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Microhabitat segregation of two coexisting tadpole species on Emei Mountain

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Abstract. Understanding mechanisms determining the coexistence between different species is one of the key issues in community ecology and biodiversity conservation. Microhabitat segregation is a way for species to coexist, which reflects the specific habitat selection of coexisting species in a finer spatial scale. Despite quantitative studies have been conducted to investigate the microhabitat segregation of coexisting species, this type of studies was not often performed on tadpoles. In this study, we assessed the habitat selection of two coexisting tadpoles (*Quasipaa boulengeri* and *Leptobrachium boringii*) in a stream on Emei Mountain, China. Our results demonstrated that *L. boringii* and *Q. boulengeri* tadpoles occupied different microhabitats. Specifically, *Q. boulengeri* tadpoles preferred deep, narrow, and weak acid stream segments with slow current velocity and low value of conductivity, while *L. boringii* tadpoles tended to occur in a wide, shallow water bodies with relatively higher pH, conductivity, and current velocity. Overall, our study supported the Hutchinson's niche concept, showing that at least one dimension of niche differentiation (i.e., microhabitat) occurred between coexisting tadpole species.

Keywords. Microhabitat selection, species coexistence, niche differentiation, environmental variables.

INTRODUCTION

Understanding mechanisms determining the coexistence between different species is one of the key issues in community ecology and biodiversity conservation (Adler et al., 2010; Hanane, 2015). Previous studies indicated that coexisting species should occupy specific ecological niche (e.g., microhabitat niche, trophic niche; Grinnell, 1917) to evade competition based on the limiting similarity theory (Macarthur and Levins, 1967). As ecological niche is an *n*-dimensional ecological space satisfying all the essential conditions that support the organisms (Hutchinson, 1957), coexisting species exhibit at least one dimension of niche differentiation in the same ecosystem (Caceres and Machado, 2013; Hanane, 2015). This phenomenon can be referred to as niche partitioning involved in several facets, such as temporal or spatial distribution, as well as the trophic habits (Baker and Ross, 1981; de Andrade et al., 2014; Schoener, 1974). Therefore, Hutchinson's niche concept primarily focuses on habitats and resources utilization of sympatric species, as well as their environmental tolerances (Rosenfeld, 2002). Accordingly, the segregation of habitats can be considered as one of the important niche partitioning forms allowing the coexistence of species (Melo et al., 2013; Schoener, 1974; Wei et al., 2000). For instance,

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Guo et al. (2012) indicated that two species of goby fish (*Rhinogobius giurinus* and *Rhinogobius cliffordpopei*) were introduced into Lake Erhai, China in 1961. They eventually had to adapt to a different ecological niche in order to coexist in the ecosystem.

At a mesohabitat scale, habitat segregation is usually tested in physiognomically homogeneous units (Heggenes and Saltveit, 2007; Kano et al., 2013; Rezende et al., 2010). However, when focusing on species at a finer spatial scale, habitat segregation is usually assessed by measuring a set of environmental parameters (Rincon, 1999). This can reflect exact habitat utilization of coexisting species in the same ecosystem, particularly in the aquatic ecosystems (Jackson et al., 2001; Jorgensen, 2004; Leger et al., 1983; Leitao et al., 2015). For instance, two turtle species coexist in freshwater streams in Southwest of Iberian Peninsula due to their divergence in habitat selection. Specifically, the European pond turtle (*Emys orbicularis*) shows a preference for temporary, shallow, well vegetated, and sandy stream segment, while the Mediterranean pond turtle (*Mauremys leprosa*) tends to occupy deeper stream segment with more rocks (Segurado and Figueiredo, 2007). Similar situation is also observed in two coexisting fish species in Anizacate River. In this ecosystem, current velocity is the key factor that facilitates the coexistence of the stream catfish (*Trichomycterus corduvense*) and the eel catfish (*Heptaterus mustelinus*), who prefers the high-velocity and the low-velocity water flows, respectively (Hued and Bistoni, 2006). Despite quantitative studies have been conducted to investigate the microhabitat segregation of coexisting species in different ecosystems (e.g., invertebrates, Mammola et al., 2016; fish, Horinouchi, 2008; Kessler and Thorp, 1993; and amphibian adults, Ayala et al., 2018), empirical studies are still relatively rare in anuran tadpoles.

