrubland while inner zones present extensive forests with interspersed natural grasslands. Several human settlements existed since ancient times in the Cilento area, however, human population density in the Park (85 municipalities, total population about 200.000) is rather low, with a median of 60 inhabitants per km^2 (ISTAT, 2023). In general, human settlements coexist in harmony with the natural environment and reflect local people's traditional lifestyles (Cerreta & Girard 2021). Traditional agro-pastoral practices have created complex patterns of agrosystems, mainly based on low-intensity annual crops, and olive groves as permanent crops; these agrosystems host significant patches of natural vegetation throughout the study area.

Data collection

We obtained data on species occurrences from three main sources: literature, citizen science, and fieldwork. To ensure complete coverage of the study area, we divided it into a grids of cells of 5×5 km, which are submultiples of the 10×10 km UTM grid, resulting in 117 cells overlapping with the Park area. Grid-based sampling provides a good trade-off between sampling bias and costs at both the single- and multi-species levels (Marta et al., 2019). During springs and summers of the last 14 years (from 2008 to 2022), we carried out at least one field survey in each cell (mean surveys per cell: 3.692) targeting historical sites known from the literature and new sites potentially suitable for herpetofauna. We identified potentially suitable sites using the Military Geographical Institute Map (IGM) (MATTM, 2017), which accurately maps the presence of water, and aerial photographs from Google Maps (https://www.google.it/maps) using the software QGIS 3.22 (https://qgis.org/).

To search for amphibians and reptiles on the field, we followed standard methodologies (McDiarmid et al., 2012; Dodd, 2009; 2016). In particular, amphibians' potential aquatic habitat types have been identified and sampled following the methods reported by Romano et al. (2010; 2012). For both, we gathered additional information about species presence from local people, primarily shepherds and farmers. We obtained bibliographic
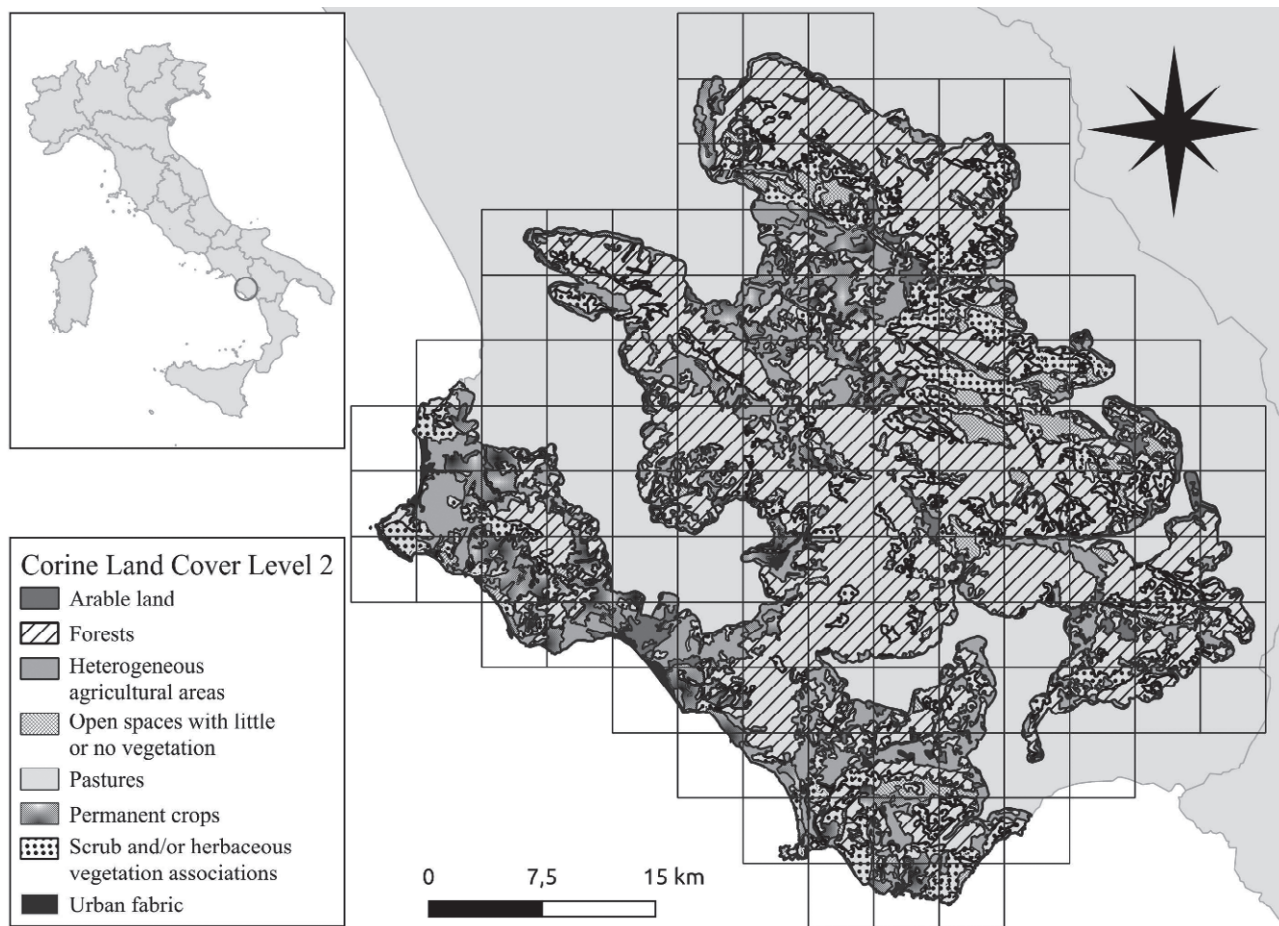
Fig. 1. Study area, the "Cilento, Vallo di Diano e Alburni" National Park, with Corine Land Cover level 2 categories and the used UTM grid of 5×5 km cells.

data from Caputo et al. (1985), Caputo and Guarino (1992), and Ruffo and Stoch (2005). We georeferenced species occurrences following the "Guide to Best Practices for Georeferencing" (Chapman and Wieczorek 2006) using QGIS and the IGM (MATTM 2017), which provides toponyms used in the above-mentioned sources. In 2021 we created a citizen science project on iNaturalist entitled "Herpetofauna of Cilento" (https://www.inaturalist.org/projects/erpetofauna-del-cilento).

Species richness and elevation range

Using the chosen grid of 5×5 km, we obtained maps of species α diversity (species number per grid cell) for amphibians, reptiles and the two combined using the QGIS function "count points inside polygons" with species as value of the "class" parameter. We used the Copernicus Digital Elevation Model (DEM) at 20 m resolu-

tion (Copernicus, 2021) to extract the elevation (m asl) of each occurrence point in QGIS. For observations that could present the species' higher elevation limits, we used a GPS Garmin device to record the elevation in the field. We reclassified the DEM into nine elevation classes (Fig. S1) of 200 meters (except for the highest class that ranged from 1600 to 1879 m asl) using QGIS. To investigate whether there are significant differences among species' elevation preferences, we used Kruskal-Wallis one-way analysis of variance (KW), followed by Mann-Whitney U test and Bonferroni correction. We tested for correlation between elevation classes and α diversity by applying the Spearman rank correlation test for herpetofauna, amphibians and reptiles, using the function cor.test in R software version 4.2.3 (https://www.r-project.org/). Furthermore, we used the Chi-square test to test the hypothesis that changes in species richness reflect only the available land area. This involved comparing the percentage of land area and the percentage of species occurrence in each eleva-

tion class. KW, Mann-Whitney U and Chi-square tests were performed as in the statistical package PAST ver.4 (Hammer et al., 2001).

Species richness and rarity

To describe species diffusion and rarity, we considered only those 5×5 km cells containing at least one record of amphibians (N=85) or reptiles (N=102) of the 117 5x5 km cells. We estimated species rarity using two methods, the Index of Species Rarity (ISR) and Rabinowitz's forms of rarity. The ISR was originally proposed by Gheu & Gheu (1980) and was calculated as follows: ISR = (1-n/N)*100, where n is the number of grid cells occupied by a species and N is the total number of grids of its class. This index ranges from 0 (very common species) to 100 (very rare species, i.e. only one record). The second method is based on Rabinowitz's approach (Rabinowitz et al., 1984) as implemented in the "Rare7" R package (Maciel and Arlé, 2020), which employs a combination of three parameters to evaluate species' rarity: (1) geographic range, (2) population abundance, and (3) habitat breadth. The combination of these three parameters results in the classification of a species as either common or as one of seven types of increasing rarity. We estimated the size of the geographic range for each species by counting the number of occupied 5x5 km cells. We used the mean number of records per cell as a proxy for local abundance, and we calculated habitat specificity by examining the number of Corine Land Cover level 3 types (CLC3) in which the species were found. To account for the effect of possible local extinctions, we calculated ISR and Rabinowitz's only using post-2000 data. We chose three thresholds to classify species in a wide or restricted range, various or uniform habitat and scarce or abundant populations: the 25th percentile of occupied cells/total number of cells, the median number of mean occurrences per cell and the median number of frequented habitat types (CLC3).

Habitat preferences

To determine the terrestrial habitat type in which the species was recorded, we used the Corine Land Cover (CLC), a European program that collects and disseminates data on land cover and land use throughout Europe (Copernicus, 2018). The CLC classifies land cover into different levels of detail, namely CLC Level 2 (CLC2) and Level 3 (CLC3). The first comprises 15 broad land cover types that distinguish between macro environmental conditions, e.g. Urban Fabric, Arable Land, For-

ests etc. The second provides more detailed information within each of the Level 2 land cover types, resulting in 44 subcategories. For instance, the Level 2 category "Forests" is further subdivided into three Level 3 categories: broad-leaved forest, coniferous forest and mixed forest. We assigned a CLC2 category to each occurrence point in QGIS 3.2 with the "join attributes by location" function. CLC2 categories were considered relevant for species' broad distribution in the study area as they identify general differences among environments. Eight CLC2 categories are present in the study area (Fig. 1). For aquatic habitat types, we classified amphibians' breeding sites into nine categories based on their characteristics (Romano et al., 2010; Romano, 2014): ditch (small running waters occurring in agricultural or urban areas), drinking trough for livestock grazing (rectangular and elongated, singular or multiple container, made of concrete or stone, filled by running water), pond (small still waters), river (perennial running waters greater than streams), spring (pools generating from a water spring), stream (running waters with a torrential regime), tank (generally a single quadrangular stone or cement cistern designed to collect water for irrigation), vernal pool (small seasonal still waters), and well (holes drilled to access groundwater) (for detailed differences among artificial water sites see Romano, 2012).

To investigate species-habitat associations, we calculated the probability of observing the species in each habitat type (aquatic for amphibians and terrestrial for both) and the significance of each possible association. In order to examine the relationship between species and habitat type, we made a 2×2 contingency table (Table 1) with the following division of species occurrences: *i*) the number of occurrences of the focal species in the focal habitat type (1,1: row 1 and column 1), *ii*) the number of occurrences of all species recorded in the habitat type (1,2), *iii*) the number of occurrences of the focal species in the focal habitat type (2,1), and *iv*) the number of occurrences of all species recorded in all habitat types (2,2). We used this table to estimate species' probability of being

Table 1. The 2x2 contingency table used to calculate odds ratios for species-habitat relationships with species' occurrences on rows and habitat types on columns.

	Habitat type	All habitat types
Species	Number of occurrences of the focal species in the focal habitat type.	Number of all occurrences of the species except those in the focal habitat type.
All species	Number of all occurrences recorded in the habitat type except those of the focal species.	Number of all occurrences recorded in all habitat types except those recorded in the focal habitat type.

observed in each habitat type as by calculating the odds ratio by dividing the product of the counts in cells 1,1 and 2,2 by the product of the counts in cells 1,2 and 2,1 and then by transforming the odds ratio in probability of observing in each habitat type using the equivalence: Probability = Odds / (Odds + 1) (McHugh, 2009; Grant, 2014), obtaining values ranging from 0 (low probability of observing the species in the habitat type) to 1 (high probability). To determine if the found habitat-species associations were statistically significant, we performed the Fisher's Exact Test for count data using the 'fisher.test' R function. Finally, we used the Chi-square test to test the hypothesis that changes in species richness between habitat types reflect the available habitat types' area. This involved comparing the percentage of land area and the percentage of species occurrence in each habitat type in the software package PAST ver.4 (Hammer et al., 2001).

Community assemblages

To investigate species association, we first defined species assemblages using distances between occurrence points and habitat types. To create amphibians sampling units, we used a 100 meter buffer from each occurrence and dissolved the resulting circles according to the occupied aquatic habitat types, merging multiple buffer polygons of the same habitat type into one single polygon. This method ensured that if two species occurred within a distance of 100 m but in two different habitat types (e.g. a stream and a stony well), they would be considered from two different species assemblages. Similarly, we created sampling units for both amphibians and reptiles using a buffer of 250 m from each occurrence and dissolving the resulting circles according to the occupied terrestrial habitat types. We used classes from the CLC3 (Copernicus, 2018), which we deem suited to describe the environmental filters behind the community assemblage process, and QGIS for spatial operations.

We created a matrix of species presence/absence per sampling unit using the function "ddply" from the "plyr" R package and "dcast" functions from the "maditr" R package. We used it to perform community dissimilarity analyses for both amphibians and reptiles using the Raup-Crick Dissimilarity Index with Unequal Sampling Densities of Species (Raup and Crick 1979) using the 'raupcrick' function (Chase et al., 2011) in the R package 'vegan' version 2.6-4 (Oksanen et al., 2022). The Raup-Crick Index is a measure of β diversity used to identify differences in species assemblages occurring in different environments. This index describes the probability that compared sampling units have non-identical species composition. This probability is calculated through

"oecosimu" simulation (R package 'vegan') (Oksanen et al., 2022), where the observed amount of shared species between the tested sampling units is used as the test statistic and compared to a community null model where the probability of selecting a species is directly proportional to its frequency (Oksanen et al., 2022). The Raup-Crick Index ranges from 0 (identical assemblages) to 1 (distinct assemblages). Finally, we performed a cluster analysis based on Raup-Crick index values using the R function 'hclust' with Ward's minimum variance clustering method (method="ward.D") to investigate how species relate to each other based on the similarity of their assemblages. We excluded two species found in only one location (*Emys orbicularis* and *Testudo hermanni*).

RESULTS

Species distribution

Following the taxonomy of Speybroeck et al. (2020), we recorded 16 species of reptiles and 11 of amphibians (Table 2). The collected dataset amounted to 2465 records (835 for amphibians and 1630 for reptiles). We georeferenced 330 records from bibliographic sources (194 and 143, for amphibians and reptiles respectively). From the iNaturalist project (at the 5th February 2023) we obtained 401 observations (130 and 271) by 180 users (excluding fieldwork personnel); finally, we collected 1734 occurrence points during fieldwork (498 and 1217). Bibliographic sources mostly covered the decades from 1980 to 2000 while most of the data were collected during the last 2 decades and recent years (mean = 2014, median = 2020). Maps showing the distribution of each species are available on Fig. S2.

Species α diversity per grid cell ranged from 1 species (3.6% of total species present in the area) to 18 (66.7%), with a median diversity value of 8 (Fig. 2). When considering amphibians and reptiles separately, cells with amphibians ranged from 0 to 9 per cell (81.8% of total), while those with reptiles ranged from 1 (6.25%) to 11 (68.75%) per cell. Interestingly, highly diverse cells were distributed throughout the territory but did not exhibit a uniform or random pattern. Instead, they tended to cluster together, particularly towards the inner regions of the territory.

Elevation distributions of amphibian and reptile species are shown in Fig. 3. The KW test showed highly significant differences among species in each of these two classes (H = 175.4, H = 375.4 for amphibians and reptiles respectively; P < 0.001 for both classes). Among amphibians, *S. salamandra* and *T. carnifex* were present at the highest elevations, with the second species also charac-

Table 2. Amphibians and reptiles that occur in the "Cilento, Vallo di Diano e Alburni" National Park with short names used in figures, number of sites of occurrence and occupied 5x5 km cells. *Species endemic to Italy.

Species	Short name	Records pre-2005	Records Post-2005	Total Records	Number of Cells
AMPHIBIA					
Salamandra salamandra	Salsal	10	19	29	11
Salamandrina terdigitata*	Salter	6	30	36	25
Lissotriton italicus*	Lisita	38	170	208	66
Triturus carnifex	Tricar	18	32	50	14
Bombina variegata	Bomvar	22	24	46	23
Bufo bufo	Bufbuf	30	69	99	47
Bufotes viridis	Bufvir	0	4	4	4
Hyla intermedia*	Hylint	4	57	61	34
Pelophylax lessonae complex	Pelles	16	52	68	37
Rana dalmatina	Randal	7	11	18	12
Rana italica*	Ranita	34	182	216	66
REPTILIA					
Emys orbicularis	Emyorb	0	1	1	1
Testudo hermanni	Tesher	0	1	1	1
Hemidactylus turcicus	Hemtur	3	14	17	12
Tarentola mauritanica	Tarmaur	3	111	114	47
Anguis veronensis*	Angver	3	33	36	30
Lacerta bilineata	Lacbil	20	152	172	56
Podarcis muralis	Podmur	14	57	71	20
Podarcis siculus	Podsic	22	678	700	103
Chalcides chalcides	Chacha	12	19	31	24
Coronella austriaca	Coraus	6	11	17	14
Elaphe quatuorlineata	Elaqua	11	72	83	56
Hierophis viridiflavus	Hievir	17	201	218	82
Natrix helvetica	Nathel	11	53	64	37
Natrix tessellata	Nettes	3	10	13	12
Vipera aspis	Vipasp	10	45	55	40
Zamenis lineatus*	Zamlin	8	29	37	27
TOTAL		328	2137	2465	

terized by a narrow elevation range. The two species are thus restricted to mountain areas in the Park. *Bombina variegata* also preferred high elevations although some sites were recorded in hilly and lowland areas. Conversely, *B. viridis* and *P. lessonae* complex occurred mainly in lowland, although the latter exhibits a significantly wider elevation range than the former. The other amphibian species generally had a wide elevation range, with *B. bufo* reaching the highest quote (1850 m asl) in a vernal pool on the Cervati mountain peak. Another species showing a wide elevation range is *R. dalmatina*, which occurs from 75 to 1600 m asl. Among reptiles, *P. muralis* and *C. austriaca* mainly occur at high elevations. Similarly, *C. chalcides* and *L. bilineata* also occur mainly at high elevation, however both are present at lower elevations as well. Lowland species are *H. turcicus, T. mauritanica* and

N. tessellata, which only occasionally reached the mid-range elevations; while *E. quatuorolineata* and *Z. lineatus* showed a wider elevation range.

The nine elevation classes occupy an area percentage ranging from 0.6% and 19.7% (Fig. 4a). Species α diversity generally decreases with increasing elevation classes (Spearman correlation test, r_s = -0.853; P = 0.005), with the middle elevation classes (200-400, 400-600 and 600-800 m) holding the highest number of species (Fig. 4a). However, when considering the two classes separately, amphibians α diversity was not significantly correlated to elevation classes while reptiles α diversity was (r_s = -0.579, P = 0.108; r_s = -0.900, P = 0.001). Amphibians and reptiles α diversity showed a different pattern along elevation classes, with the first showing high richness at all classes except for the last two, and the latter a clear
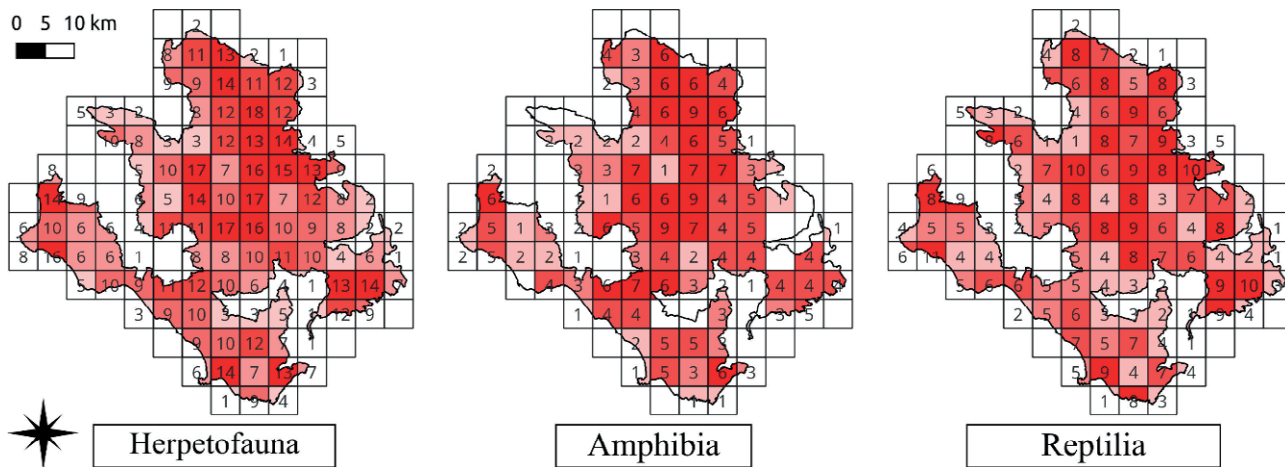
Fig. 2. Maps of species richness for the whole herpetofauna in the study area (a), and for amphibians (b) and reptiles (c) separately.
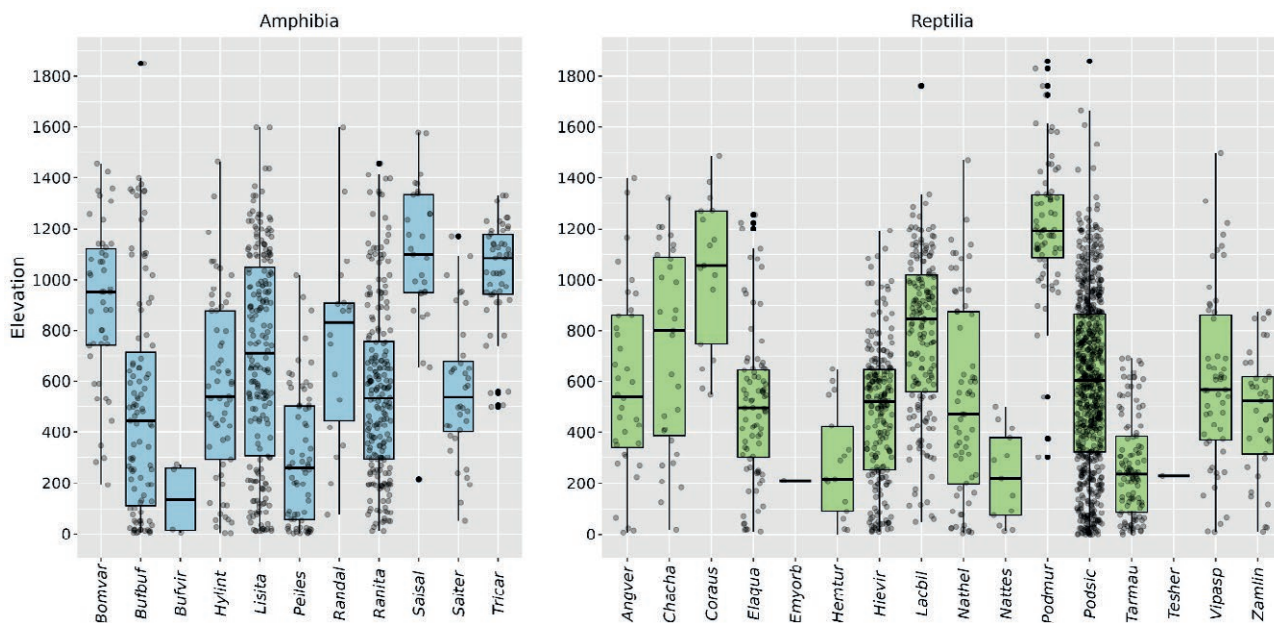

Fig. 3. Box plots of the altitudinal distribution of amphibians and reptiles in the "Cilento, Vallo di Diano e Alburni" National Park. The box represents the interquartile range (H-Spread), the median is the horizontal line inside the box; the lines extend from the lowest to the highest value within 1.5 H-Spread from the end of the box. Black circles at the end of the lines represent outliers. Species abbreviations as in Table 2.

almost monotonic decline in species richness after the 400-600 m class (Fig. 4a) with an important inflection after 1400-1600 m class. The results of the Chi-square test indicate that the species richness in the various elevation classes was proportional to the elevation class extent (Chi-square values were 13.34, 7.44 and 9.35 for amphibians, reptiles, and both, respectively; df = 9 and P > 0.05 for all tests).

Species rarity

The Index of Specific Rarity (Fig. 5), based on distribution and frequency, revealed a wide range of values among reptiles, with *P. siculus* as the most common species and others, i.e., *T. hermanni* and *E. orbicularis,* occurring in only in a single location. Snakes displayed varying degrees of rarity, with *H. viridiflavus* being relatively common and *C. austriaca* being the least common

snake. Among amphibians, *B. viridis* is localized in only 4 sites*, R. dalmatina,* and *S. salamandra* exhibited relatively restricted distribution, whereas the two Italian endemic *R. italica* and *L. italicus* were widely distributed with a high number of sites.

Rabinowitz's classification provided additional insights (Fig. 6). Common species were a minority for both amphibians and reptiles, with 3 (27%) and 6 (37%) species respectively classified in this category. On the opposite end of the spectrum, the seventh form of rarity had 2 (18%) species of amphibians and 7 (31%) of reptiles.

Habitat preferences

The most frequented habitat type was Forests (35.50% of occurrences) followed by Scrub/herb vegetation (20.73%), Heterogeneous agrosystems (17.20%), Scarce Vegetation (10.71%), Permanent crops (8.84%), Urban fabric (4.06%), Arable land (2.47%) and Pastures (0.49%). (Fig. 4b). The habitat type that hosts the highest number of amphibian species is Forests, while Forests, Heterogeneous agrosystems, and Scrub/herb vegetation have the highest number of reptile species occurrences (Fig. 4b). The median number of frequented terrestrial habitat types (i.e., where the species was observed at least once) was 6.5 for reptiles and for 6 amphibians, out of 8 available terrestrial habitat types (Fig. S3). In addition, the median num-

Fig. 4. Percentage comparisons between herpetological species richness and (a) the area availability of altitudinal ranges, and (b) the area availability of Habitat types in the "Cilento, Vallo di Diano e Alburni" National Park.
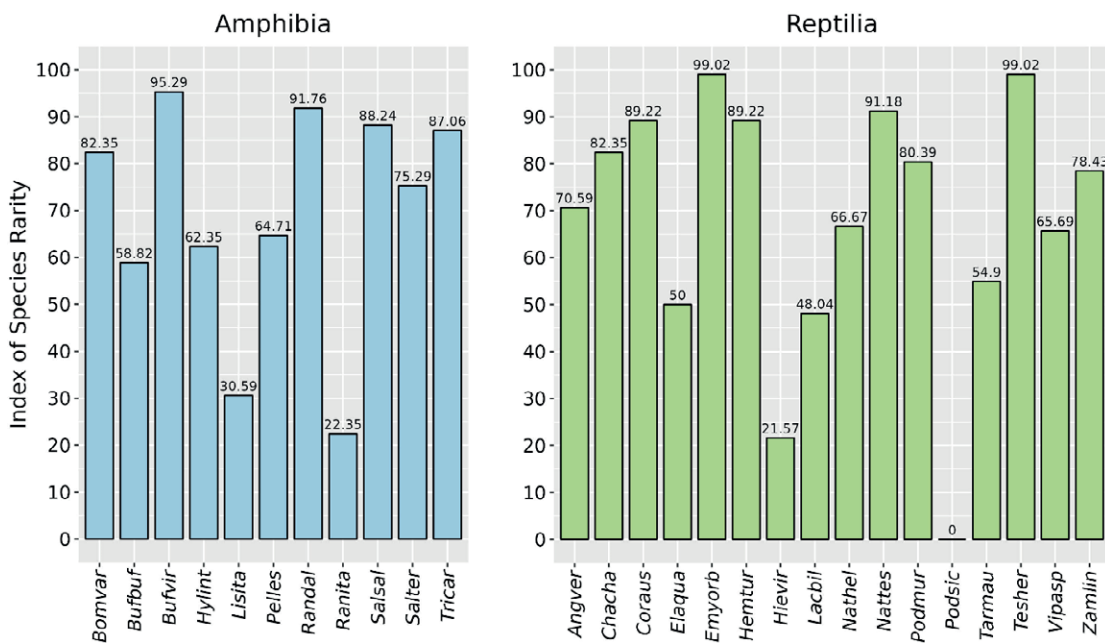
Fig. 5. Bar plots of the Index of Specific Rarity (ISR) of amphibians and reptiles in the "Cilento, Vallo di Diano e Alburni" National Park. The higher the ISR value, the rarer the species. Species abbreviations as in Table 2.
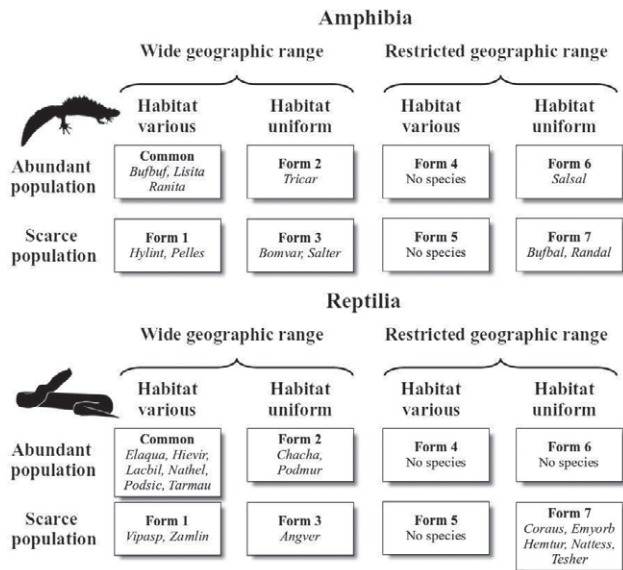
Fig. 6. Rabinowitz's index of rarity of amphibians and reptiles in the "Cilento, Vallo di Diano e Alburni" National Park, as implemented in the "Rare7" R package (Maciel and Arlé, 2020), The index provides a classification of a species as either common or as one of seven types of increasing rarity. Species abbreviations as in Table 2.
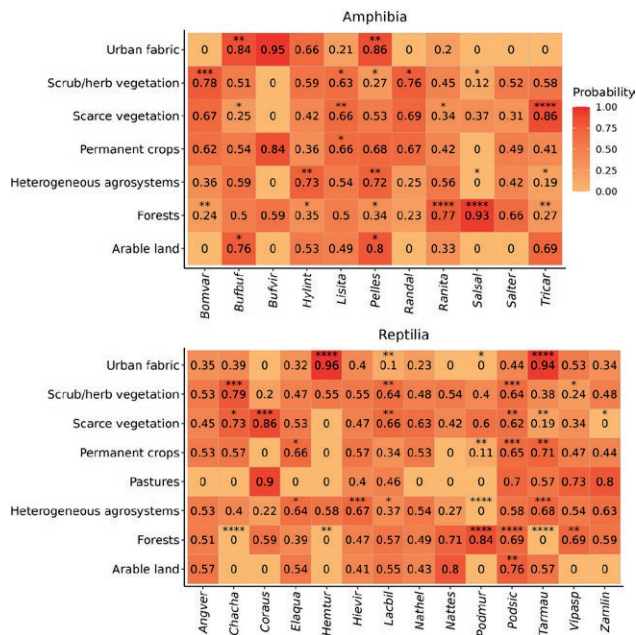

Fig. 7. Probability (Odds ratio / (Odds ratio + 1; see text) of amphibian and reptile preference for terrestrial habitat types (i.e. Corine Land Cover level 2 classes) in the study area. Statistically significant preferences are shown with asterisks: * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001. Species abbreviations as in Table 2.

ber of aquatic habitat types frequented by amphibians was 6 out of 9. However, the probability of observing a species in each habitat type was not the same, furthermore, not every species-habitat association was statistically significant according to the Fisher exact test (Figs. 7, 8).

The results of the Chi-square test indicate that the species richness was not proportional to the amount of habitat type area (Fig. 4b; Chi-square values were 31.8, 43.6 and 38.3 for amphibians, reptiles, and both, respectively; df = 8 and P < 0.01 for all tests). We identified clear signals of habitat type preference (high probability of observing the species) or avoidance (low probability for all species). Reptiles showing significant habitat associations were 11 out of 16 while amphibians were 9 out of 11. The median number of significant associations was 2 for reptiles and 3 for amphibians. The species with the highest number of significant associations was, among reptiles, *P. siculus* (5) and, among amphibians, the *P. lessonae* complex (5). Regarding aquatic habitat types, all amphibians showed significant associations (median 2) with *Lissotriton italicus* and *P. lessonae* complex showing the highest number among species (6).

Species association

The Raup-Crick Index highlighted high community similarity between pairs of species. Among amphibians,
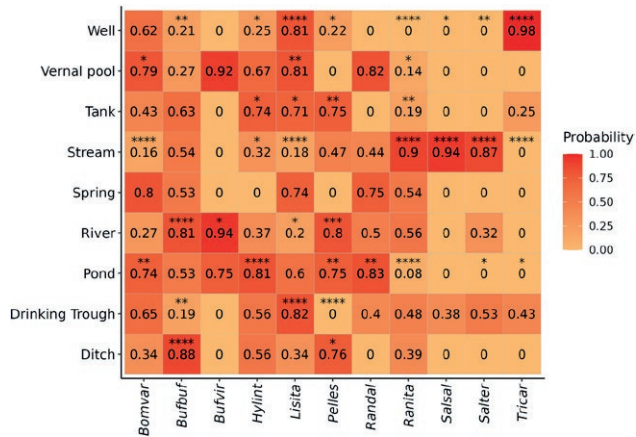

Fig. 8. Probability (Odds ratio / (Odds ratio + 1; see text) of amphibian preference for aquatic habitat types. Statistically significant preferences are shown by asterisks: * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001. Species abbreviations as in Table 2.

the pairs with the lowest Raup-Crick Index were *Triturus carnifex* and *Lissotriton italicus* (0.002), *Salamandrina terdigitata* and *Rana italica* (0.01), *Triturus carnifex* and *Bombina variegata* (0.12), while among reptiles, *Vipera aspis* and *Elaphe quatuorlineata* (0.001), *Tarentola mauritanica* and *Hemidactylus turcus* (0.001) and *Natrix tessellata* and *Natrix helvetica* (0.003) (Fig. S3).
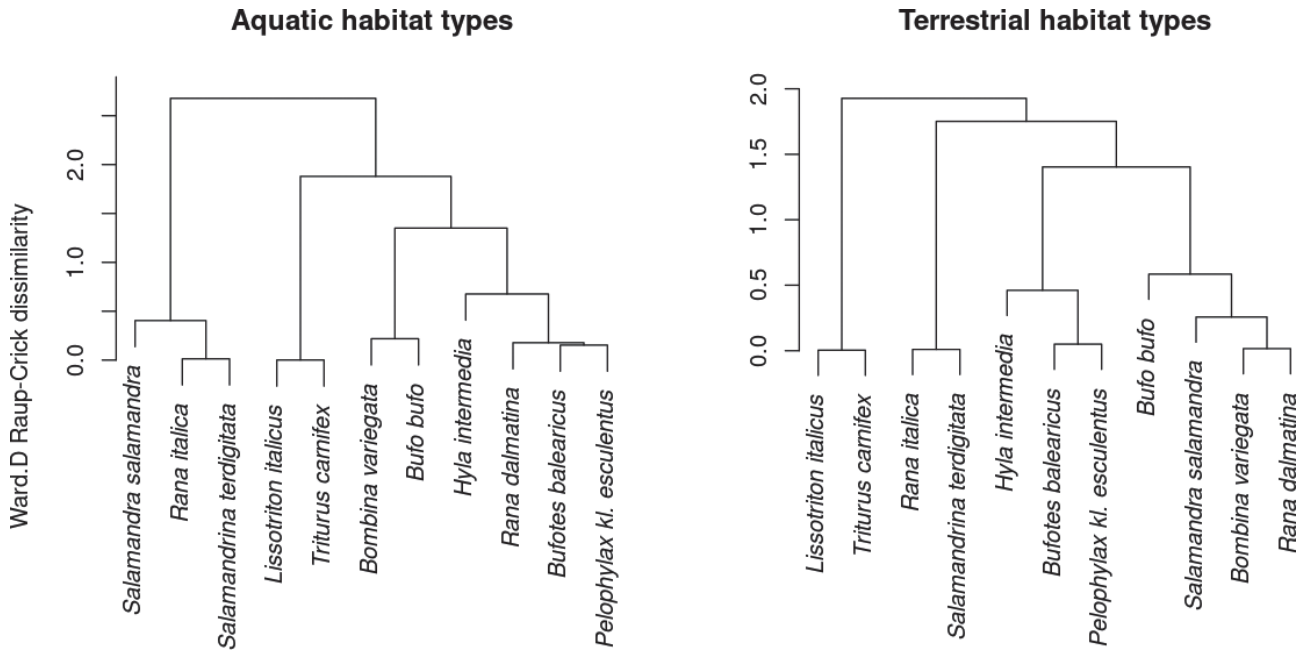
Aquatic habitat types

Terrestrial habitat types

Fig. 9. Cluster analysis of amphibian species similarity assemblage based on Raup-Crick index values using the Ward's minimum variance.
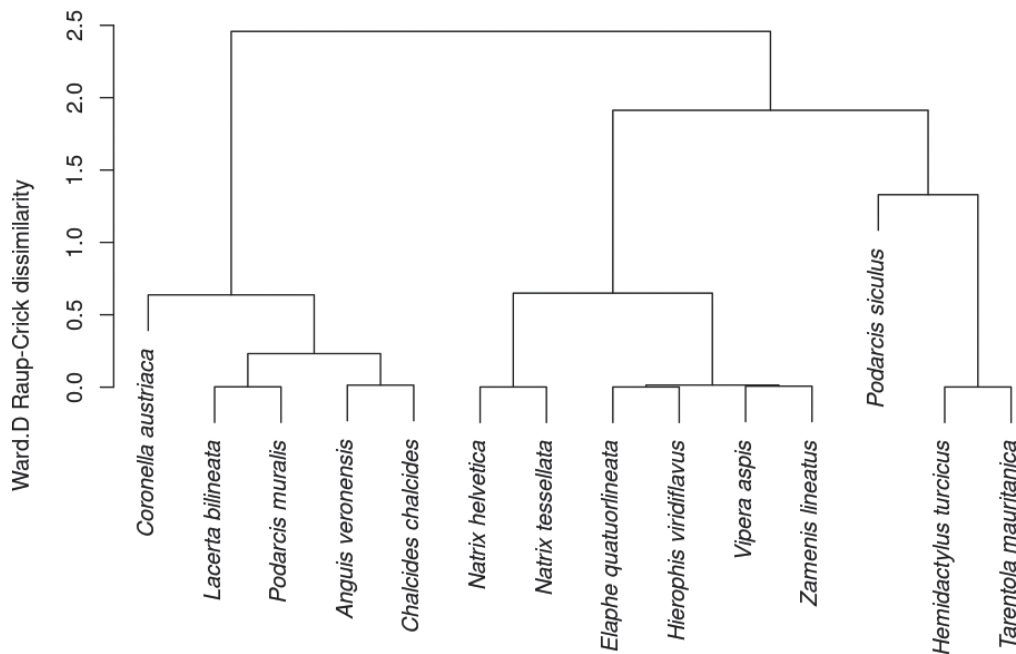

Fig. 10. Cluster analysis of reptile species similarity assemblage based on Raup-Crick index values using the Ward's minimum variance.