Tadpoles can exhibit plasticity in terms of functional traits (Jordani et al., 2019; Zhao et al., 2019), behavior (Freitas et al., 2019; Zhao et al., 2019), and metabolism (Freitas et al., 2019; Wang et al., 2019) in response to environmental change. In addition, tadpoles could influence the composition and abundance of plankton and periphyton, which has cascading effects on primary productivity in aquatic ecosystems (Alford and Wilbur, 1985; Seale, 1980; Strauss et al., 2010). More importantly, tadpoles are the larval stage of amphibian adults, which face multiple threats (e.g., habitat loss, climate change, and pollution, Alford, 2011). Therefore, identifying the microhabitat selection of coexisting tadpoles may bring important insights to tadpole biodiversity conservation, and suggest priorities for the improved management of mountain stream ecosystems.

In the present study, we evaluated the habitat selection of two coexisting tadpoles, *Quasipaa boulengeri* and *Leptobrachium boringii* in a mountain stream. Specifically, we first compared the difference of variables of microhabitat occupied by *L. boringii* and *Q. boulengeri* tadpoles. We then explored the distribution pattern of the two tadpoles in the stream. Based on previous studies (e.g., Winston, 1995; Xu et al., 2020), we predict that the coexisting tadpoles of two different species occupy different microhabitat.

MATERIAL AND METHODS

Study area and species description

Field work was carried out in Heilongjiang stream, Qingyinge of Emei Mountain, Sichuan Province, China (Fig. 1). The vegetation of this area is mostly composed by evergreen broadleaved forest. The elevation of this region is about 680m a.s.l., and the weather is characterized by subtropical monsoon climate. The mean annual temperature and the mean annual precipitation is about 17.29 °C and 1555.3 mm (Gu and Li, 2008; Ling, 2005). Several tadpoles belonging to different species were detected during our field work in this stream (e.g., *Megophrys omeimontis*, *Megophrys minor*, *Odorrana graminea*, *Q. boulengeri*, *L. boringii*, *Leptobrachella oshanensis*, and *Odorrana schmackeri*). The dominant species were *L. boringii* and *Q. boulengeri*, which occupied 90% of the proportion of the individuals. We focused on the divergences in habitat selection occurring between these two dominant species, as it would be more physiological than that showing how non-dominant species can coexist (Lyons et al., 2005; Barrett et al., 2008).

Data collection

Based on the distribution of target tadpoles and accessible for the sampling, one kilometer of the segment of the stream was selected as the transect. The transect was fixed and extended through a gorge, with strong variability in its physical variables due to the complex terrain and different vegetation cover rate. We divided this transect into three parts based on the blocking of rocks (i.e., approximate 200m of the upstream transect, 450 m of the medium-stream transect, and 350 m of the downstream transect). Tadpole sampling was carried out after sunset (between 20:30 and 23:00) from 22nd to 27th in August 2018. We searched at the both edges (about 1m from the bank) of the stream where the tadpoles distributed, with one part of one side being sampled per night. Specifically, we divided each side of the transect into 1000 squares (1 × 1 m), and two persons searched these plots of 1 m^2 intensively from downstream to upstream using torch (220 lm). Once the target tadpoles were detected, the related square was recorded as one valid quadrat. After that, all the tadpoles located in the quadrat were collected by sweeping all potential microhabitats for tadpoles (i.e., water column and edge of rocks with and without vegetation) using hand nets (mesh size: 2 mm). We assumed that tadpoles likely did not have chances to move from one side to the other