The cluster analyses based on The Raup-Crick Index produced a clear indication of species associations (Fig. 9 a,b; Fig. 10). Considering aquatic habitat types, among amphibians are recognizable two main clusters (Fig. 9a): one including species associated with running waters and one including all the remaining species that preferred lentic or very slow-running waters. Considering the terrestrial habitat types, the association among species is

similar but shows some differences (Fig. 9b). Reptile species relationships are more complex (Fig. 10) with almost all snakes clustered together, with *N. helvetica* and *N. tessellata* grouped in a subcluster because both were found mainly in acquatic environments. The last cluster includes *P. siculus*, which may be considered almost ubiquitous, and the two geckos that are associated with urban and open areas and that avoid forests.

DISCUSSION

Species distribution

Protected areas are designated areas that are managed to conserve natural ecosystems and biodiversity (Rodríguez-Rodríguez and Martínez-Vega, 2022) and can provide critical refuge for species sensitive to habitat loss and fragmentation. However, the effectiveness of protected areas in conserving biodiversity depends on their ability to represent the diversity of species that occur within their boundaries. Using an extensive dataset based on decades-long fieldwork, citizen science and bibliographic sources, we investigated the distribution, ecology and community assembling of amphibians and reptiles in the "Cilento, Vallo di Diano e Alburni" National Park. Our work painted a comprehensive picture of the herpetofauna diversity inside the park, which has been overlooked in the past, and highlighted how the Park represents a hotspot area for amphibians and reptiles in Southern Italy. We found all the species that are potentially present in the area according to their known regional distribution (Guarino et al., 2012). They greatly differed in distribution and rarity. *Emys orbicularis* (classified by the IUCN as Endangered) and *Testudo hermanni* (Critically Endangered, Rondinini et al., 2022) are localized in only one site; in particular, the presence of a wild population of *T. hermanni* has not been confirmed, as only an individual in the wild was found, and the possibility of an escape from captivity cannot be ruled out. With the exception of these two species, the other ones have been classified in some category of threat by the IUCN (Rondinini et al., 2022), are quite extensively distributed inside the park. The conservation value of the study area is thus significant not just locally but also at a national scale, as several rare endemic species and subspecies (Table 2) were recorded (Fig. S2 and Fig. 5 and 6).

The highest values of α diversity for both amphibians and reptiles are found in inland areas, particularly hilly and mountainous regions. However, some coastal or sub-coastal zones also exhibit high values of species richness (Fig. 2). Defining elevation ranges at a local scale can help determine the potential presence of certain species, making it easier to detect and monitor them within pro-

tected areas. Higher species richness is observed between 200 and 800 m for both amphibians and reptiles, while richness decreased with increasing elevation. Numerous studies have shown that animal and plant richness is highest at mid-elevations, and three hypotheses have been proposed to explain this pattern (reviewed in Rahbek, 1995). The "ends are bad" hypothesis suggests that there are limiting factors at high and low elevations, while the "middle is good" hypothesis cites optimal productivity at mid-elevations. The third hypothesis suggests that lower elevations experience more disturbance, leading to reduced diversity. Studies on herpetofauna suggest either a consistent decrease in richness with increasing elevation or a hump-shaped relationship with a peak at a certain elevation (Fauth et al., 1989; Nathan and Werner, 1999; Fu et al., 2006; Fischer and Lindenmayer, 2005; Wiens et al., 2007). Reptiles generally exhibit decreasing patterns (McCain, 2010), while preliminary studies on amphibians suggest mid-elevation peaks for salamanders and varied patterns for frogs. Amphibians and reptiles in the CVDA National Park follow a similar trend but reptiles begin to decrease at lower elevations than amphibians and more linearly (Fig. 4a). Indeed, the first mountain elevation zone is a hot spot for amphibian diversity, while almost all reptile species straddle between lowland and hilly elevation zones (Fig. 4a). Besides species richness, the conservation value of an elevation range is also determined by which species are present within it (Fig. 3). Species' elevation preferences suggest that lowland areas are important for the conservation of *B. viridis;* hilly and low mountain areas (500-1000 m asl) are focal for *R. dalmatina,* and mountain ranges (above 900 m asl) are crucial for the conservation of *S. salamandra*. Regarding reptiles, *H. turcicus* and *N. tessellata* will benefit from additional sampling efforts toward lower elevation areas. Conversely, a targeted conservation effort for *C. austriaca* will mainly need to take into account mountain areas. Finally, it is noteworthy to mention that our data provide new upper elevation limits for seven species in the Campania Region in comparison to the limits reported by Guarino et al. (2012). Specifically, our findings indicate that *B. bufo* can be found at elevations up to 1850 m (compared to 1450 m), *H. intermedia* up to 1465 m (compared to 1180 m), *R. dalmatina* up to 1600 m (compared to 1350 m), *L. italicus* up to 1600 m (compared to 1450 m), *P. muralis* up to 1860 m (compared to 1597 m), *P. siculus* up to 1860 m (compared to 1600 m), and *C. austriaca* up to 1485 m (compared to 1375 m).

Habitat preferences, species rarity and association

This study encompassed all types of habitats within the study area to investigate the distribution and ecology

of the herpetofauna. In terrestrial environments, amphibians and reptiles exhibit a richness that is proportional to the availability of habitat type area (Fig. 4b). Amphibian richness is proportional to the availability of Forests, Pastures, and Urban fabric but it is higher than expected in Scarce vegetation and Permanent crops. These habitat types, as a rule, are not suitable to ecological requirements of amphibians, however, it should be noted that in the Cilento area, where non-intensive agriculture is prevalent, they host numerous artificial water sites used for irrigation and watering of cattle and by amphibians as breeding sites. Reptiles appear to positively select Urban fabric and counter-select Forests, shaded environments which offer reduced basking opportunities only on their margins and ecotonal zones (Jaggi & Baur, 1999). The majority of reptile species in the CVDA National Park are active thermoregulators, while only a few species do not actively thermoregulate and prefer shaded habitats, such as the cover specialist *Anguis veronensis*. The preference for Urban fabric can also be attributed to the characteristics of the Cilento's landscape, in which urban settlements are sparsely distributed inside natural environments, with numerous ruined buildings. Even the continuous Urban fabric of main towns includes numerous dry stone walls, small vegetable gardens, and family chicken coops which offer high availability of shelters and trophic resources.

Considering species' habitat preferences, among amphibians (Fig. 7a) *B. bufo* and *P. lessonae* complex were the only two species that showed a positive association with Urban fabrics, consistent with their ecology on the Italian territory (Sindaco et al., 2006). *Lissotriton italicus* exhibited a positive association with Permanent crops (mainly olive groves), Scrub and Scarce vegetation. This endemic species spawns frequently in drinking troughs, stony wells and irrigation tanks (Romano et al., 2010; 2012), all man-made elements found in rural landscapes. Consequently, as expected for some species of amphibians, the importance of a particular terrestrial habitat type is determined by its association with certain aquatic habitat type preferences. Other species, such as *S. salamandra*, showed a negative correlation with open environments, while others, like *B. variegata* and *T. carnifex,* are negatively associated with Forests, in agreement with their general ecology (Sindaco et al., 2006). The habitat type with the most positive associations is Scarce vegetation, but this habitat type is also negatively correlated with the occurrence of many other species, along with Urban fabrics and Forests (Fig. 7b).

The relationship between amphibians and aquatic habitat types displays a considerable degree of interspecific variation (Fig. 8). *Triturus carnifex* and *S. salamandra* are the most stenoecious species because they are strictly associated with only one habitat type (wells and streams, respectively) avoiding all other aquatic habitat types. While this is consistent with the knowledge of the Fire salamander on the national territory, it does not hold true for the Italian crested newt (Sindaco et al., 2006). Indeed this species appears to be relatively adaptable to several aquatic environments, preferring permanent or semi-temporary water bodies, in plains or moderately elevated areas (Sindaco et al., 2006; Vanni et al., 2007). Conversely, in the CVDA National Park, *T. carnifex* emerges as a stenoecious species, considering both aquatic (Fig. 8) and terrestrial habitat types (Fig. 7a), and for the elevation range (Fig. 3a).

On the other hand, *Lissotriton italicus* and *P. lessonae* complex showed a significant positive association with many aquatic environments. On the whole, ponds are the aquatic habitat type with the highest level of positive species association while wells are characterized by the highest rate of negative associations (Fig. 8).

Species association revealed interesting patterns. Two primary groups can be distinguished among amphibians based on the types of aquatic habitats they occupy (Fig. 9a): the first includes *Salamandra salamandra, Salamandrina terdigitata* and *Rana italica* that share running waters. The latter two are in a subcluster because they breed typically in syntopy as already known in the Apennines (Romano et al., 2007; 2012). The second cluster includes all the other species although is a much more branched group, where *T. carnifex* and *L. italicus* cluster together, *Bufo bufo* and *Bombina variegata* seems to share similar aquatic habitat types; the other anuran species are associated because they share still or very slow-running waters. Clearly, aquatic habitat preferences dictate amphibian association, furthermore they partially reflect phylogeny, as close species clustered together (e.g. geckos, newts, and water snakes).

Considering the terrestrial habitat types for amphibians (Fig. 9b), the two newts are associated, as for aquatic habitat types, because they often breed in stony wells and concrete tanks used in agriculture or pasture areas. The second cluster includes those species (*S. terdigitata* and *R. italica*) that preferred forest habitat types (from beech to maquis forests). The third species group is associated mainly with urban or suburban areas but also arable lands. The last cluster includes species occurring in a variety of habitat types and in particular in Forests, Scrub/herb vegetation but also Heterogeneous agrosystems (low-intensity), although *S. salamandra* significantly avoids agricultural areas (see Fig. 7a).

The complexity of relationships among reptile species is greater (Fig. 10). *Coronella austriaca* is the only colubrid markedly separated by the other snakes (that clustered together) and clusters with saurians experiencing a

great variety of habitat types but in particular with those associated with Scarce vegetation (see also Fig. 7b).

ISR identified species rarity considering their distribution only and was consistent with the Rabinowitz's forms of rarity that identified species potentially more vulnerable to habitat loss despite being relatively widespread, due to their restricted habitat breadth (Fig. 6). Species characterized by at least two of the three parameters used to assess rarity should be considered priority species for increased sampling effort and, when needed, conservation actions. Example of these species are, among amphibians, *B. variegata, S. salamandra, S. terdigitata, B. viridis* and *R. dalmatina*, and, among reptiles, *E. orbicularis, T. hermanni, C. austriaca, N. tessellata, H. turcicus,* and *A. veronensis.* The assessment of species rarity can however be influenced by low detection rates, a common issue for herpetofauna that can greatly vary among species, and uneven sampling effort across grid cells, which could artificially increase rarity estimations (Dodd, 2009, 2016; Gomez-Rodriguez et al., 2012; McDiarmid et al., 2012). However, our conclusions on species distribution and rarity can be considered robust, as our dataset derives from numerous, repeated surveys carried out by different observers in different seasons. In this perspective, opportunistic and ancillary data (i.e. those from citizen science) can provide a significant contribution, especially to improve the knowledge on distribution and ecology of rare and cryptic species (Hauser and Heise-Pavlov, 2017; Marshall et al., 2023).

Conclusions and conservation notes

Based on the analyzed data presented here, the "Cilento, Vallo di Diano e Alburni" National Park emerges as a protected area with high herpetological value, ensuring high levels of diversity. Our findings agree with the ecological information available in Italy, while for some species, we identified some peculiar characteristics in their local ecology, divergent from the rest of their range, that confirm their plasticity and adaptation to local conditions. Species showing population and range declines at national scale exhibit a good conservation status inside park boundaries. However, during fieldwork, we noticed many road kills of all reptile species excluding geckos. This threat should be addressed with specific surveys and assessments inside the CVDA National Park.

Surveys repeated after a period of about 15 years on the same sites revealed dramatic habitat alterations for many amphibians. In historical sites, *B. variegata*, experienced local extinctions while *S. terdigitata* and *T. carnifex* are close to disappearing. Habitat degradation was caused by two opposite factors: the abandonment of traditional agro-pastoral activities resulting in the lack of maintenance of artificial aquatic sites that lose permeability or are filled by vegetation (Fig. S4), and the collection of water from spring through pipelines, that drastically reduced water availability in synergy with climate change. In the CVDA National Park, the management of artificial aquatic sites is crucial for amphibian conservation (Romano, 2012), similarly to other parts of the Apennines (Temple and Cox, 2009; Romano et al., 2012; Buono et al., 2019). Finally, our study confirms the importance of incorporating the opportunistic data from citizen science into research and monitoring plans.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < http://www.unipv.it/webshi/appendix > Manuscript number 14562.

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New record and dietary ecology of an endangered amphibian species, *Micryletta nigromaculata* Poyarkov, Nguyen, Duong, Gorin & Yang, 2018, from Vietnam

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Abstract. The Black-spotted Paddy Frog (*Micryletta nigromaculata*) was recently discovered from northern Vietnam. Due to its restricted distribution in limestone karst forests and habitat degradation, the endemic species has been listed as "Endangered" in the IUCN Red List. However, knowledge about the natural history of this species is scarce, including data on its dietary ecology. Recent field surveys revealed new populations of *M. nigromaculata* occurring in Son La Province, northwestern Vietnam based on morphological analysis. Using the stomach-flushing method, we analyzed stomach contents of 45 individuals (21 males and 24 females) from a total of 52 captured frogs because stomachs of six males and one female (13.5%) were empty. The food spectrum of *M. nigromaculata* comprises 12 types with a total of 691 prey items (687 invertebrate items and four unidentified items), belonging to six insect orders, larvae and Araneae. Similar to other species of the family Microhylidae, *M. nigromaculata* is also identified as an ant (Formicidae) – termite (Rhinotermitidae) eating specialist with distinctly higher percentages of occurrence frequency (F = 65 %), number of prey items (N = 80 %), and importance (Ix = 75 %) than those of remaining types. The Simpson diversity index of 0.939 shows a high abundance of food prey in the wild, and the Evenness index of 0.536 suggests biased consumption of feeding selection for *M. nigromaculata*.

Keywords. Formicidae, prey items, Rhinotermitidae, Son La Province, stomach contents.

INTRODUCTION

Inhabiting both freshwater and terrestrial ecosystems, amphibians play a paramount role in maintaining the stability of food webs and energy flow between their

habitats (Duellman and Trueb, 1994; Wells, 2007). In particular, amphibians transfer the energy intake by consuming many invertebrates, even small vertebrate species, to higher trophic levels by serving as an essential food source for several predatory animal groups (such as reptiles and

mammals), accounting for a major part of animal biomass in ecosystems (Burton and Likens, 1975; Toledo et al., 2007; Oliveira et al., 2013). Thus, understanding the trophic niche may provide additional insights into species' natural history and biotic interactions (Schoener, 1974; Toft, 1980; Prado et al., 2005; Wells, 2007; Duré et al., 2009). To investigate this matter in amphibians, the stomach content is often obtained to identify which prey was eaten and their relative importance in the species' diet (Maneyro et al., 2004; Caldart et al., 2012; Le et al., 2018). In terms of conservation assessments, these analyses provide crucial data on conditions and resources required for species survival under human impacts (e.g., habitat loss and degradation and overexploitation for trade and food consumption) that can imperil the stability in animal communities with intermittent trophic chains (Clavel et al., 2011). However, detailed information on the diet niche of many amphibian species is still lacking.

The paddy frogs of the genus *Micryletta* Dubois 1987 currently consist of 13 species, distributed from northeastern India and China through the Indochina region and expanding southward to west Malaysia and Sumatra (Frost, 2023). Remarkably, as many as eight species of *Micryletta* have been discovered in the last five years (Frost, 2023). The Black-spotted Paddy Frog, *Micryletta nigromaculata* Poyarkov, Nguyen, Duong, Gorin & Yang 2018, was originally described in Hai Phong and Ninh Binh provinces, northern Vietnam (Poyarkov et al., 2018). The species was subsequently listed as Endangered in the IUCN Red List of Threatened Species due to habitat loss and degradation (IUCN, 2023).

As a result of our recent field surveys in Son La Province, individuals of *Micryletta* were collected and their morphological characteristics resemble the description of *Micryletta nigromaculata* by Poyarkov et al. (2018). We herein confirm to record the species for the first time from Son La Province. In addition, to better understand the natural history of dietary ecology of the highly threatened species, we analyzed the stomach content of 52 captured individuals to identify prey items and determined their relative importance in the species' diet.

MATERIALS AND METHODS

Field surveys and sampling

Field surveys were conducted in June, July, and September 2017 in two localities of Son La Province, northwestern Vietnam: the first site in Son La City (21°18.659'N, 103°46.956'E, at an elevation of 550 m) and the second one in Phong Lai Commune, Thuan Chau District (21°36.458'N, 103°33.980'E, at an elevation of

Table 1. The number of paddy frogs captured from Son La Province, northwestern Vietnam.

Locality	Date	Number of individuals		
		Male	Female	Total
Son La City	10-13 June 2017	5	3	8
	6-9 July 2017	3	4	7
	5-7 September 2017	2	0	2
Phong Lai Commune	16-19 June 2017	6	9	15
	15-18 July 2017	8	6	14
	11-13 September 2017	3	3	6
Total		27	25	52

680 m) (Table 1, Fig. 1). The survey transects were set up with lengths ranging from 2.0 to 3.0 km, in limestone karst forests and open areas of grass and shrubs. We captured frogs by hand between 20:00 and 24:00h and used a stomach-flushing technique to obtain stomach contents without sacrificing them (Griffiths, 1986; Leclerc and Courtois, 1993; Solé et al., 2005; Norval et al., 2014). Prey items were preserved in 70% ethanol and deposited at the Faculty Environmental of Science, University of Science, Vietnam National University, Hanoi, Vietnam. Frogs were subsequently released at the collecting site after measurements of snout-vent length (SVL) and mouth width (MW) with a digital caliper to the nearest 0.01 mm taken. In total, 52 frogs, including 27 males and 25 females, were caught at two sites (Table 2).

For taxonomic identification, four individuals were collected for voucher specimens. After having been photographed in life, animals were anesthetized and euthanized in a closed vessel with a piece of cotton wool containing ethyl acetate (Simmons, 2002), fixed in 85% ethanol and subsequently stored in 70% ethanol.
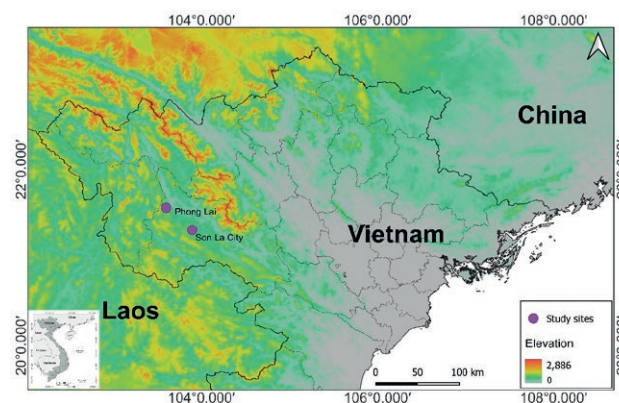

Fig. 1. Map of surveyed sites in Son La Province, northwestern Vietnam: (1) Son La City and (2) Phong Lai Commune.

Stomach content analysis

In the laboratory, prey items were identified under microscopes (Olympus SZ 700) following taxonomic literature of invertebrates (i.e., Naumann et al., 1991; Johnson and Triplehorn, 2005). The maximum length (L) and width (W) of each prey item were measured to the nearest 0.01 mm using either a digital caliper or a calibrated ocular micrometer fitted to a microscope (Hirai and Matsui, 2001). The volume (V, mm³) of prey items was calculated using the formula for a prolate spheroid ($\pi = 3.14$; Magnusson et al., 2003): $V = 4\pi/3 \times (L/2) \times (W/2)^2$. The index of relative importance (Ix), was used to determine the importance of each food type, which was calculated following the formula: $Ix = (\%F + \%N + \%V)/3$ (Caldart et al., 2012), where %F (F – Frequency of occurrence) is a percentage of stomach containing each prey type, %N (N – Number) is a percentage of number of each prey item in all.

We used the reciprocal Simpson's heterogeneity index, 1-D, to calculate dietary heterogeneity: $D = \Sigma[n_i(n_i - 1)]/[N(N - 1)]$; where n_i is the number of food items in the ith taxon category and N is the total number of prey items (Krebs, 1999). We used another index to estimate prey evenness. The evenness index is calculated from the equation: $J' = H'/H_{max} = H'/lnS$; where S is the total number of prey taxa and H' is the index of taxon diversity. The value of H' is calculated from the equation: $H' = -\Sigma(p_i \times lnp_i)$; where p_i is the proportion of food items belonging to the ith taxon for the total food items of the sample (Magurran, 2004; Muñoz-Pedreros and Merino, 2014).

Shapiro-Wilk's test was used to check the assumption of normality. Wilcoxon tests were performed to determine differences or similarities in phenotypic characteristics of the species, and sizes and volume of ingested food between males and females, and between populations. Females were identified by having longer snout-vent length (SVL ≥ 21.0 mm), whereas males were characterized by a single internal vocal sac and shorter SVL (< 21.0 mm). All statistical analyses were performed by using the software environment R.3.1.2 (RStudio Team, 2018).

RESULTS

A new record of Micryletta nigromaculata *in Son La Province*

Pham et al. (2016) reported *Micryletta inornata* (Boulenger, 1890) from Co Ma Commune, Thuan Chau District, Son La Province, Vietnam. However, the specimens were re-identified herein as *M. nigromaculata*. This is also the first record of *M. nigromaculata* in Son La Province, which is approximately 200 km from the type locality of the species in Cuc Phuong National Park of Ninh Binh Province.

Morphological characteristics of paddy frogs collected in Son La Province match well with the diagnosis of *Micryletta nigromaculata* (Poyarkov et al., 2018): size small (SVL: 14.8–27.7 mm, n = 52); head wider than long; snout obtusely rounded in profile; eyes equal to or shorter than snout; the interorbital distance wider than upper eyelid; tibiotarsal articulation of adpressed limb reaching the level of eye center; dorsal surface granular with small round tubercles; supratympanic fold present; outer metatarsal tubercle absent; dorsum coloration brown to reddish-brown; body flanks brown with dark-brown to black patches or spots edged with white, a large black blotch in the inguinal area on each side; lateral sides of head immaculate reddish brown lacking white patches; and throat in males whitish with light-gray marbling (Fig. 2).

Regarding sexual dimorphism, males have a shorter snout-vent length (SVL: 19.03 ± 1.95 mm, ranging 14.8 – 20.8 mm, n = 27) and a narrower mouth width (MW: 5.6 ± 0.73 mm, ranging 4.4 – 6.7 mm, n = 27) than those of females (SVL: 24.73 ± 1.4 mm, ranging 21.5 – 27.7 mm and MW: 6.77 ± 0.49 mm, ranging 6.0 – 7.8 mm, n = 25; all P-values < 0.0001).

In terms of natural history, paddy frogs were found between 19:00 and 24:00 at elevations between 550 and 680 m a.s.l. The surrounding habitat was the limestone karst forest of small hardwoods, shrubs, and grasses (fig. 2). The relative humidity was approximately 70 – 85% and the air temperature ranged from 25 to 30ºC. Other sympatric amphibian species were also observed, including *Leptobrachella* sp., *Microhyla butleri* Boulenger 1900, *M. heymonsi* Vogt 1911, and *Polypedates megacephalus* Hallowell 1861.

Dietary ecology

A total of 52 individuals (27 males and 25 females) of *M. nigromaculata* were captured in Son La City (17 individuals) and Thuan Chau District (35 individuals) of Son La Province. All caught individuals were checked for stomach contents, except for seven frogs (13.5%), whose stomachs were empty. Based on the obtained contents, we identified a total of 691 ingested food items (687 invertebrates and four unidentified items) belonging to 12 different prey types of six insect orders: Blattodea, Coleoptera (Coccinellidae, Staphylinidae, Tenebrionidae, and Other Coleoptera), Diptera (Syrphidae, Other Diptera), Hymenoptera (Formicidae), Isoptera (Rhinotermitidae), and Orthoptera (Gryllidae), Insect larvae, and Spiders (Araneae).

Fig. 2. Natural habitat (above) and individuals (below) of *Micryletta nigromaculata* found in Son La Province, northwestern Vietnam.

In terms of size measurements, the item-mean width (W_m) of ingested prey was 1.33 ± 0.12 mm (0.2 – 5.0 mm), the item-total width (W_t) was 15.7 ± 2.56 (0.5 – 66.0 mm), the item-mean length (L_m) was 2.8 ± 0.19 mm (0.64 – 7.23 mm), and the item-total length (L_t) was 35.24 ± 6.6 (2.0 – 184.8 mm) (Fig. 3, Table 2). We calculated the mean volume of 44.02 ± 8.41 mm³ (0.26 – 224.6 mm³). There was only a significant difference in the W_m

of consumed prey between males and females (P-value = 0.045), whereas remaining prey characteristics (i.e., W_t, L_m, L_t and volume) were relatively similar between intraspecific sexes and localities (all P-values > 0.05; Fig. 3, Table 2). Our multiple linear regression analyses showed that the sizes and volume of ingested prey are not significantly correlated with phenotypic traits of SVL and MW of the species (Table 2).

Table 2. Sizes and volume of prey consumed by *Micryletta nigromaculata* in relation to phenotypic characteristics. Total = values computed on all individuals; Female = data for all females; Male = data for all males; Sex comparison = Wilcoxon's test for between-sex comparison; Locality comparison = Wilcoxon's test for between-localities comparison; SVL = Pearson's correlation between prey measures and snout-to-vent length; for all test, statistic and associated P-value are reported; significant values are bolded. Measures abbreviations: W_m = item-mean width; W_t = item-total width; L_m = item-mean length; L_t = item-total length, Volume = item volume.

|	Total	Female	Male	Test (P-values)	Test (Locality)	SVL	HW
W_m (mm)	1.33 ± 0.12 (0.2 – 5.0)	1.11 ± 0.09 (0.5 – 2.67)	1.58 ± 0.23 (0.2 – 5.0)	**W = 166** **P = 0.045**	W = 150.5 P = 0.34	r = -0.18 P = 0.25	r = 0.02 P = 0.92
W_t (mm)	15.7 ± 2.56 (0.5 – 66.0)	18.5 ± 4.28 (0.5 – 66.0)	12.5 ± 2.4 (1.0 – 50.7)	W = 237.5 P = 0.75	W = 229.5 P = 0.27	r = 0.14 P = 0.34	r = 0.05 P = 0.72
L_m (mm)	2.8 ± 0.19 (0.64 – 7.23)	2..81 ± 0.25 (1.07 – 7.23)	2.78 ± 0.31 (0.64 – 5.8)	W = 257.5 P = 0.90	W = 179 P = 0.84	r = 0.06 P = 0.69	r = 0.24 P = 0.11
L_t (mm)	35.24 ± 6.6 (2.0 – 184.8)	46.32 ± 11.52 (2.0 – 184.8)	22.6 ± 4.01 (2.0 – 72.0)	W = 262 P = 0.83	W = 254.5 P = 0.07	r = 0.23 P = 0.14	r = 0.14 P = 0.35
Volume (mm³)	44.02 ± 8,41 (0.26 – 224.6)	40.8 ± 10.9 (0.26 – 188.4)	47.7 ± 13.3 (0.3 – 224.84)	W = 232 P = 0.66	W = 222 P = 0.36	r = -0.06 P = 0.70	r = -0.02 P = 0.91
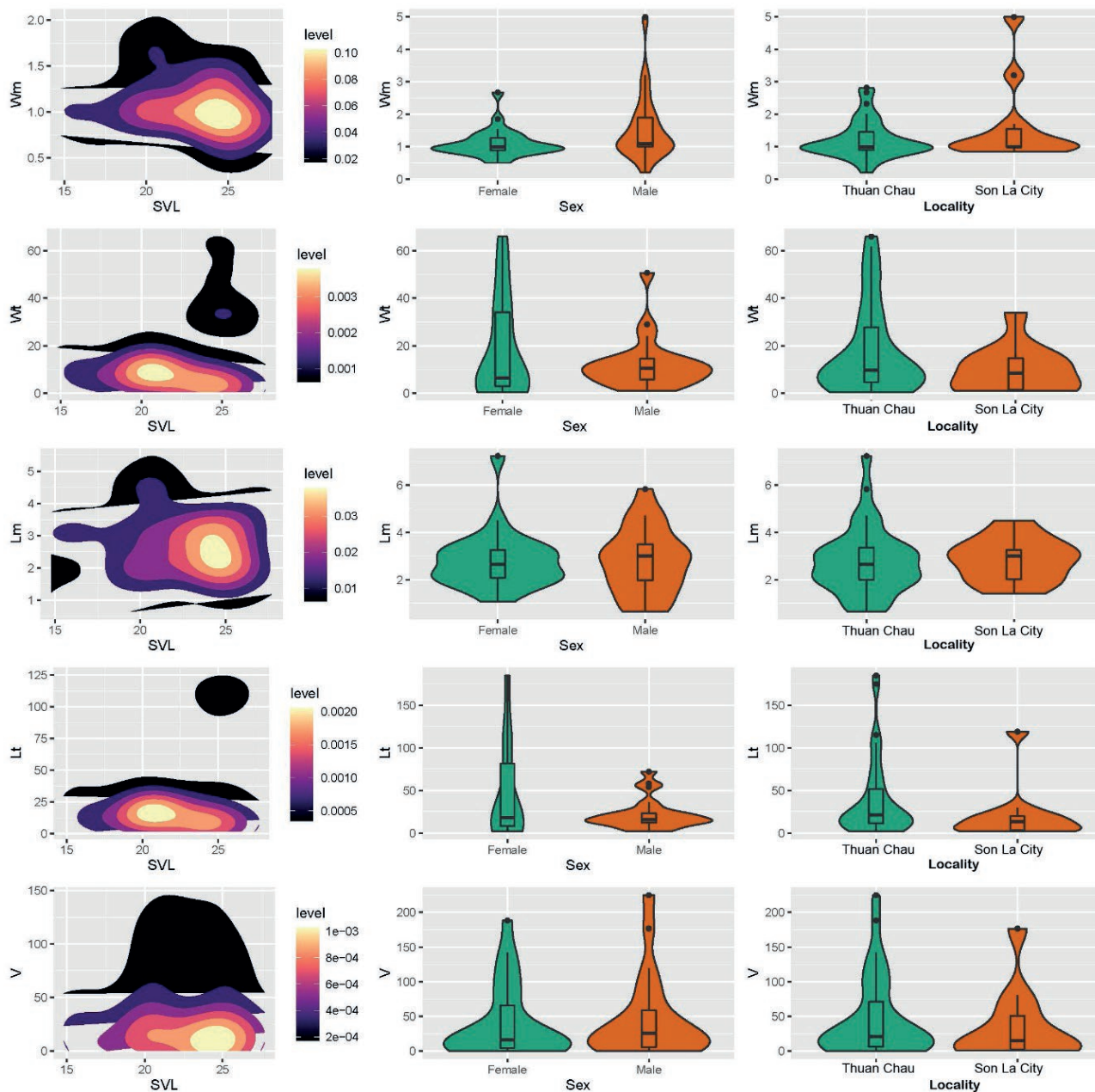
Fig. 3. Size and volume of prey consumed by *Micryletta nigromaculata* in relation to snout-vent length (left column), to sex (middle column), and locality (right column).

The prey of *M. nigromaculata* mainly comprised two invertebrate families of Formicidae and Rhinotermitidae, which accounted for more than 65% of the frequency of occurrence (F) and 80% of the number of prey items (N) (Table 3). The two invertebrate families were also the most important prey of the species, accounting more than 75% of Ix (Table 3).

The dietary breadth of *M. nigromaculata* from Son

La Province, expressed by the Simpson's index of diversity was 0.939, and evenness was 0.536 (Table 4). Males of *M. nigromaculata* had higher values of both Simpson's index (0.926) and evenness (0.783) than those of females (Table 4). The population of *M. nigromaculata* in Son La City consumed less diverse prey than the population in Thuan Chau District (Table 4).

Table 3. Dietary composition of *M. nigromaculata* in Son La Province: F = frequency of occurrence, N = number of items, V = volume (mm³), Ix = index of relative importance of each prey type (n = 45 stomach contents)

Prey type	F	%F	N	%N	V	%V	Ix
Araneae	1	1.41	1	0.14	1.57	0.08	0.54
Blattodea	1	1.41	1	0.14	20.12	1.02	0.86
Coleoptera							
Coccinellidae	2	2.82	2	0.29	9.42	0.48	1.19
Staphylinidae	3	4.23	3	0.43	4.31	0.22	1.63
Tenebrionidae	1	1.41	1	0.14	2.74	0.14	0.56
Other Coleoptera	6	8.45	9	1.30	219.35	11.07	6.94
Diptera							
Syrphidae	1	1.41	1	0.14	5.65	0.29	0.61
Other Diptera	1	1.41	3	0.43	36.24	1.83	1.22
Hymenoptera							
Formicidae	36	50.70	476	68.89	632.57	31.94	50.51
Isoptera							
Rhinotermitidae	11	15.49	91	13.17	901.84	45.53	24.73
Orthoptera							
Gryllidae	2	2.82	2	0.29	12.04	0.61	1.24
Insect larvae	2	2.82	97	14.04	14.26	0.72	5.86
Unidentified	4	5.63	4	0.58	120.67	6.09	4.10
Total	71	100	691	100	1980.77	100	100

Table 4. Diversity and Evenness indices of *Micryletta nigromaculata*'s prey, expressed with value (95% Confidence limits).

	Simpson 1-D	Evenness
Species (*Micryletta nigromaculata*)	0.939 (0.930 – 0.9438)	0.536 (0.495 – 0.573)
Male	0.926 (0.909 – 0.933)	0.783 (0.684 – 0.825)
Female	0.8896 (0.877 – 0.899)	0.50 (0.471 – 0.562)
Son La City	0.761 (0.670 – 0.818)	0.566 (0.466 – 0.697)
Phong Lai Commune	0.9255 (0.9158 – 0.932)	0.5647 (0.516 – 0.599)

DISCUSSION

In this study, *M. nigromaculata* was first recorded in Son La Province, which is approximately 200 km and 350 km far distance from the type locality of the species in Ninh Binh Province and Hai Phong City, respectively. From Son La Province, the species has been recorded at significantly higher elevations up to 700 m a.s.l., whereas the species was previously known at elevations of 90 – 150 m a.s.l. only (Poyarkov et al., 2018). Our finding agrees well with the anticipation of Poyarkov et al. (2018) that this species can occur at different elevations in other adjacent ecosystems of limestone karst in northern Viet-

nam. With the first-time record of *M. nigromaculata* in Son La Province, we recommend additional field surveys should be conducted in other karst ecosystems in northern Vietnam to discover new populations of the species and fully understand the biogeographical aspect.

A total of 12 different prey types belonging to six invertebrate orders, larvae and spiders (Araneae) were identified as the food spectrum of *M. nigromaculata*, in which ants (Formicidae) and termites (Rhinotermitidae) were determined as the most dominant and important prey of the species. Anurans, especially in the family Microhylidae have been widely recognized as ant or sometimes termite specialists (Toft, 1980; Solé et al., 2002; Isacch and Barg, 2002; Berazategui et al., 2007; Atencia et al., 2017; Lopes et al., 2017).

In this study, we did not find any intersexual difference in the sizes and volume of prey eaten by *M. nigromaculata*. Competitive interactions among conspecifics for sharing resources could take place between males and females (Schoener, 1974; Irschick et al., 2005; van Schingen et al., 2015). Our estimation based on the number of food items for indices of Simpson diversity (more than 0.88) showed a high abundance of prey at the studied sites, but not evenly among food types (0.536 – Evenness index). Furthermore, females' prey (Evenness index = 0.5) was more biased than those in males (0.783). All of these can reduce intersexual competition. Pham et al. (2022) studied the diet niche of *Microhyla butleri* and *M. heymonsi* in Son La Province and showed that both sympatric species primarily consumed ants and termites as well. However, there were significant differences in the trophic niche between them to limit interspecific competition. In particular, 11 prey types of *M. butleri* were not found in the food spectrum of *M. heymonsi* and nine prey types were not presented in vice versa. Furthermore, they preferred to consume significantly different prey in the sizes and volume (Pham et al., 2022).

Regarding human impacts, karstic forest degradation caused by infrastructure development (e.g., road expansion, development of residential areas, and limestone quarrying) has been documented in Son La City (Pham pers. obs), which could explain the low number of *M. nigromaculata*. Only 17 individuals of *M. nigromaculata* were captured in Son La City during three surveys, compared to half of captured animals (n = 35) in less disturbed habitats in Phong Lai Commune. Wild populations of the species in Son La Province ergo have been severely threatened by habitat loss and limestone extraction (Pham pers. obs.). Such human impacts particularly destabilize its wild populations and food availability. The potential of finding new populations beyond the known areas of occupancy in northern Vietnam, as Poyarkov et

al. (2022) anticipated, could reduce the risk of extinction to this endangered species (IUCN, 2023). However, similar to what is happening in Son La Province, unsustainable human activities (particularly limestone extraction and tourist activities) over karst ecosystems in northern Vietnam, can extensively impact undiscovered populations of the range-restricted species in unique karstic habitats (Clemens et al., 2006; Poyarkov et al., 2022). Therefore, conservation measures would urgently be required, though *M. nigromaculata* has not been targeted for the pet trade and food consumption, and its geographical distribution is potentially extended. However, to date, no conservation plan has been developed to safeguard the highly threatened species as well as its natural habitats. Besides the investigation of dietary ecology, we highly recommend further studies to identify the fully geographical extent, assess population status and determine other ecological traits of *M. nigromaculata*. Only when the background data becomes available, appropriate conservation measures can be designed to better protect the species from current extinction risks.