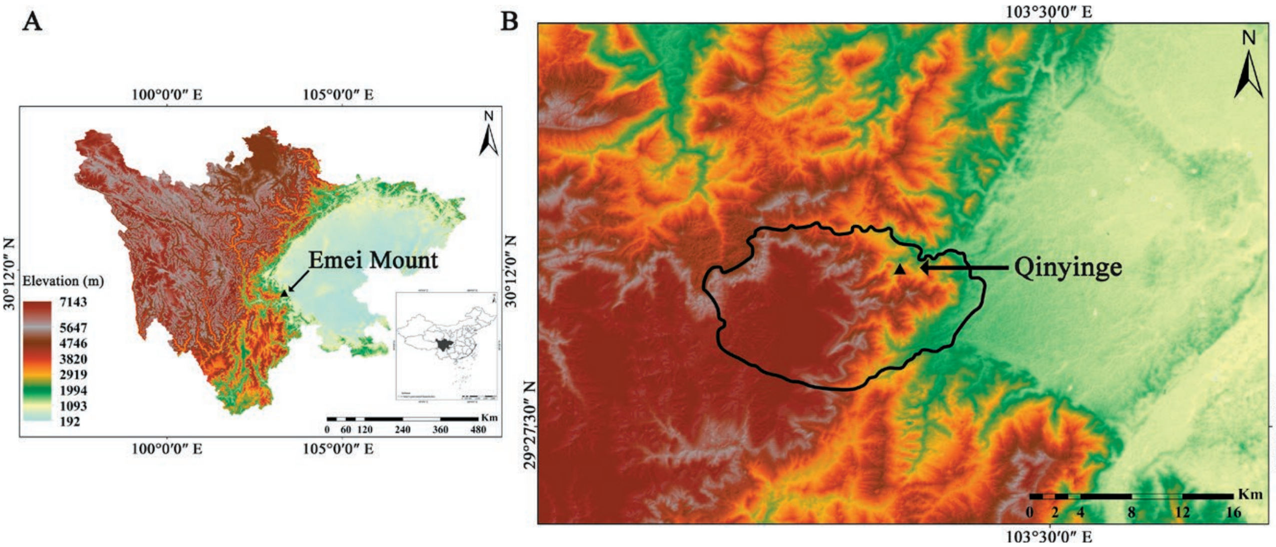

Fig. 1. Geographical location of the study area. The solid line describes the range of Emei Mountain.

of the river, or to cross the rocks limiting the sections during the survey period. The two target tadpoles can be easily identified based on their external morphology. Specifically, there is a light "*Y*" symbol at the back of *L. boringii* tadpole, while *Q. boulengeri* tadpole has flat head and back, thick caudal muscle, and I:4+4/1+1:II labial tooth row (Fei et al., 2012, Fig. S1). Collected tadpoles were kept in 500 ml plastic bottles with freshwater from the stream separately. The stage of each tadpole was determined based on Gosner (1960).

In each quadrat where we found tadpoles, a set of eight environmental factors were measured. Details of the factors and the related measurement approaches are as follows: substrate type was divided into two groups (i.e., gravel and a mix of gravel and humus, associating with different food resources in the quadrats), water temperature was measured to the nearest 0.1 °C by a thermometer (KTJ - TA318, China, Shenzhen), river width was measured to the nearest 0.1 m using a tape meter, the maximum depth of the quadrat was measured to the nearest 0.1 cm with a ruler, current velocity was measured to the nearness 0.1 m/s using a portable current meter (LS1206B, China, Nanjing), pH, conductivity (to the nearest 0.1μS/cm) and dissolved oxygen (to the nearest 0.1 mg/l) were measured using a portable fluorescence photometer (Star A, 520M - 01A, Thermo Fisher Scientific, USA). During the measurements, the researcher remained outside the stream to do not affect the recorded parameters (Ferreira et al., 2015).

Statistical analyses

Shapiro-Wilk test was used to test the normality of the seven environmental variables of microhabitats. In order to compare the difference of variables of microhabitat occupied by *L. boringii* and *Q. boulengeri* tadpoles, we conducted Student's t tests for variables which followed a normal distribution, or Mann-Whitney U test for those were not normal distributed. After that, we used detrended correspondence analysis (DCA) to explore the distribution pattern of the two tadpoles (linear model or single peak model). Considering the DCA axis lengths is less than 3 (i.e., the species distribution was fitted well with the linear model), we finally chose redundancy analyses (RDA) to quantify the environmental determinants of the distribution of the two target tadpoles. All statistical analyses were performed in R version 3.6.1 (R development core team 2020) using the packages *stats*, *spaa*, and *vegan*.