ACKNOWLEDGMENTS

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Population dependent behavioral responses among color morphs of Common wall lizard (*Podarcis muralis*)?[§]

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§ *Presented at the XI International Symposium on the Mediterranean Lacertid Lizards, Lipari 27-28th September 2021*
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Abstract. Color polymorphisms are common across reptile species and are frequently correlated with variation in behavior and other functional traits. Here, we tested whether the ventral color morphs (yellow, orange and white pure morphs, as well as yellow-orange and white-orange mosaic morphs) of Common wall lizards *(Podarcis muralis)* exhibit differential temporal changes in their reaction to exposure to a novel environment. We conducted 15 min long trials of a novel environment test (open field test) in a set of populations from the eastern Pyrenees. Locomotion, boldness and freezing behavior changed over time, in agreement with a previous study carried out in central Pyrenees, but without highlighting a clear behavioral difference among color morphs. Only yellow-orange lizards showed a distinct correlation pattern between locomotion and body size compared to the other morphs. Carefully standardized studies involving more populations are needed to unravel the complex interactions between morphological and behavioral traits among lizard color morphs.

Keywords. Behavior, color polymorphism, locomotion, open field test, Common wall lizard, *Podarcis muralis*, selection.

INTRODUCTION

Coexistence of multiple categorical color phenotypes within a single population (color polymorphism) has been reported throughout the animal kingdom, including lizards (Sinervo and Lively, 1996; Svensson, 2017). Maintenance of alternative genetically determined color phenotypes (i.e., color morphs) is often explained by complex evolutionary processes involving multiple selective forces (Sinervo and Lively, 1996; Svensson, 2017). Such

morphs should be either characterized by equal fitness, or if fitness differences exist, rare morphs should be promoted by selection (Sinervo and Lively, 1996). This is usually explained as a consequence of the co-variation between color and other phenotypic traits, which results in development of alternative strategies involving complex combinations of behavioral, physiological, morphological or life history characteristics (Coladonato et al., 2020; Galeotti et al., 2013; Sacchi et al., 2007; Sinervo and Lively, 1996; Svensson et al., 2001; Thompson et al., 2023). In

reptiles, variation in color within populations (including those that may be polymorphic) is often interpreted as an adaptive compromise between conflicting selective pressures exerted by social, antipredator, and thermoregulatory functions (Cooper and Greenberg, 1992).

Functional traits that sometimes covary with color, such as locomotor behavior, can influence the fitness of alternative color morphs and play a major role in regulating selective processes (Grant and Liebgold, 2017; Sreelatha et al., 2021). Animals with more active locomotion may be more exposed to predators and, hence, suffer increased risk of mortality (Werner and Anholt, 2015). However, high locomotor activity can be advantageous for finding resources (foraging or basking spots) as well as for social, and reproductive interactions (Arnold and Bennett, 1988; Boratyński, 2020; Huyghe et al., 2007; Pačuta et al., 2018; Sinervo et al., 2000). Animals with high level of boldness (prone to undertake risky behaviors) and low level of neophobia may explore an unfamiliar space (novel environment) more likely and faster (Damas-Moreira et al., 2020; White et al., 2013). On the contrary, reluctant to explore a novel environment (freezing), shy animals may avoid exploring unfamiliar space, reducing probability to forage under high predation risk (Evans et al., 2019). Consequently, some individuals can familiarize with novelty much faster than the others on exposure to a new environment and over short time intervals, differing in their locomotion and freezing behaviors (Pačuta et al., 2018; Sreelatha et al., 2021). Even though the locomotion of lizards is strongly dependent on environmental temperature (Braña, 1991; Paladino, 1985), they may develop alternative strategies corresponding to these behaviors. An interplay between the alternative behavioral strategies and morphological, physiological and life history traits might eventually affect the variable fitness optima of color morphs (Pérez i de Lanuza and Font, 2015; Sacchi et al., 2017, 2018; Sinervo and Svensson, 2002).

The Common wall lizard, *Podarcis muralis* (Laurenti, 1768)*,* exhibits ventral color polymorphism characterized by three pure color morphs (yellow, white, orange) and two intermediate (i.e., mosaic) morphs (yellow-orange and white-orange; Aguilar et al., 2022; Pérez i de Lanuza et al., 2013, 2018; Scali et al., 2013). Previous studies have identified cases where these color morphs use alternative resources, optimizing their fitness to local adaptive optima (Abalos et al., 2016; Coladonato et al., 2020). In the Eastern Pyrenees region, the local frequencies of yellow and yellow-orange morphs seem to be constrained by climatic factors, whereas white, orange and white-orange morphs are widely distributed over species range (Pérez i de Lanuza et al., 2018). At smaller spatial scales, orange animals prefer more humid micro-habitats than other morphs (Pérez i de Lanuza and Carretero, 2018; Scali et al., 2016). The local diversity of the morphs can also depend on the intensity of sexual selection (i.e., sex-ratio, emerging rare yellow and yellow-orange morphs in male-biased populations; Pérez i de Lanuza et al., 2017). Thus, the color polymorphism within populations of *P. muralis* appears to be locally maintained by a combination of environmentally dependent and sexual selections, showing a great geographical dependence (Calsbeek et al., 2010; Galeotti et al., 2013; Pérez i de Lanuza et al., 2013, 2018; Pérez i de Lanuza and Carretero, 2018; Sacchi et al., 2007).

In a previous study, focused on the behavioral strategies of the three pure morphs of *P. muralis* from Val d'Aran (central Pyrenees), we found that the morphs differed in locomotion and freezing behavior, and showed significant changes throughout a 15-minute experimental trial (Sreelatha et al., 2021). Yellow animals showed a risk sensitive strategy, by exploring the novel environment quickly and gradually decreasing the locomotion over time, moving to potentially safe areas. Orange and white lizards showed a risk aversive strategy, with the opposite pattern. Such variation in locomotion was repeatable over consecutive experimental trials (Sreelatha et al., 2021). Here, we extend our approach by testing if *P. muralis* color morphs differ in behavioral strategies in populations from an independent, more anthropized area within the same region. We quantified behavioral traits of the several morphs by exposing them to a novel environment. By doing so, we aimed at generalizing our previous findings, which suggested behavioral differences among morphs. Additionally, we also explore eventual maladaptive behaviors in the mixed morphs, which are often less frequent in natural populations than expected, assuming random pairing (Pérez i de Lanuza et al., 2013).

MATERIALS AND METHODS

Sampling was conducted in small towns of the Cerdanya plateau (Fig. S1), eastern Pyrenees, about 90 km eastwards the area of the previous study (Val d'Aran; Fig. S1), during the breeding season in June 2019. We captured 94 adult male lizards, including both pure (orange: N = 24, white: N = 24, yellow lizards: N = 26) and mosaic (white-orange: N = 9 and yellow-orange: N = 11) color morphs. After capture, lizards were acclimatized to the laboratory conditions for at least 24h (with water *ad libitum*) before the experimental trials (Pačuta et al., 2018). The snout-vent length (SVL; ± 0.01 mm) was recorded as a measure of lizard size. We restricted the

analysis only to adult lizards (i.e., SVL ≥ 56 mm; Pérez i de Lanuza et al., 2013).

We measured the behavioral responses to the novel environment using an open field arena (1×1 m, walls: 0.25 m, white in color) in a temperature-controlled room (30 ± 1.5 °C, around the preferred body temperature of the animal; Braña, 1991) that was unfamiliar to the lizards (Montiglio et al., 2010; Sreelatha et al., 2021). A video camera (Casio EXILIM EX-F1, filming at 50 frames per second) was placed directly above the open field for recording the experimental trials. The trials were started by gently placing the animal in the middle of the open field arena, covered by an opaque plastic cup placed upside down. For minimizing the disturbance to the animal, nobody was present in the trial room during the experimental trials. After 2 minutes of acclimatization time, the cup was gently removed, and the animal was allowed to explore the open field arena. The activity of the animal was recorded with the video camera for another 15 minutes. At the end of each trial, the animal was removed from the arena and the arena was thoroughly cleaned with 70% ethanol. Two arenas were used in rotation. All the animals were subjected to the trials no later than 48h from their time of capture, and then released to their exact point of capture.

Using the Ethovision XT 14 software (Noldus, The Netherlands), animals were automatically tracked from the videos to record their movement and duration spent in the different parts of the arena (center: 0.6 × 0.6 m; border zone excluding corners: 0.6 × 0.2 m; corners: 0.2 × 0.2 m; Sreelatha et al. 2021). We measured 14 behavioral variables (Table S1); total distance moved in the arena, distance moved in center, corners and border zone (cm), duration of time lizards spent in the center, corners and border zone (s), number of visits to the center, corners and border zone, duration of the mobility (s), number of times the mobility was initiated, mean and maximum velocity (cm/s; Noldus et al., 2001; Sreelatha et al., 2021). To evaluate the short-term responses of the morphs in the measured locomotor and behavioral traits on exposure to a novel environment, the 15-minute duration was divided into 2.5 minutes intervals (six time intervals, Sreelatha et al., 2021). An alternative analysis with 5 min interval duration was also conducted producing comparable results (Table S2). All the measured traits in different scales were standardized by scaling and centering prior to the analysis. To summarize the data, the 14 correlated behavioral traits were reduced to a few uncorrelated components using principal component analysis (PCA) with varimax rotation on data combining information from six-time intervals for each animal. The first four rotated principal components (RCs), each explain-

ing > 5% of the variance in the data with eigenvalues > 1, were considered for further analyses (Table S1, Fig. 1).

In order to determine if the behavioral traits change during the trial duration and vary among the color morphs, we conducted linear mixed model analyses fitted with the function "lmer" in the "lme4" (Bates et al., 2015) package in R v.3.5.0 (R Core Team, 2018). We used four different models with each of the four principal components as dependent variable. Morph (five levels) and time (six-time intervals, ordered categorical variable) were included as factorial predictors while SVL (scaled and centered) was included as a continuous predictor. Two-way interactions between morph and SVL, as well as morph and time, were tested. To reduce the complexity of the model we used a stepwise backward elimination of non-significant interactions ($P > 0.05$). Individual ID was encoded as a random factor.

RESULTS

The first four rotated-principal components explained 85% of variance observed in the data. The first rotated-principal component (RC1: Table S1; Fig. 1) explained 44% of the variance and was positively correlated with variables representing locomotion, such as total distance moved, distance moved in the corners and border zone, number of visits to the corners and borders,
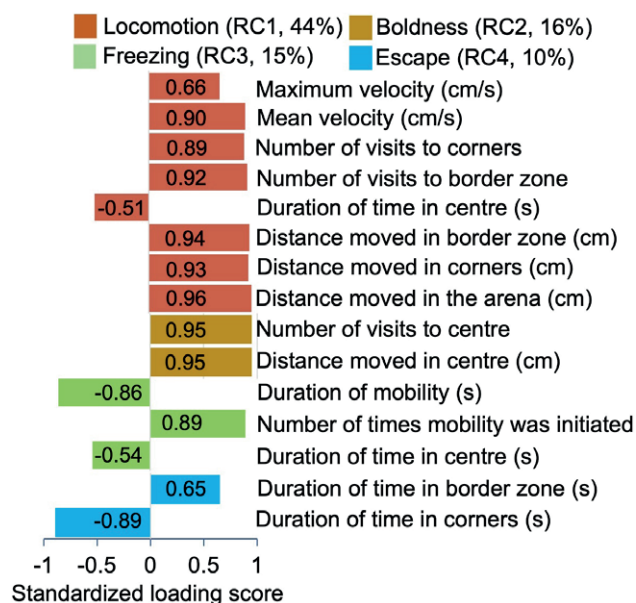

Fig. 1. Results from principal component analysis on behavioral variables obtained after tracking *Podarcis muralis* lizards in an open field arena. The analysis was conducted on 15-minute-long trials, divided into 2.5 minute intervals.

Table 1. Differences in behavioral traits (rotated principal components) among the five color morphs (orange, white, yellow, yellow-orange and white-orange) of *Podarcis muralis*. The analysis was conducted on 15 min long trials, divided to 2.5 min intervals. Models included individual ID as random factor. MS refers to mean square, and $F_{n,d}$ to F statistic with degrees of freedom for numerator and denominator.

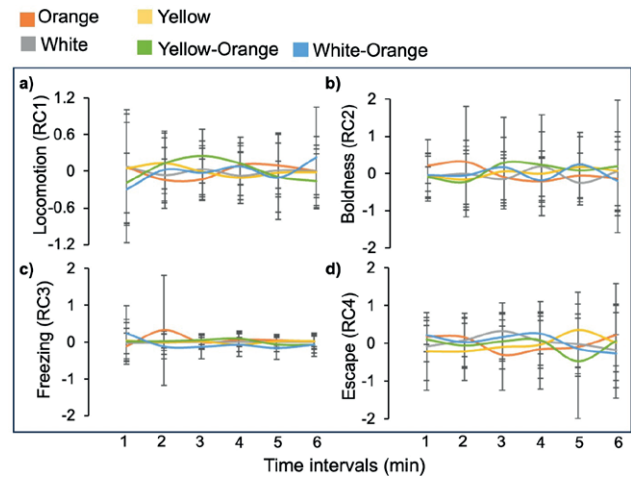| Dependent variables	Predictors	MS	$F_{n, d}$	P
Locomotion (RC1)	Morph	0.27	$0.78_{4, 84}$	0.54
	Time	6.77	$19.57_{5, 465}$	<0.001
	SVL	0.00	$0.01_{1, 84}$	0.97
	SVL: Morph	1.12	$3.23_{4,84}$	0.02
Boldness (RC2)	Morph	1.44	$1.75_{4, 58.54}$	0.15
	Time	0.98	$1.19_{5, 465}$	0.31
	SVL	0.99	$1.20_{1, 86.88}$	0.28
Freezing (RC3)	Morph	0.08	$0.80_{4,88}$	0.53
	Time	92.84	$968.88_{5, 465}$	<0.001
	SVL	0.11	$1.16_{1, 88}$	0.28
Escape (RC4)	Morph	0.64	$0.81_{4, 88}$	0.52
	Time	0.15	$0.19_{5, 465}$	0.97
	SVL	0.63	$0.80_{1, 88}$	0.37

Fig. 2. Changes over time of the behavioral traits among color morphs (orange, white, yellow, white-orange and yellow-orange) of *Podarcis muralis* lizards. The residual scores of behaviors are averaged (± SD) within morph and time interval. The 15-minute trials were divided to 2.5-minute time intervals during the analyses: (1) 0.0–2.5 min; (2) 2.5–5.0 min; (3) 5.0–7.5 min; (4) 7.5–10.0 min; (5) 10.0–12.5 min; (6) 12.5–15.0 min. In order to obtain the residual scores of locomotion, we adapted a mixed model including the scores of principal components as dependent variables, morphs and time as predictors and individual ID as random factor.

number of times the mobility was re-initiated, and mean and maximum velocity of mobility. The second rotated-principal component (RC2: Table S1; Fig. 1) explained 16% of the variance and was positively correlated with variables representing boldness, notably distance moved in the center and number of visits in the center. The third rotated-principal component (RC3: Table S1; Fig. 1) represented 15% of the variance and was positively correlated with variables linked to freezing, i.e., number of times the mobility was re-initiated, and negatively correlated with the duration of mobility. The fourth rotated-principal component (RC4: Table S1; Fig. 1) explained 10% of the variance and correlated with variables signifying the effort of animals to escape from the arena, i.e., duration of the time spent near borders, and negatively correlated with the time spent in corners.

Locomotion changed significantly over time (RC1: $F_{5,465}$ =19.57; P < 0.001; Table 1), decreasing significantly from the fourth time interval till the sixth (Table S3), irrespectively of the color morph (morph by time interaction: $F_{20,445}$ =1.07; P = 0.38; Fig. 2). There was correlation between body size and locomotion of the different color morphs (SVL by morph interaction: $F_{4,84}$ =3.20; P = 0.016; Tables 1, S3; Fig. S2). However, only in the yellow-orange animals locomotion decreased significantly with body size ($F_{1,22}$ = 5.52; P = 0.040; Table S4; Fig. S2). Analysis showed that boldness did not change over the trial duration (RC2: $F_{5, 465}$ = 1.19; P = 0.31; Table 1; Fig. 2) while freezing behavior was positively correlated with

trial duration (RC3: $F_{5, 465}$ = 968.88, P < 0.001, Table 1, Table S5, Fig. 2). The levels of escape behavior remained similar during the trial ($F_{5, 465}$ = 0.19, P = 0.97; Table 1; Fig. 2). The responses for boldness (RC2: $F_{4, 58.54}$ = 1.75; P = 0.15; Table 1), freezing (RC3: $F_{4, 88}$ = 0.80; P = 0.53; Table 1) and escape behaviors (RC4: $F_{4, 88}$ = 0.81; P = 0.52; Table 1) did not depend on morph types. Analysis after excluding mosaic morphs from the data showed similar results (Table S6).

DISCUSSION

Our results indicate that males of *Podarcis muralis* shifted locomotion and freezing behaviors with time on exposure to a novel environment, but without marked differences among the morphs. The only significant difference detected is in the correlation between locomotion and body length, where the yellow-orange mosaic morph showed a different (negative) relation compared to the other morphs. Pure and mosaic color morphs shifted freezing behavior similarly with time on exposure to a novel environment. The efficiency of locomotion might be dependent on the body size of animals (Lindstedt, 1987). Thus, the significant interaction between morph and body size perhaps suggest that growing individuals

may change their behavioral strategies with age (Makai et al., 2020). This also suggests that investigating behavioral mechanisms behind color polymorphism can be very complex, requiring much higher sample size than in this and many other studies. Meanwhile, negative results should be treated with caution.

In our previous study we showed behavioral differences among pure morphs of *P. muralis* from Val d'Aran, particularly in locomotion, freezing and escape behaviors. Yellow lizards were expressing a risk sensitive strategy, while white and orange lizards follow risk aversive strategy (Sreelatha et al., 2021). Results from the same lizard species but from another population (Cerdanya plateau) presented here suggest differential behavioral responses, indicating that these morph specific behavioral patterns are not consistent between the two populations. The substantial difference in the levels of anthropization and human disturbance, as well as other unmeasured parameters between the study areas could account for these different outcomes. The environmental context of such behavioral differences should be investigated with a wider population dataset. Previous studies indicate that higher disturbance implies shier behavior towards predator-simulated attacks, but also suggest that such behaviors are modulated by habitat (Carretero et al., 2013). Open and more disturbed habitats in Cerdanya area may be shifting behavior of the lizards towards shyness and, hence, masking any differences among morphs. This advises for caution while generalizing the results of the color morphs of *P. muralis* (Labra and Leonard, 1999). To confirm these hypotheses, further studies are needed comparing populations or eventually manipulating human disturbance and predation pressure.

Finally, mosaic morphs in Cerdanya area apparently follow the same behavioral patterns as pure morphs in locomotion, boldness, freezing, and escape behavior, discarding eventual negative selection. Our field observations indicate that *P. muralis* populations from Val d'Aran and Cerdanya differ in the proportion of yellow animals, relatively common in Cerdanya but rare in Val d'Aran. This may lead to reconsideration of the role of alternative strategies and local adaptation in maintaining color polymorphism, incorporating the processes overlooked heretofore. To provide firm conclusions further experiments are needed where standardized conditions are carefully maintained for individuals originating from variable populations and localities. Sample sizes of hundreds of individuals from variable habitats and morphs are needed for carefully designed tests targeting specific behaviors. Finally, extensive investigation with multiple populations and species from variable habitats is needed for generalizing any conclusions.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> Manuscript number 14610.

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Molecular genetic characteristics of *Darevskia portschinskii* lizard populations based on microsatellite markers analysis

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Abstract. The Caucasian rock lizard species *Darevskia portschinskii* is one of the bisexual species participating in interspecific hybridisation as the paternal ancestor with the maternal ancestors *D. mixta* and *D. raddei* resulting in the successful formation of the parthenogenetic *D. dahli* and *D. rostombekowi*, respectively. Populations of *D. portschinskii* have been previously divided into two subspecies, *D. p. portschinskii* and *D. p. nigrita* according to their geographical distribution and the morphological data, but they have not been characterised genetically. Here, we used ten microsatellite markers to determine the genetic structure of the *D. portschinskii* populations. The utility of the developed microsatellite markers for investigating the genetic variability within and among populations with a heterogeneous spatial distribution was demonstrated. Our results showed that the intra- and interspecific differentiation of the studied populations were consistent with the morphological data on the subspecies status of the *D. p. portschinskii* and *D. p. nigrita* populations. A potential applicability of the developed microsatellite markers to study genetic diversity of *Darevskia* species and subspecies complexes is suggested.

Keywords. *Darevskia* lizards, *Darevskia portschinskii*, taxonomy and population genetics, microsatellite loci, genetic polymorphism, genetic differentiation.

INTRODUCTION

The Caucasian rock lizard *Darevskia portschinskii* was described for the first time by K.F. Kessler in 1878 based on samples from the Tbilisi region (Kessler, 1878). The main range of this species is concentrated along the right bank of the Kura River valley and the ravines of its right tributaries within Georgia, northern Armenia, and northwestern Azerbaijan at altitudes of 300-1700 meters above sea level. There is a large, isolated population in the valley of the middle part of the Iori River (left tributary of the Kura River) on the southern slopes of the Tsivi-Gombori mountain range, as well as in the ravine of the Akera River (part of the Araks River basin). The species inhabits

relatively arid zones along the banks of rivers and mountain slopes with xerophytic shrubs and herbaceous vegetation (Darevsky, 1967).

The increased interest in the study of *D. portschinskii* populations is due to the fact that this species acts as a paternal taxon in interspecific hybridisation with *D. mixta* (maternal species), resulting in the formation of unisexual (parthenogenetic) *D. dahli* (Uzzel and Darevsky, 1975), and is one of the examples of sympatric speciation in rock lizards of the genus *Darevskia* within the framework of the theory of reticulate or reticular evolution (Dobzhansky, 1937; Borkin and Darevskiy, 1980). Furthermore, in part of its range, *D. portschinskii* is sympatric with the parthenogenetic *D. dahli*, and in such

zones there is the possibility of interspecific hybridisa-
tion and the appearance of triploid hybrids (Darevsky,
1967), whose evolutionary potential as participants in
new stages of reticular evolution has not yet been estab-
lished. However, unlike other studies on triploid hybrids
between parthenogenetic and sexual species (Danielyan
et al., 2008; Freitas et al., 2019; Freitas et al., 2022), the
ongoing processes of hybridisation and triploid hybrids
from sympatry sites between *D. dahli* and *D. portschinskii*
are less known and only few information about hybrid
zone from previous years are available (Darevsky, 1967;
Petrosyan et al., 2020a).

Darevskia lizards, including *D. portschinskii*, show
strong heterogeneity in their geographical distribution
with many isolated populations due to the high topo-
graphic and climatic variability of the Caucasus region
(Petrosyan et al., 2020b). According to morphological
analysis and geographical distribution, *D. portschinskii*
lizards were divided by Bakradze (1976) into two sub-
species, *D. p. portschinskii* and *D. p. nigrita*. *D. p. nigrita*
(originally designated *Lacerta portschinskii nigrita*) was
found in the gorge of the river Mashavera (Georgia), as
well as in the gorge of the middle reaches of the river
Dzoraget (Armenia, outskirts of the city of Stepanavan).
Populations of *D. p. nigrita* subspecies live at an altitude
of 1400 - 1500 m, whereas other populations have been
found at lower altitudes. Lizards of the *D. p. nigrita* sub-
species have a much darker colorations than those of the
D. p. portschinskii subspecies, with two distinct longitudi-
nal rows of pale ocelli running along the upper edge of
the temporal stripes. In addition, they are characterised
by a brighter orange-yellow in vivo colouration of the
underside of the body of males. *D. p. portschinskii* liz-
ards are characterised by gray, grayish-beige or brownish
colouration above, and longitudinal rows of light ocelli
along the edges of the temporal stripes are absent or
weakly expressed.

Thus, the classification of subspecies of *D. portschin-
skii* populations was based solely on morphological and
geographic data. At the same time, genetic studies on
this problem have not yet been carried out. Therefore,
the main objective of this study is the molecular genetic
characterisation of *D. p. portschinskii* and *D. p. nigrita*
populations based on the analysis of the variability of
microsatellite markers and obtaining interrelated esti-
mates of the phenetic and genetic classification of these
populations. To solve this problem, ten di- and trinu-
cleotide microsatellite loci were searched in and selected
from the genome assembly of *D. valentini* (Ochkalova
et al., 2022), and their orthologs were also found in the
draft genomic assemblies of *D. unisexualis* and *D. rad-
dei*. Based on these loci, a PCR analysis system was

developed for the molecular genetic characterisation of
Darevskia species, including *D. portschinskii*. As an out-
group for comparison, *D. valentini* lizards with a firmly
established species status, belonging to the same 'rudis'
clade as *D. portschinskii* (Arribas, 1999; Rato et al.,
2021), were taken.

MATERIALS AND METHODS

Sample collection

Tail fragments of 43 *D. portschinskii* lizards were col-
lected in three main areas: in the vicinity of the villages
of Zuar and Marts, and in the Dzoraget region (Fig. 1
and Table 1). After sampling the biomaterial, the lizards
were released into their habitats. Total genomic DNA
were isolated by the standard phenol-chloroform meth-
od using proteinase K. As an outer group we used eight
previously obtained DNA samples of *D. valentini* liz-
ards from the populations of Lchashen, Tezh, and Adis
(Armenia, 40°51'N, 44°90'E; 40°70'N, 44°61'E; 40°30'N,
44°73'E, respectively).

Developing of the microsatellite markers

The search for microsatellite loci in the *D. valentini*
genome (Ochkalova et al., 2022) was carried out using
a pipeline that included several programs and Python
scripts. First, we searched for microsatellite repeats with a
given monomer length of two and three nucleotides and
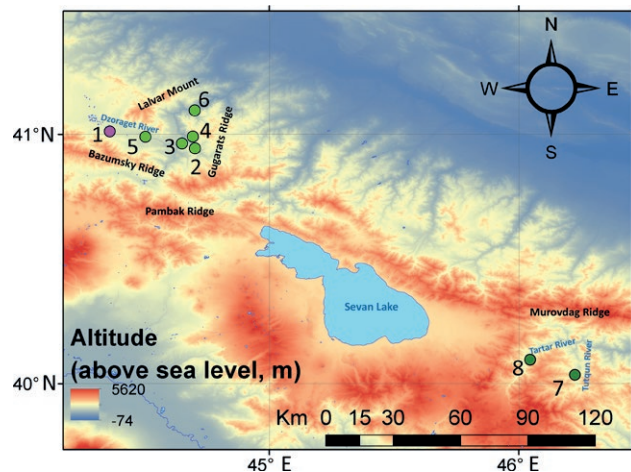with a number of at least ten repeats in the genome of *D.*

Fig. 1. Sampling localities of *D. portschinskii* used in the study, as
reported in Table 1. The licensed ArcGIS Desktop 10.4.1 program
(http://desktop.arcgis.com) was used to create the map.

Table 1. Samples of *D. portschinskii* used in the present study. The geographic localities are shown in Fig. 1.

Species/ Subspecies	Population (n)	Locality	Map locality	Sample size	Coordinates	
D. p. nigrita	"Dzoraget" (12)	Dzoraget	1	12	40°95'N	44°70'E
D. p. portschinskii	"Marts" (14)	Marts	2	10	40°95'N	44°70'E
		Dzekh	3	1	40°96'N	44°65'E
		Karinj	4	1	40°97'N	44°69'E
		Karmir Ageg	5	1	40°98'N	44°55'E
		Zarni Monastery (Haghpat)	6	1	41°09'N	44°71'E
	"Zuar" (17)	Zuar	7	16	40°04'N	46°23'E
		Karvachar	8	1	40°11'N	46°04'E

valentini using the MISA perl script (Thiel et al., 2003). Then, using BedTools v2.30.0 (Quinlan and Hall, 2010), all sequences that met the following criteria were selected: 1) the size of the sequences adjacent to the 5' and 3' ends of the microsatellite is at least 100 bp, 2) the microsatellite and flanking regions do not contain N, and 3) the flanking regions do not contain repeats and sequences that hinder primer hybridisation. A similar search found orthologous loci in the draft genome assemblies of *D. unisexualis* and *D. raddei*. For the selection of PCR primers, the Primer3 program was used (Untergasser et al., 2012; Koressaar and Remm, 2007) at a given length of the desired product, including a microsatellite repeat of at least 100 bp, a distance from the primers to the microsatellite of at least 10 bp, a primer length of 20–25 bp, and a melting point of 58-62 °C. The most suitable primer pairs were tested for the uniqueness of the amplified product by local *in silico* search in the *D. valentini* genome using BLAST+ 2.12.0 (Camacho et al., 2009). Ten microsatellite loci were selected for molecular genetic analysis (supplementary Table S1).

Amplification procedure.

PCR was carried out in a volume of 20 μl per 50 ng of DNA using the GenPakPCRCore kit (Isogene Laboratory) according to the manufacturer's protocol under the following temperature conditions: denaturation at 94 °C - 3 minutes, amplification for 30 cycles (denaturation: 94 °C - 1 minute, annealing: 60 °C - 40 seconds, elongation: 72 °C - 30 seconds), last cycle - 5 minutes at 72 °C. The concentration of each primer was 0.2 μM. One of the primers for amplifying individual loci was labelled with a fluorescent dye at the 5' end. Table S1 shows the characteristics of the loci and primers used in the work. The PCR amplification products were fractionated in 0.8% agarose gel followed by DNA fragment isolation using the GeneJET Gel Extraction Kit (ThermoScientific).

Fragment analysis.

Fragment analysis was performed at the Synthol company using the NANOFOR-05 genetic analyser. The size of the amplified fragments was determined using the Peak Scanner v1.0 software (Applied Biosystems). Each amplification product was considered as a biallelic locus, heterozygous in the presence of two fragments of varied sizes and homozygous if a single fragment was detected.

Genetic analysis.

Number of alleles/genotypes observed, Simpson's index (1-D), and alleles distribution (Evenness) for all microsatellite loci were estimated using *poppr* (Kamvar et al., 2014) package. For analysis of allelic richness (A_R), observed and expected heterozygosity (H_{obs}, H_{exp}), we used the *hierfstat* (Goudet, 2005). The deviation from Hardy-Weinberg equilibrium for each locus was tested with allele randomizations (1000 permutations per test) with the package *pegas* (Paradis, 2010). The degree of genetic differentiation between samples was determined by calculating F_{ST} (Nei, 1987) and a modified version of Hedrick's G_{ST} (Hedrick, 2005; Meirmans and Hedrick, 2011) using *mmod* package (Winter, 2012), taking into account the tendency of the standard G_{ST} test to underestimate the degree of differentiation between a small number of populations. An UPGMA tree based on the matrix of Nei (1972) pairwise genetic distances (implemented in *poppr*) was used to visualise the relationships among all individuals and populations studied. Support for each node was tested by 1000 bootstrap replicates. The association index used to assess multilocus linkage disequilibrium was determined using the *poppr* package (Kamvar et al., 2014).

Two approaches have been used to infer clusters or subpopulations from a sample by genetic mixture analysis. Firstly, to determine the population structure of *D. portschinskii*, we used discriminant principal component

analysis (DAPC) (Jombart et al, 2010) implemented in the *adegenet* package (Jombart 2008; Jombart 2011). A sample of *D. valentini* was added as an outgroup, while the analysis was carried out without taking into account the a priori population structure of *D. portschinskii* known by geographic origin. Before the DAPC analysis, we checked all loci and found 214 missing values in the total sample (Fig. S1). The sc7287 and sc1872 loci contained more than 5% missing values and were corrected. To avoid bias, the missing data were replaced by mean allele frequencies. The optimal number of genetic clusters in populations obtained by the k-means algorithm was determined using the Bayesian information criterion. The probability of the relationship of specific individuals to genetic groups was calculated.

An additional analysis of the genetic structure of the species *D. portschinskii* was carried out using the Bayesian approach implemented in the STRUCTURE 2.3.4 program (Pritchard et al., 2000). Taking into account the probability of the origin of populations from a common ancestor, we used the admixture model (allele frequencies correlated among populations), which does not exclude the possibility of crossing. The posterior probabilities were estimated using a Markov chain Monte Carlo method (MCMC) based on 1,000,000 iterations of this chain, following a burn-in period of 200,000 iterations. Since the sample of *D. portschinskii* comes from eight collection points (Fig. 1, Table 1) combined into three known populations ("Marts", "Zuar", and "Dzoraget"), we conditioned our data on different values of K ranging from 1 to 8 initially containing the optimal value, but the calculations did not include information on the population structure (option LOCPRIOR=0). Ten runs were performed for each K to test the stability of the results. The best value of K that captured

the main structure in the data was determined by the ΔK value estimation method according to Evanno et al. (2005) using the web-based program Structure Harvester (version 0.6.8) (Earl and vonHoldt, 2012). CLUMPAK (Kopelman et al., 2015) with defaults parameters (LargeKGreedy algorithm, random input order and 2000 replicates) was used for merging the replicate runs, and graphical representation of the results obtained by STRUCTURE.

RESULTS

Molecular genetic characteristics of microsatellite loci

Comparative data on the analysis of allelic polymorphism on 10 microsatellite loci, expected heterozygosity (H_{exp}), distribution of alleles (Evenness), and the Simpson diversity index (1–D) for the loci used, obtained on the samples of *D. portschinskii* and *D. valentini*, are presented in Table 2. It can be seen that the loci differ in a wide range in the number of alleles and genotypes in these samples. The average values of the above indicators allow us to use these loci to assess the intraspecific polymorphism of these species. The smallest number of alleles, as well as the smallest values of the Simpson index (1–D) and expected heterozygosity (H_{exp}) were obtained at the sc10877 and sc12962 loci. The alleles of the microsatellite loci form distinct genotypes, the number of which varies depending on the locus and the subspecies (Table 2).

Genetic polymorphism in populations of D. portschinskii

Darevskia portschinskii portschinskii is characterised by a larger number of alleles and genotypes compared

Table 2. Genetic variability of the loci used in a mixed sample of *D. portschinskii* and *D. valentini* individuals. 1-D - Simpson index, H_{exp} - expected heterozygosity (Nei's, 1978, gene diversity), Evenness - distribution, A_R - allelic richness.

Locus	Number of alleles	Number of genotypes	1-D	H_{exp}	H_{obs}	Evenness	A_R
sc12962	4	5	0.2609	0.3034	0.30	0.53	3.9785
sc4525	14	15	0.3830	0.8060	0.79	0.68	13.2006
sc138	9	14	0.5417	0.7737	0.76	0.72	8.8333
sc149	12	18	0.4348	0.8408	0.83	0.72	11.7690
sc12560	14	24	0.7234	0.8802	0.87	0.80	13.4590
sc7287	13	22	0.5854	0.8277	0.81	0.69	13.0000
sc1872	10	10	0.3095	0.4640	0.46	0.43	9.8804
sc4045	11	16	0.3750	0.7535	0.74	0.62	10.6804
sc10877	3	3	0.0638	0.2969	0.29	0.58	2.9984
sc6476	24	31	0.7083	0.9102	0.90	0.67	22.5750
Overall	11.40 ± 5.85	15.50 ± 8.56	0.44 ± 0.21	0.69 ± 0.24	0.68 ± 0.23	0.64 ± 0.11	11.04 ± 5.46

Table 3. Genetic characteristics of the loci used in the three analysed samples. 1-D - Simpson index; H_{exp} and H_{obs} - expected and observed heterozygosity (Nei's, 1978, gene diversity); Evenness - distribution, A_R - allelic diversity.

Samples	Locus	Number of alleles	Number of genotypes	H_{obs}	H_{exp}	1-D	Evenness	A_R
D. valentini	sc12962	2	2	0.2500	0.2321	0.22	0.61	1.9917
	sc4525	10	7	1.0000	0.9286	0.88	0.88	9.2333
	sc138	4	4	0.0000	0.8214	0.72	0.93	3.9917
	sc149	7	6	0.6250	0.8839	0.81	0.87	6.6167
	sc12560	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc7287	6	5	1.0000	0.7679	0.73	0.78	5.6167
	sc1872	6	6	0.8570	0.6667	0.63	0.60	6.0000
	sc4045	7	8	0.7500	0.8661	0.80	0.84	6.6167
	sc10877	2	2	0.3750	0.3214	0.30	0.71	2.0000
	sc6476	12	8	1.0000	0.9554	0.90	0.89	10.8583
	Overall	5.7±3.56	4.9±2.56	0.58±0.40	0.64±0.34	0.60±0.31	0.79±0.12	5.39±3.2
D. portschinskii	sc12962	3	4	0.2778	0.2117	0.31	0.60	2.2669
	sc4525	4	8	0.2083	0.2222	0.70	0.92	3.7402
	sc138	8	12	0.6319	0.6349	0.76	0.72	5.3348
	sc149	9	13	0.3889	0.5082	0.78	0.72	5.7252
	sc12560	13	23	0.8699	0.8344	0.85	0.77	7.0208
	sc7287	10	17	0.4806	0.7845	0.77	0.67	5.7787
	sc1872	4	4	0.2235	0.2520	0.23	0.47	2.3147
	sc4045	7	9	0.2917	0.4236	0.65	0.69	4.9570
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	16	23	0.6528	0.7896	0.86	0.73	7.4568
	Overall	7.5±4.7	11.4±7.7	0.4±0.26	0.47±0.29	0.59±0.30	0.7±0.12	4.56±2.15
D. p. nigrita	sc12962	2	3	0,8333	0.5076	0.50	1.00	2.0000
	sc4525	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc138	3	3	0.7500	0.5076	0.50	0.82	2.833
	sc149	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc12560	7	9	0.9167	0.7992	0.77	0.78	6.6230
	sc7287	6	9	0.6000	0.8556	0.80	0.92	6.0000
	sc1872	2	2	0.5455	0.4091	0.40	0.83	2.0000
	sc4045	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	6	9	0.5833	0.7538	0.72	0.73	5.7880
	overall	3±2.4	3.9±3.6	0.42±0.38	0.38±0.36	0.37±0.34	0.85±0.1	2,92±2.31
D. p. portschinskii	sc12962	2	2	0.0000	0.0638	0.07	0.45	1.9887
	sc4525	4	7	0.3125	0.3329	0.65	0.82	4.0000
	sc138	8	11	0.5729	0.6975	0.81	0.82	7.8159
	sc149	9	12	0.5833	0.7612	0.83	0.81	8.8619
	sc12560	11	17	0.8466	0.8518	0.85	0.82	10.4047
	sc7287	9	12	0.4208	0.7490	0.71	0.60	9.0000
	sc1872	3	3	0.0625	0.1735	0.12	0.43	2.9574
	sc4045	7	9	0.4375	0.6339	0.72	0.75	6.7587
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	14	16	0.6875	0.8075	0.86	0.72	12.8615
	Overall	6.8±4.2	9±5.7	0.39±0.30	0.51±0.33	0.56±0.35	0.69±0.16	6.56±3.92
D. p. portschinskii ("Marts")	sc12962	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc4525	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc138	7	7	0.3333	0.7424	0.72	0.64	5.8643

(Continued)

Table 3. (Continued).