RESULTS

In total, 27 quadrats were sampled and measured for environmental variables, in which 13 quadrats were occupied by *L. boringii* tadpoles, and 14 quadrats were occupied by *Q. boulengeri* tadpoles. These two tadpoles were not detected at the same time in each of the sampled quadrat. We overall captured 74 individuals of *L. boringii* from stage 24 to 37 and 193 individuals of *Q. boulengeri* from stage 25 to 43.

Student's t test indicated that there was no significant difference of dissolved oxygen (t = -1.674, P = 0.107) in the microhabitats that were occupied by *L. boringii* and *Q. boulengeri* tadpoles. Mann-Whitney U tests revealed that pH, conductivity, river width, water depth, and current velocity were significantly different ($P < 0.05$; Fig. 2; substrate type cannot be tested as it was a categorical variable). Specifically, *L. boringii* tadpoles occupied quadrats that had higher values of pH, conductivity, river width, and current velocity, but lower values of water depth. In contrast, *Q. boulengeri* tadpoles occupied quadrats that had lower values of pH, conductivity, river width,

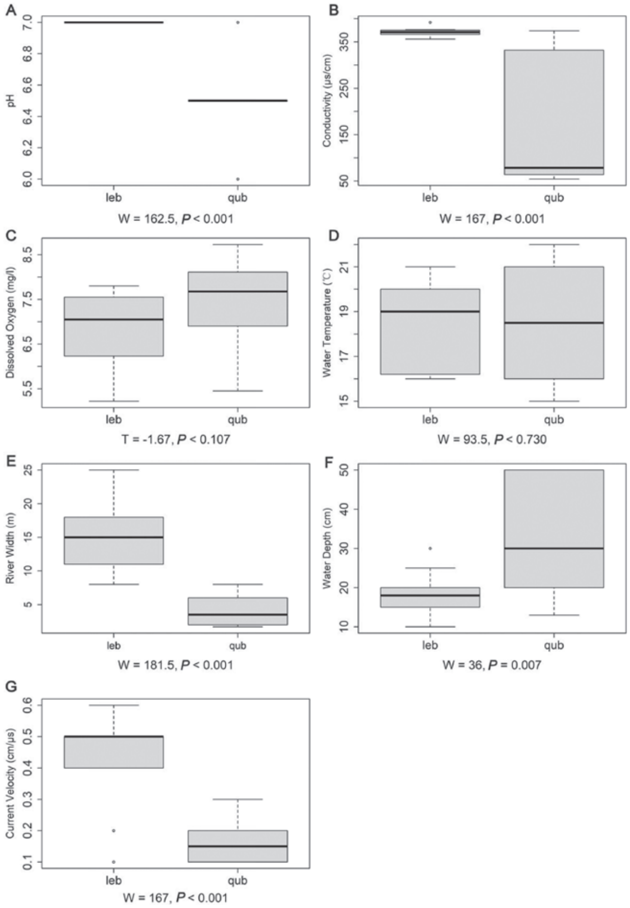

Fig. 2. Comparison of environmental variables of microhabitats between *L. boringii* tadpoles and *Q. boulengeri* tadpoles. “leb”: *L. boringii* tadpoles, “qub”: *Q. boulengeri* tadpoles.

Table 1. The influence of eight environmental factors tested by RDA analyses on the two tadpole species.

Environmental Variables	RDA1	RDA2	r^2	*P*
pH	-0.918	0.397	0.524	**0.001**
Conductivity	-0.968	0.252	0.573	**0.001**
Dissolved oxygen	0.997	0.082	0.139	0.160
Water temperature	-0.650	0.760	0.012	0.869
River width	-0.847	0.531	0.596	**0.001**
Water depth	0.793	-0.609	0.266	**0.014**
Current velocity	-0.737	0.676	0.600	**0.001**
Substrate type	-0.799	0.600	0.632	**0.001**

Significant effects are indicated in bold.

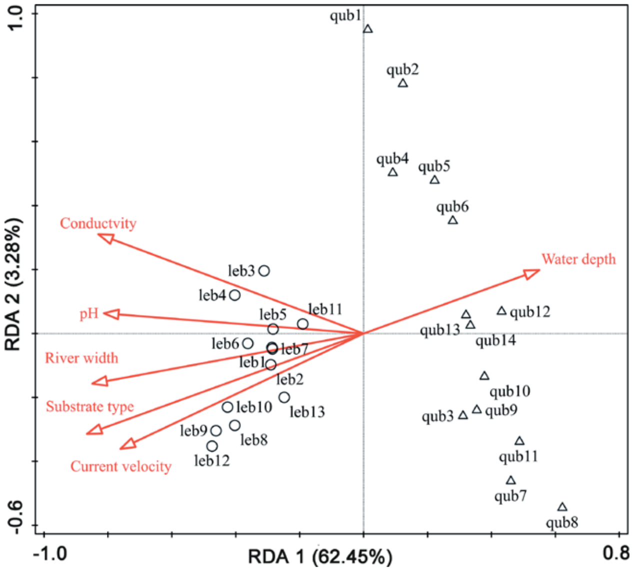

Fig. 3. Redundancy analyses of the relationships between environmental factors and the distributions of model tadpoles. The length of an environmental vector indicates the degree of correlations. Only significant variables are depicted ($P < 0.05$), “leb”: *L. boringii tadpoles*, “qub”: *Q. boulengeri* tadpoles.

and current velocity, but higher values of water depth. In addition, no significant difference existed in terms of water temperature values ($W = 93.5$; $P > 0.05$).

The RDA model was significant when testing the environmental determinants of the distribution of *L. boringii* and *Q. boulengeri* tadpoles ($P = 0.001$). The first two axes accounted for 65.75% of the variation (62.45% and 3.28%, respectively). Our results revealed that five environmental factors including substrate type, river width, current velocity, pH, and conductivity had highly significant effects on the distribution of *L. boringii* tadpoles and *Q. boulengeri* tadpoles ($P = 0.001$, Table 1), and the influence of water depth was significant ($P < 0.05$, Table 1). Obvious dissimilarity of the distribution of *L. boringii* and *Q. boulengeri* tadpoles could be observed in the RDA sequence diagram (Fig. 3). Specifically, most of the quadrats occupied by *L. boringii* tadpoles were distributed in the second and third quadrant densely, which were positively associated with current velocity, river width, pH, conductivity, and substrate type. However, they were negatively correlated with water depth. In contrast, most of the quadrats occupied by *Q. boulengeri* tadpoles exhibited opposite distribution pattern, which were positively correlated with water depth, and negatively associated with current velocity, river width, pH, conductivity, and substrate type.

DISCUSSION

Discrepant preferences for microhabitat utilization on a small scale is often considered to be responsible for the coexistence of sympatric species (Dammhahn and Goodman, 2014; Dammhahn et al., 2013; Escoriza et al., 2018; Wei et al., 2000; Yang et al., 2019). Our results demonstrated that despite *Q. boulengeri* and *L. boringii* tadpoles co-occurred in the same stream, microhabitat segregation existed between them. Specifically, *L. boringii* tadpoles tended to occur in a wide, shallow water bodies with relatively higher pH, conductivity, and current velocity, which was consistent with previous study (Wei et al., 2017). In contrast, *Q. boulengeri* tadpoles were apt to occupy deep, narrow stream segments with low pH, conductivity and current velocity. Therefore, our results indicated that these two tadpoles occupied totally different microhabitat in this stream. Indeed, due to the poor movement ability, tadpoles' microhabitats are largely determined by the selection of breeding habitats by breeding adults (Biesterfeldt et al., 1993). Therefore, the occupation of the microhabitat of the two tadpole species were in accordance with previous observation showing that female *Q. boulengeri* spawned in puddles under the stream waterfall, while female *L. boringii* spawned at streams segments with more rocks and slow water flow (Fei et al., 2012).