Samples	Locus	Number of alleles	Number of genotypes	H_{obs}	H_{exp}	1-D	Evenness	A_R
	sc149	7	8	0.6667	0.8750	0.87	0.92	6.5059
	sc12560	9	10	0.8182	0.8864	0.88	0.84	7.8177
	sc7287	7	7	0.3750	0.9018	0.87	0.85	7.0000
	sc1872	3	3	0.1250	0.3571	0.34	0.57	3.0000
	sc4045	5	4	0.5000	0.6402	0.63	0.66	4.5310
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	13	10	0.7500	0.9167	0.91	0.75	9.8641
	Overall	5.4±3.98	5.2±3.65	0.36±0.32	0.53±0.40	0.52±0.40	0.75±0.13	4.76±3.17
D. p. portschinskii ("Zuar")	sc12962	2	2	0.0000	0.1250	0.12	0.50	1.9879
	sc4525	4	6	0.6250	0.6604	0.64	0.77	4.0000
	sc138	5	5	0.8125	0.6562	0.64	0.74	4.9637
	sc149	5	6	0.5000	0.6484	0.62	0.71	5.0000
	sc12560	8	11	0.8750	0.8188	0.79	0.80	7.6129
	sc7287	5	6	0.4667	0.6071	0.58	0.71	4.8644
	sc1872	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc4045	6	8	0.3750	0.6271	0.60	0.60	5.8387
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	6	7	0.6250	0.7000	0.68	0.74	5.7371
	Overall	4.3±2.3	5.3±3.2	0.43±0.33	0.48±0.31	0.47±0.30	0.70±0.10	4.20±2.21

to *D. p. nigrita* at eight of the 10 loci analysed (P<0.05). At the sc12962 locus, the number of alleles in both subspecies was the same; however, in the *D. p. nigrita* alleles form three genotypes, and two in the subspecies *D. p. portschinskii*.

In the analysed sample of each species and subspecies, the sc12962 locus is represented by an equally small number of alleles, two alleles each. The sc10877 locus, represented by only three alleles, turned out to be the least polymorphic: in individuals of *D. portschinskii*, this locus was represented by one allele, and in the sample of *D. valentini*, by two alleles. The sc1872 locus is represented by 10 alleles; however, the Simpson index and expected heterozygosity were less than 0.5 (Table 2). This is because the alleles of this locus are unevenly distributed between species (Table 3): six alleles were found in the sample of *D. valentini* individuals, and four alleles were found in the sample of *D. portschinskii*. The expected and observed heterozygosity (H_{exp}) values in the total analysed sample range from 0.3 to 0.9, and the average value of this parameter is 0.68 (Table 2). However, each of the studied species differs in the main characteristics of the loci (Table 3). The sc10877 locus turned out to be species-specific: in all studied individuals of *D. portschinskii*, the locus is monomorphic, and all individuals are homozygotes (Table 3). According to these data, the sc10877 locus cannot be used to analyse the intraspecific

polymorphism of *D. portschinskii*, but it can be used for interspecific comparisons. The number of alleles at the studied loci varies significantly in distinct groups. Some loci, which have one or two alleles, are characterised by low diversity, low expected heterozygosity (H_{exp}), and uneven distribution of alleles. For example, loci sc4525, sc149, and sc4045 in the subspecies *D. p. nigrita* are represented by one allele. Characteristics of the loci also vary between populations of *D. p. portschinskii*. In the "Marts" population, the mean number of alleles is higher than in "Zuar" population, but the mean number of genotypes in both populations is nearly the same (Table 3). Observed heterozygosity in "Marts" population is lower than in "Zuar". This could be explained by formation of individuum-specific homozygous genotypes. In total, "Marts" is more polymorphic than the "Zuar" population, which may be related with higher sampling localities (Table 1).

Combinations of alleles at ten analysed loci for each individual of *D. portschinskii* form multilocus genotypes (MLG). Supplementary Fig. S1 shows the accumulation curve of MLG. For 48 analysed individuals (eight individuals of *D. valentini* and 40 individuals of *D. portschinskii*) the curve "reaches" a plateau starting from the genotype consisting of seven loci. Thus, this curve makes it possible to identify the minimum number of loci required to separate population samples. Based on the results obtained, a Nei's UPGMA tree (bootstrap 1000)
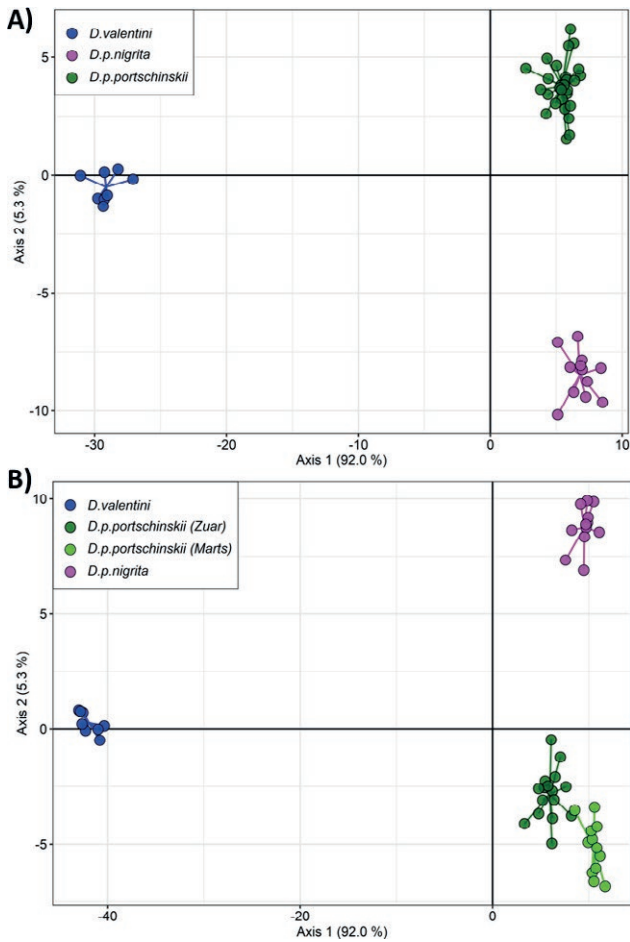
Fig. 2. Clustering of *D. portschinskii* and *D. valentini* individuals based on discriminant principal component analysis (DAPC). A) K=3; B) K=4.

was constructed, demonstrating a significant division of the *Darevskia* lizards sample into two groups corresponding to *D. valentini* and *D. portschinskii* and supposed but non-significant subdivision of *D. portschinskii* into two clusters according of subspecies status, *D. p. portschinskii* and *D. p. nigrita* (Fig. S2).

Genetic differentiation in D. portschinskii *populations*

The standardised association index for the subspecies *D. p. nigrita* (-0.0247, P = 0.816) reflects the free recombination of alleles within this subspecies (Fig. S3A). For the subspecies *D. p. portschinskii*, the value of this parameter is 0.0677 (P = 0.001), which indicates the absence of allele recombination between the Zuar and Marts populations (Fig. S3B) and reflects the geographical remoteness of these groups. The value of the standardised association

Table 4. Values of the parameters of intraspecific and interspecific differentiation of *D. portschinskii* and *D. valentini*.

	F_{ST}	G_{ST}
D. portschinskii – *D. valentini*	0.298	0.797
D. p. portschinskii – *D. valentini*	0.303	0.782
D. p. nigrita – *D. valentini*	0.443	0.908
D. p. portschinskii – *D. p.nigrita*	0.286	0.556
D. p. portschinskii ("Zuar") – *D. valentini*	0.357	0.819
D. p. portschinskii ("Marts") – *D. valentini*	0.328	0.798
D. p. portschinskii ("Zuar") – *D. p. nigrita* ("Dzoraget")	0.353	0.624
D. p. portschinskii ("Marts") – *D. p. nigrita* ("Dzoraget")	0.332	0.617
D. p. portschinskii ("Marts") – *D. p. portschinskii* ("Zuar")	0.222	0.461

index shows the free recombination of alleles within each group: 0.000293 (P = 0.624) and -0.00396 (P = 0.655) for the Zuar and Marts groups, respectively (supplementary Fig. S4). The level of statistical significance P > 0.05 does not allow us to reject the hypothesis of the absence of linkage of alleles between loci and their independent recombination within each subspecies. A value of 0.0953 (P = 0.001) for *D. portschinskii* as a whole (i.e. for both subspecies) indicates no allele recombination between subspecies (Fig. S3C), i.e. these subspecies are isolated and crosses between their individuals are absent.

The main values of the parameters of intra- and interspecific differentiation, obtained by pairwise comparison of individuals of *D. portschinskii*, its subspecies, and the species *D. valentini*, are shown in Table 4. The F_{ST} parameter changes insignificantly during interspecific and intraspecific comparison. The G_{ST} value between *D. portschinskii* and *D. valentini* is 0.797, while between the subspecies of *D. portschinskii* it is 0.556. *Darevskia portschinskii portschinskii* in this study is represented by two geographically distant populations, "Zuar" and "Marts", although individuals of this subspecies from the population "Marts" are geographically closer to the population of the subspecies *D. p. nigrita* "Dzoraget" (Fig. 1). The values of inter- and intraspecific G_{ST} and F_{ST} indices are shown in Table 4. According to the data obtained, the values of these parameters and the level of differentiation between the "Marts" and "Zuar" populations are lower than that between the "Dzoraget" - "Marts" and "Dzoraget" - "Zuar" populations.

The use of the DAPC method on the entire sample of *D. portschinskii* and the outgroup *D. valentini* made it possible to determine the presence of three genetic groups (K = 3) with a clear division into subspecies according to the taxonomic status, which form a three non-overlapping graphs array (Fig. 2A). The same analysis with K =
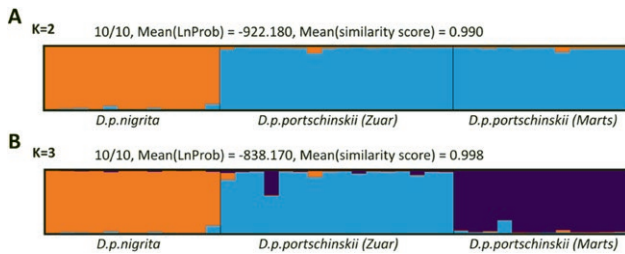
Fig. 3. Genetic structure of the species *D. portschinskii* according to the results of clustering in the STRUCTURE program. K=2 allows differentiating *D. portschinskii* into the subspecies *D. p. nigrita* and *D. p. portschinskii* (A), while K=3 allows the exact definition of two distinct populations within *D. p. portschinskii* (B).

4 shows differentiation on the groups, corresponding to *D. valentini*, *D. p. nigrita*, and *D. p. portschinskii* "Marts" and "Zuar" (Fig 2B.) The populations "Mars" and "Zuar" are geographically isolated, individuals can't mate, so there is no exchange of alleles between individuals. The partial overlapping between these two groups can be explained by gene flow before their isolation from each other (Cota et al., 2017), and can reflect their belonging to the same subspecies. The DAPC on the samples of *D. portschinskii* species only determines the presence of three genetic groups (K = 3) with a clear division into populations "Marts", "Zuar", and "Dzoraget" (Fig. S6).

At the same time, the probability of attracting individuals from *D. valentini* species to the corresponding cluster was 100% [95% confidence interval 67.6-100%]. The same accuracy of classification is observed for the subspecies *D. p. nigrita*. Evanno's method was used to determine the optimal number of clusters *K* (supplementary Fig. S7A) for the analysis of interpopulation subdivision of the species *D. portschinskii* in the STRUCTURE program. Already at the value of K=2, two large clusters are distinguished, corresponding to the subspecies *D. p. nigrita* and *D. p. portschinskii* (Fig. 3A). The optimal number of clusters is K=3, at which the separation of *D. p. portschinskii* into two populations "Marts" and "Zuar" (Fig. 3B) is observed. However, an increase in the number of clusters, and performing the same analysis with adding *D. valentini* (K = 4), does not affect the reliable division of *D. portschinskii* into two groups with subspecies status (Fig. S8). The slight admixture between subspecies can be explained by gene flow before their isolation from each other (Cota et al., 2017).

DISCUSSION

The study of intraspecific diversity of *D. portschinskii* is of particular interest in connection with the participa-

tion of its populations in interspecific hybridisations of various types, with the formation of unisexual (parthenogenetic) forms, *D. dahli* and *D. rostombekowii* (Uzzel and Darevsliy, 1980), and possible hybridisation of *D. portschinskii* with the related parthenogenetic species. Until now, the population classification of this species has been based only on morphological and geographical data that suggested the subspecies taxonomic status of *D. p. portschinskii* and *D. p. nigrita* populations (Bakradze, 1976). The aim of our work was to perform molecular genetic characterization of *D. portschinskii* populations based on microsatellite markers and to state the value of their genetic differentiation. For this, 40 lizards with wide morphological variation from different geographic locations were genotyped using 10 microsatellite loci, each present in the *D. valentini*, *D. raddei*, and *D. unisexualis* genomes. As follows from the data obtained, the value of one of the main indicators of intraspecific differentiation F_{ST} for *D. portschinskii* is 0.298 (Table 4), which is higher than shown for a number of vertebrate species. For example, the value of F_{ST} parameter between some subspecies of *Darevskia bithynica* lizards is 0.048 (Koç et al., 2017), for the introduced American mink is 0.144 (Korablev et al., 2018), and for the toothed smelt is 0.0701 (Semenova et al., 2019). This suggests that the populations studied in this work are not only geographically isolated, but also diverged to the subspecies level (Mikulícek et al., 2007). The values of the F_{ST} parameter when comparing the species *D. valentini* and *D. portschinskii* differ insignificantly and are comparable with those obtained when comparing the subspecies *D. p. portschinskii* and *D. p. nigrita* (Table 4). However, it seems more correct to use the G_{ST} criterion according to Hedrick (2005) (Table 4), as it more accurately reflects intraspecific and interspecific relationships in the *Darevskia* group. The use of F_{ST} and G_{ST} criteria to assess intraspecific diversity and differentiation of *D. portschinskii* shows similar results (Table 4). Populations belonging to the same subspecies *D. p. portschinskii*, despite significant geographical remoteness, are characterised by greater genetic similarity than populations of different subspecies *D. p. portschinskii* and *D. p. nigrita* living at a relatively close distance. According to the data obtained, *D. p. nigrita* is characterised by a lower level of intrapopulation polymorphism than *D. p. portschinskii*, which is probably due to the small range of this subspecies. This is consistent with the earlier results of the analysis of the genetic diversity of allozyme loci in the species *D. portschinskii*, which showed that small disjunct populations isolated from the main range of the species are characterised by a low level of genetic polymorphism compared to non-isolated contiguous populations (MacCalloch et al., 1997). In general, our genetic data on the

population differentiation of *D. portschinskii* confirms the morphological data on the subspecies status of the *D. p. portschinskii* and *D. p. nigrita* populations. It is evident that 10 developed microsatellite loci were effective for studying intraspecific diversity of investigated *Darevskia* species: we also suggested a potential applicability of the developed microsatellite markers for study other *Darevskia* lizard species and subspecies. In general, they are more polymorphic than tetranucleotide microsatellite markers developed by us previously (Korchagin 2007), and which are widely used for genetic study of *Darevskia* lizards (Badaeva et al., 2008; Girnyk et al., 2018; Vergun et al., 2014, 2020; Ryskov et al., 2017; Tarkhnishvili et al, 2017; Freitas et al., 2019; Koç, et al., 2017; Kropachev et al., 2023). We suppose that they can be useful to study different *Darevskia* lizard species.

The study of evolutionary relationships at the population, subspecies, and species levels requires the analysis of rapidly evolving markers. Microsatellites are characterised by a high mutation rate and genetic polymorphism (Jarne and Lagoda, 1996; Estoup and Corneut, 2000; Ellegren, 2004; Badaeva et al., 2008), and a number of studies confirms the successful use of microsatellites for the reconstruction of phylogenetic relationships of such taxa at the level of species/subspecies/population (Estoup et al., 1995; Harr et al., 1998; Petren et al., 2005; Richard and Thorpe, 2001; Peréz et al., 2002; Orsini et al., 2005; Chirhart et al., 2005; Hughes et al., 2005; Kankare et al., 2005; Knaden et al., 2005). Here, we first present phylogenetic relationships within *D. portschinskii* based on the polymorphism of developed trinucleotide (sc12962, sc138, sc149, sc4045) and dinucleotide (sc12560, sc6476, sc4525, sc10877, sc7287, sc1872) microsatellite loci. The main genetic characteristics of these loci were determined, and their suitability for molecular genetic study of *Darevskia* species was demonstrated. In particular, it has been shown that they are effective for studying the intraspecific diversity of *Darevskia* lizards: the number of alleles is sufficient to reliably separate morphologically different populations; multilocus genotypes formed by alleles of only seven microsatellite loci make it possible to construct various types of phylogenetic trees.

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-262 9.unipv.it/webshi/appendix/index.html> manuscript number 14756.

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First data on the consumed prey by *Speleomantes italicus* from the Republic of San Marino

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Abstract. We here present the first study on the consumed prey by an epigean population of the Italian cave salamander (*Speleomantes italicus*) from the Republic of San Marino. Using the harmless technique of stomach flushing, we aimed to provide the first data on the diet and on the degree of individual specialization that characterizes the studied population. We performed two-night surveys (one in autumn 2022 and one in spring 2023) in which we opportunistically searched for salamanders in forested areas and dry walls. Captured salamanders were sexed, weighed, photographed, measured and stomach flushed. Overall, we inspected the stomach contents of 67 individuals, recognizing 1,018 prey items belonging to 28 different prey categories. The examined population was mainly composed by generalist individuals, and no differences in number and diversity of prey consumed were observed among salamanders. Contrarily to what has been observed in other *Speleomantes* species, flying prey provided a minor contribution to the diet of the investigated population. Our study produced the first information on the diet of *Speleomantes italicus* from the Republic of San Marino, representing the starting point for future assessments on the dietary habits of this specific population.

Keywords. Diet, *Hydromantes*, Plethodontidae, salamander, stomach flushing, trophic niche, Urodela.