It has been recognized that tadpole functional traits were correlated with their selection of microhabitats in water bodies (Fatorelli et al., 2015; Glos et al., 2017), as well as other factors (e.g., predatory occurrence and strategy, Mogali et al., 2020). Based on our previous study (Xu et al., 2020), these two tadpole species have distinct phenotypic functional traits, which also reflected their adaptation of different environment in the water bodies (Zhao et al., 2017). Specifically, *Q. boulengeri* tadpoles have flattened bodies and stubby tails, associating with their selection of deep and slow flowing water bodies. *L. boringii* tadpoles have long and muscular tails, which can be useful for them to swim when water velocity is high. These external functional traits reflect the food acquisition and locomotion of tadpoles, which are critical for them to obtain nutrients to survive, and to facilitate the movement in water bodies (Schoenfuss and Blob 2007). In the present study, substrate type was one of the main environmental factors determining the distribution of the two tadpoles, which probably related to their feeding preferences. That is, *Q. boulengeri* tadpoles tended to choose a substrate with gravels which can provide them adherent algae and benthos. In contrast, *L. boringii* tadpoles preferred a mixture of humus and gravels where organic detritus and invertebrates are more abundant. However, more work such as stable isotope analyses are needed to verify our inferences.

Overall, the present study evaluated the microhabitat segregation of two sympatric tadpole species in a mountain stream. Our observations supported Hutchinson's niche theory demonstrating that the existence of at least one dimension of niche differentiation between coexisting species. Therefore, specific microhabitat features should be incorporated into the conservation strategies for different species. Beyond our studies, future work could focus on the roles that coexisting species played in communities and ecosystems. Furthermore, as mountain streams are vulnerable to anthropogenic disturbance, how increasing fishing pressure on tadpoles from tourists may affect ecosystem functioning can be also tested.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <htttp://www.unipv.it/webshi/appendix> manuscript number 9758

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Morphometric differentiation and sexual dimorphism in *Limnomedusa macroglossa* (Duméril & Bibron, 1841) (Anura: Alsodidae) from Uruguay

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Abstract. Intersexual morphological differences within a species occur in many traits, including body size and shape. Many processes that cause geographic variability in morphology have been proposed: population structure, phenotypic plasticity (environmental effects on development), and natural and/or sexual selection. Several hypotheses can explain patterns of sexual dimorphism in anurans, including natural or intra/inter-sexual selection, and differences in life history strategies between sexes. *Limnomedusa macroglossa* is considered a habitat specialist restricted to rocky outcrops in Brazil, Argentina, Paraguay, and Uruguay. We evaluated the extent of sexual (size and shape) dimorphism in *L. macroglossa* from Uruguay based on morphometrics and secondary sexual characteristics, while taking into account geographic variation. Sexual dimorphism in body size of adults was found, but multivariate analyses did not demonstrate the existence of significant differences in shape. There were also significant differences in body size and hind leg measurements among six hydrographic basins as a result from the phenotypic plasticity correlated with local temperature, representing a clinal variation along the latitudinal gradient of Uruguay. The sexual dimorphism found in body size is probably the consequence of higher growth rates and/or late sexual maturity in females, which favors larger body size for accommodating larger ovaries, and thus, higher reproductive output.

Keywords. Sexual dimorphism, clinal variation, morphometrics, *Limnomedusa macroglossa*, Uruguay.

INTRODUCTION

Morphology is one of the main components of the phenotype that can be studied through qualitative as well as quantitative characteristics. In particular, morphology can be assessed via morphometrics to quantitatively describe, analyze and interpret morphological variation within and between species (Kaliontzopoulou, 2011; Rohlf, 1990). Morphological quantitative traits are usually polygenically inherited and show considerable plasticity in relation to environmental factors (Babik and Rafinski, 2000). Furthermore, plasticity can lead to geographic variability in morphology. In that sense, many processes have been proposed, such as: biogeographical barriers that partially isolate populations, effects of environmental parameters (precipitations and temperature) on growth rates, and action of sexual selection resulting in sexual dimorphism (Schäuble, 2004). Body size is a strongly plastic morphological trait (Green, 2015) fundamental in physiological and ecological contexts. Traditionally, snout-vent length (SVL) has been used as the gold standard to measure body size in frogs (Kupfer, 2007). Among anurans, analyses of intraspecific geographical variability in morphology have often revealed extensive variation in body size (Schäuble, 2004). Due to the limited dispersal ability and high philopatry in frogs, it is common to find

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intraspecific differences in morphology among geographically separated populations, particularly in body size, caused by genetic divergence among isolated populations (Baraquet et al., 2012; Castellano et al., 2000). In addition to geographic distance, landscape features could account for spatial morphological variation. For instance, hydrographic basins could act as physical barriers promoting isolation and spatial structuring among populations as a result of changes in altitude, slope and landscape features among basins. Moreover, climate and food availability may also vary geographically, leading to differences in the ability to grow, resulting in morphological variation (Lovich and Gibbons, 1992; Hartmann, 2016).