European plethodontid salamanders (genus *Speleomantes*) are eight allopatric species mainly distributed in Italy and in a small area of the French Provence (Lanza et al., 2006); among the mainland species, *S. italicus* also occurs in the Republic of San Marino (Casali et al., 2005). *Speleomantes* are facultative cave-dwellers inhabiting different typologies of subterranean environments (e.g., caves, mines), but also forested areas and even dry walls (Costa et al., 2016; Lanza et al., 2006; Manenti, 2014), where suitable

microclimatic conditions (relatively cold temperature and high humidity) occur (Ficetola et al., 2018).

Speleomantes are generalist predators feeding on a wide number of invertebrate species (Lunghi et al., 2018a; Salvidio, 1992; Vignoli et al., 2006). Within subterranean environments, *Speleomantes* can forage throughout the year (Lunghi et al., 2022a), while in surface ones they are generally active only during favorable seasons (e.g., in spring and autumn) (Costa et al., 2016;

Salvidio et al., 2012). The main method used for studying the dietary habits of the genus is the stomach flushing (Salvidio, 1992). The use of stomach contents therefore turned out to be an optimal and sustainable method allowing to assess *Speleomantes* trophic niche, but also to evaluate the degree of diet specialization that individuals have within populations (Lunghi et al., 2020a; Lunghi et al., 2020d; Salvidio et al., 2015; 2017). However, this methodology was never adopted to study the trophic niche of *Speleomantes* populations occurring in the Republic of San Marino.

We here present the first data on the dietary habits of a population of *S. italicus* occurring in the Republic of San Marino, focusing on the diversity of prey consumed and on the diet specialization of individuals. We selected a population occurring within an urbanized area, hypothesizing that the related environmental conditions may cause a divergence of dietary habits compared to populations occurring in natural areas.

We performed two-night surveys in the Republic of San Marino, searching for individuals of *Speleomantes italicus* in a patched forested area of broad-leaved trees, and inside dry walls occurring within an urbanized area. The first survey was performed on 21st November 2022 between 8:00 p.m. and 0:00 a.m. (minimum air temperature Tmin 3.1 °C, maximum air temperature Tmax 11.2 °C, precipitations 1.5 mm), while the second one on 7th April 2023 between 9:00 p.m. and 1:00 a.m. (Tmin 4.7 °C, Tmax 11.6 °C, precipitations 0 mm) (climatic data are retrieved from https://www.3bmeteo.com). All operators worked using disposable gloves and used clean and disinfected instruments. Salamanders were opportunistically searched and captured individuals were processed as follow. Adult males were recognized by checking the presence of male secondary sexual characters (i.e., mental gland) (Lanza et al., 2006). Adult females and juveniles were later distinguished on the basis of snout-vent length (SVL); individuals with SVL ≥ 50 mm were considered adult females, while the other juveniles (Lunghi, 2022). Salamanders were then weighed on a digital scale (0.01 g) and placed on the portable studio to shoot a picture which will be used to estimate individuals SVL in the lab using the ImageJ software (https://imagej.nih.gov/ij) (Lunghi et al., 2020c). All pictures were visually checked in order to individually recognize salamanders using their dorsal pattern (Lunghi et al., 2019); no individual was captured twice. Finally, we performed stomach flushing on salamanders (discarding juveniles with SVL < 40 mm) to inspect the residuals of their last foraging activity. Stomach contents were preserved in 70% EtOH until their recognition. Prey items were counted and recognized following (Lunghi et al., 2018a). Briefly, prey items were generally recognized at order level, distinguishing between larval and adult stages when morphology and ecology of the two life stages strongly diverge. For two groups (Coleoptera and Hymenoptera) we were able to reliably recognize prey items from a specific family, respectively Staphylinidae and Formicidae; these families were used as independent prey categories (Lunghi et al., 2018a).

We pooled together data from the two seasons as the high divergence of the number of recognized prey between seasons (1:10) would affect the reliability of such comparison. We used Generalized Linear Mixed Models (GLMMs) (Douglas et al., 2015) to evaluate whether salamanders consumed a larger number or higher diversity (Shannon index) of prey on the basis of their size (SVL) or sex (adult males, adult females, juveniles). GLMMs were built using the log-transformed number of consumed prey or the Shannon index as dependent variable, while the SVL and sex were used as independent variables; date of survey was used as random factor. Likelihood ratio test was used to assess the significance of variables in GLMMs (Kuznetsova et al., 2016). We then estimated the degree of individual diet specialization (IS) (Zaccarelli et al., 2013). We calculated IS and transformed using the index (V = 1 – IS) proposed in Bolnick et al., (2007), where values tending to 1 indicate a high degree of individual diet specialization. Bootstrapping (repeated 9999 times) was used to test whether the observed index of individual diet specialization diverged significantly from a scenario in which all individuals randomly chose their prey. To avoid over estimations of IS, the dataset used in this analysis only included individuals from which at least 3 prey items were recognized.

We inspected the stomach contents of 67 individuals of *Speleomantes italicus* (46 from autumn and 21 from spring) including 26 adult females, 29 adult males and 12 juveniles (Table S1). Only 2 individuals from autumn had empty stomach. We overall recognized 1,018 prey items (average ± SD per individual; 15.66 ± 13.23) belonging to 28 groups of prey (Table 1).

During autumn, the captured individuals consumed 921 prey items belonging to 27 categories, where three groups of prey (Diptera larvae, Entomobryomorpha, and Hemiptera) accounted for 62.48% of the consumed prey. In spring the captured salamanders consumed 97 prey items belonging to 17 prey categories, where two groups (Diptera and Araneae) accounted for 44.33% of the consumed prey. These proportions only considered prey categories with relative importance > 10% (Table 1). The number of consumed prey was not affected by both salamander size ($F_{1,60} = 1.72$, P = 0.195) and sex ($F_{2,60.02} = 0.11$, P = 0.897). Similar results were obtained for the diversity of consumed prey (size, $F_{1,60} = 0.76$, P = 0.387;

Table 1. List of the prey residuals recognized from stomach contents of *Speleomantes italicus* from the Republic of San Marino. In brackets, the relative importance (%) of each category of consumed prey calculated for each season.

Prey order	Number of recognized prey (relative importance, %)	
	Autumn 2022	Spring 2023
Pulmonata	24 (2.61)	0
Sarcoptiformes	50 (5.43)	8 (8.25)
Trombidiformes	1 (0.11)	1 (1.03)
Araneae	25 (2.71)	14 (14.43)
Pseudoscorpiones	33 (3.58)	3 (3.09)
Opiliones	11 (1.19)	1 (1.03)
Lithobiomorpha	6 (0.65)	1 (1.03)
Geophilomorpha	7 (0.76)	0
Scolopendromorpha	1 (0.11)	0
Polydesmida	37 (4.02)	1 (1.03)
Isopoda	8 (0.87)	2 (2.06)
Symphypleona	14 (1.52)	2 (2.06)
Poduromorpha	6 (0.65)	0
Entomobryomorpha	221 (24)	7 (7.22)
Blattodea	0	1 (1.03)
Psocodea	1 (0.11)	0
Hemiptera	121 (13.14)	8 (8.25)
Hymenoptera	9 (0.98)	0
Hymenoptera_Formicidae	24 (2.61)	6 (6.19)
Coleoptera	12 (1.3)	4 (4.12)
Coleoptera_Staphylinidae	10 (1.09)	0
Coleoptera_larva	12 (1.30)	0
Lepidoptera	2 (0.22)	0
Lepidoptera_larva	29 (3.15)	1 (1.03)
Diptera	24 (2.61)	29 (29.90)
Diptera_larva	231 (25.08)	8 (8.25)
Dermaptera	1 (0.11)	0
*Speleomantes*_skin	1 (0.11)	0
Total	921	97

sex, $F_{1,60.03} = 0.3$, P = 0.742). Overall, 11 individuals consumed < 3 prey items; these individuals were excluded from diet specialization analysis. The studied population of *S. italicus* showed a very low proportion of specialized individuals (V = 0.28, P < 0.001).

This study provides the first information on the diet of *Speleomantes italicus* from the Republic of San Marino. The unfavorable climatic conditions (i.e., dryness) occurring during our spring survey may have negatively affected the activity of both predators (salamanders) and prey (invertebrates), limiting our data collection and therefore hampering the possibility to provide meaningful and robust comparisons between the two seasons. In the

future, multiple seasonal surveys and data collection on prey availability may allow to produce a more complete knowledge on the trophic niche for this population and evaluate the potential effect of local microclimate on prey availability (Centomo et al., 2023; Lunghi et al., 2018b; Salvidio, 1992).

All the 28 prey categories recognized from this population were already known for the species (Lunghi et al., 2022b), although divergences in the relative proportion can be observed. The first evident divergence is the proportion of consumed flying prey. In the present population flying prey (Ephemeroptera, Hymenoptera, Mecoptera, Plecoptera, Lepidoptera, Diptera) only accounted for less than 19% of the overall consumed prey (Table 1), with a relatively low increase in their proportion in spring (Table 1). This is quite interesting as *Speleomantes* seems to have a preference for flying prey independently from the habitus of the considered population (i.e., epigean or subterranean) (Roth 1982), hypothesis supported by the large proportion of this type of prey within *Speleomantes* diet (71.5% in Lunghi et al., 2018a; 94% in Lunghi et al., 2021; 52.7% in Lunghi et al., 2022b; 85% in Salvidio et al., 1994; 53% in Vignoli et al., 2006; but see Salvidio, 1992). Furthermore, *Speleomantes* are known to increase the consumption of this prey type at the beginning of the warm season, particularly when the abundance of Diptera in caves is higher (Lunghi et al., 2020b; 2022a). However, we must remark that our data is strongly biased towards autumn, a season in which the consumption of non-flying prey generally increases compared to spring (Lunghi et al., 2018b; 2022a); indeed, further samplings are needed to clarify this unexpected result. Another curiosity emerging from this dataset is the very large proportion of consumed Entomobryomorpha, especially in autumn, where they represented 24% of the total consumed prey (Table 1). Although Entomobryomorpha are relevant components of terrestrial ecosystems and constitute a significant proportion of the animal biomass (Handschin 1955; Kampichler and Bruckner 2009), the reason for this high proportion is difficult to hypothesize (especially at this level of taxonomic rank) and probably lies in the composition of the prey available in these survey periods.

The sampled population was characterized by a high proportion of generalist individuals (V = 0.28), characteristic also supported by the results of GLMMs analyses, where neither sex nor size did affect both number and diversity of consumed prey. A high proportion of diet specialization has been observed in spring within an epigean population of *S. italicus*, where the population seems to not be in competition with the sympatric *Salamandra salamandra* (Lunghi et al., 2022c). A high

proportion of specialists may arise due to competition, or because the wide trophic availability allows individuals to specialize on the preferred prey (Araújo et al., 2011). If we consider only the data collected in spring (Table 1), individuals from the Republic of San Marino consumed 17 prey categories while individuals from the other epigean population 35 (Lunghi et al., 2022c), supporting the hypothesis that a wider trophic availability may be responsible for diet specialization of individuals. The two populations strongly diverge in the type of habitat they occupy, one located within an urbanized area and the other in a fully forested area, meaning that probably the area interested by less urbanization may show higher prey diversity (Buczkowski and Richmond 2012; Chapman 2003). This is an interesting hypothesis that deserves to be investigated in the future.

With this study, we provided the first information on the dietary habits of *Speleomantes italicus* from the Republic of San Marino. Our results should be considered the starting point to further investigate the trophic niche of this *Speleomantes* population and to test the multiple hypotheses raised by this study.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-262 9.unipv.it/webshi/appendix/index.html> manuscript number 14748.

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The original authorship of *Chalcides ocellatus tiligugu* (Squamata: Scincidae)

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Abstract. The current herpetological literature considers Gmelin (1789) as the authority for making the name *Chalcides ocellatus tiligugu* available. However, the subspecies had already been introduced by Hermann (1783) who referred in his short description to a publication by Cetti (1777). This short note shows that in accordance with the rules of nomenclature the correct notation for this Sardinian scincid lizard should be *Chalcides ocellatus tiligugu* (Hermann, 1783).

Keywords. *Chalcides ocellatus*, nomenclature, authorship, Tiligugu, Sardinia.

The taxon *Chalcides ocellatus* (Forskål, 1775: 13) currently comprises six subspecies including the nominotypical *C. o. ocellatus* that had been described based on a specimen originating from Egypt (Uetz et al. 2023). Only two years later Cetti (1777: 21) described a new lizard from Sardinia in detail and provided the names "Tiligugu" or "Tilingoni". The latter name means "earthworm" in old Sardo and Algherese Catalan. Cetti was apparently unaware of Forskål's earlier publication but he compared his lizard to the diagnoses provided by Linnaeus (1758) for the 43 species included in the genus *Lacerta*. Cetti (1777) initially excluded all lizards with a compressed tail ("cauda compressa") followed by those species those with a ringed tail ("cauda verticillata"). The last two "classes" according to Linnaeus (1758) contained lizards ("lacertae") with a round, smoothly scaled tail, namely species with a tail longer than the body and those with a tail shorter than the body. Cetti's specimen had a short tail in comparison to the body length and was similar in general appearance to *Lacerta scincus* Linnaeus, 1758 (now *Scincus scincus*). Cetti (1777) concluded that his species was related to the apothecary / medicinal skink ("medicinale

scinco") but not identical with it. Cetti went on to make further comparisons between his specimen and *Lacerta scincus* to proof that his species was new. In a way he followed the current rules of nomenclature prescribed in Article 13.1.1 of the International Code of Zoological Nomenclature (ICZN 1999. "the Code") that requires that new names need to include "a description or definition that states in words characters that are purported to differentiate the taxon". However, Cetti's (1777) work did not apply the Linnean (1758) binominal nomenclature consistently and therefore the name was not made available according to Art. 11.4 of the Code.

In the 13th edition of Linnaeus' Systema Naturae edited by Gmelin several new species in the class Amphibia were introduced, one of which was *Lacerta tiligugu*. Gmelin (1789: 1073) provided a short diagnosis that referred to Cetti (1777): "L.[acerta] cauda tereti mediocri conica, digitis pedum 5 marginatis, totidemque unguiculis. Cetti. anfib. di sard. p. 21. Habitat in Sardinia, 8 pollices longa. Corpus crassum, supra fuscum punctis nigris dense aggregatis varium, subdus albidum; pedes brevissimi, posteriores longiores, cauda 3 1/2 pollices lon-

ga.". [L. [acerta] with a smooth conical tail, 5 toes, and as many claws. Cetti. anfib. di sard. p. 21. Inhabits Sardinia, 8 inches long. Body thick, above brown with densely aggregated black dots variegated, underneath whitish; extremities very short, hind legs longer, tail three and a half inches long.]

Gmelin (1789) did not have a specimen at hand and only repeated a set of characters he had extracted from Cetti's (1777) work. As Gmelin's work consistently employed binominal nomenclature, Gmelin (1789) is considered as the original author of the taxon *Lacerta tiligugu* in the herpetological literature. However, an earlier, widely overlooked publication by Hermann (1783) had already mentioned "Lac.[erta] tiligugu" briefly. Hermann (1783: 263) provided a very short comparison between *Lacerta scincus* Linnaeus, 1758 and *L. tiligugu*: "Namque in Lac. Scinco jam pedes sunt perbreves, breviores in Lac. Tiligugu (o), qui in octopollicaris animali quinque aut sex lineis non sint longiores. [Already in Lac. scincus the legs are very short, [but even] shorter in Lac. Tiligugu (o), where they are not longer than five or six lines in an eight-inch animal]".

The (o) refers to a footnote on the same page where Hermann referenced Cetti's (1777) publication: "Nova scinco accedente specie, descripta a Cetti. Anfibi de Sardegna p.21. tab. 1. [A new species of skink, described by Cetti. Anfibi de Sardegna p.21 tab. 1.]". Hermann (1783), in contrast to the later work by Gmelin (1789), additionally refers to an illustration in Cetti (1777). This illustration (Fig. 1) clearly shows a specimen with a short, round tail and the general appearance of a scincid lizard. While the body proportions are reasonably accurate, the length of extremities are clearly exaggerated if it comes to scincid lizards inhabiting Sardinia. In a footnote Cetti (1777: 27) heavily complained about the illustrator and assumed that he altered the illustration intentionally, even employing the word "vizioso [vicious]": "allungamento tanto piu vizioso, che altera l'animale apunto in uno de suoi principali caratteri, che è la grandissima brevità delle sue gambe [the enlargement (of the extremities) is all the more vicious, as it alters the animal precisely in one of its main characteristics, which is the very shortness of its legs]".

Hermann (1783) not only antedates Gmelin (1789), the publication also consistently applied Linnean binominal nomenclature and is therefore an available work in accordance with Art. 11.4 of the Code. Despite the very short and ambiguous description of *Lacerta tiligugu*, Hermann's reference to Cetti's (1777) description and illustration qualifies as an indication in the sense of Art. 12.2.1: "a bibliographic reference to a previously published description or definition even if the descrip-
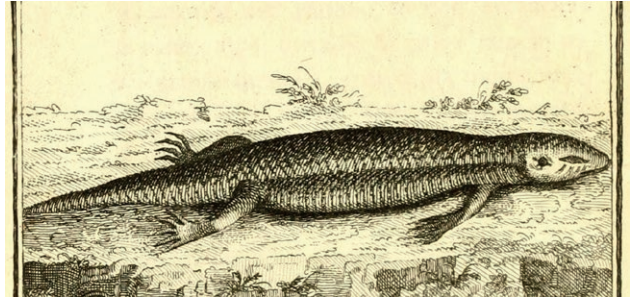

Fig. 1. The original illustration of the "Tiligugu" from Cetti (1777).

tion or definition is contained in a work ... that is not consistently binominal", and Art. 12.2.7: "the proposal ... of a new species-group name in association with an illustration of the taxon being named, or with a bibliographic reference to such an illustration". Consequently, the specimen depicted in Cetti (1777) on pl. 1 preceding p. 21 constitutes the holotype by monotypy of *Lacerta tiligugu*. In agreement with the mentioned articles of the Code Hermann (1783) made the name *Lacerta tiligugu* nomenclaturally available. Intuitively, Hermann should not be seen as the original author of the taxon. He did not have a specimen at hand and relied completely on Cetti's earlier description just repeating some of the characters provided by Cetti (1777) and translating those in a shortened form into Latin: "Otto pollici incirca ha questo animale di lunghezza dalla estremità del muso a quella della coda" (Cetti 1777); "octopollicaris animali" (Hermann 1783). "Il tiligugu ha cortissime gambe ..., le anteriori sono appena lunghe cinque linee, le posteriori sono appena lunghe linee sei" (Cetti 1777); "quinque aut sex lineis" (Hermann 1783).

Hermann (1783) even used the name "tiligugu" provided by Cetti (1777) as the species epithet. However, in such cases Art. 50.1.1 of the Code (ICZN 1999) prescribes that "if it is clear from the contents that some person other than an author of the work is alone responsible **both** [emphasis by the author] for the name or act and for satisfying the criteria of availability other than actual publication, then that other person is the author of the name or act." Consequently, Cetti cannot be seen as the author because he did not use a Linnean binomen such as "*Lacerta tiligugu*" although this could be implied through his comparison with the taxa included in *Lacerta* by Linnaeus (1758). In line with currently accepted taxonomy (Kornilios et al. 2010) the correct nomenclatural notation for this Sardinian and North African scincid lizard subspecies therefore needs to be *Chalcides ocellatus tiligugu* (Hermann, 1783).

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Cover: *Epidalea calamita*, amplexus. Hinojos, Huelva province, Spain. Photo by Iván Gomez-Mestre.

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