Another source of intraspecific variation could be sexual dimorphism; the occurrence of morphological differences between individuals of different sex within a species, may affect several traits like body size, shape and sometimes, secondary sexual characteristics (Wells, 2007). Several factors can influence sexual dimorphism including female reproductive strategy, sexual selection, and competition for resources (Fathinia et al., 2012). Sexual dimorphism may have important consequences for animal ecology, and is a key aspect for understand the evolution of life history traits (Kupfer, 2007). In particular, sexual size dimorphism (SSD) is defined as the difference in body length or mass of sexually mature males and females (Fairbairn, 1997; Kupfer, 2007; Nali et al., 2014). Several evolutionary processes have been proposed to explain patterns of sexual dimorphism in anurans. On one hand, the usually biased SSD in favor of females (Shine, 1979) is hypothesized as the result of a fecundity advantage driven by natural selection: bigger females can harbor more eggs, and then produce larger clutches (Arak, 1988; Wells, 2007). Whereas in males, natural selection operates against of bigger body sizes, because higher vulnerability of prolonged breeders to predators increase their cost of reproduction in terms of survival at small body sizes (Camargo et al., 2008). On the other hand, some authors argue that sexual dimorphism is a consequence of sexual selection. In this sense, Darwin envisioned that sexual selection depends on the struggle between males to access females, and recognized two mechanisms: intrasexual selection, through competition between members of the same sex (usually males) for access to mates, where large males defeat small ones in aggressive encounters and displace them from territories; and intersexual (epigamic) selection, where members of one sex (usually females) choose members of the opposite sex, by comparing traits of potential mates and select those that are more attractive (Darwin, 1871; Shine, 1979; Woolbright, 1983; Arak, 1988; Lovich and Gibbons, 1992). However, some authors proposed that sexual dimorphism is a function of differences in life history strategies between the sexes, as well as the result of a variety of selective forces. In this sense, SSD can be explained in terms of disparate age structure between sexes in reproductive populations; that is, females were larger because they were older than the males, which mature earlier at smaller size. In fact, Monnet and Cherry (2002) found that age differences between breeding males and females appear to have a major influence on the extent of dimorphism. Female anuran fecundity appears to be correlated with body size in all anuran species in which this phenomenon has been investigated, and, as anurans display indeterminate growth (Halliday and Verrell, 1986), this could be expected to lead to faster growth rates and delayed reproduction in females (Monnet and Cherry, 2002).

Limnomedusa Fitzinger 1843, is the most basal genus within the family Alsodidae (Frost et al., 2006; Pyron and Wiens, 2011). The only species of the genus, “rapids frog” *Limnomedusa macroglossa* (Duméril and Bibron, 1841), is a generalist insect predator of medium to large size, with shades of brown-and-gray and conspicuous glands in the back, and an immaculate white belly (Maneyro and Carreira, 2012). As secondary sexual characteristics, males present a single vocal sac and dark nuptial pads on their fingers. It is a habitat specialist, with a restricted distribution in rocky outcrops of basaltic origin and superficial soils, with or without vegetation (Maneyro and Carreira, 2012). Regarding its geographic variation in Uruguay, larval dispersion appears to be connecting separate major basins via watercourses, although it is also likely that adults disperse between habitat patches by land. As a corollary, an isolation pattern by distance was established, which maintains population stability and genetic diversity in northern populations (Fernández, 2016).

Recently, de Olivera et al. (2018) found a correlation between body size and ovarian mass in populations of *Limnomedusa macroglossa* from Uruguay, suggesting a fecundity advantage for larger females since they can accommodate larger ovaries. Moreover, they also reported a prolonged pattern of reproduction for this species, which is usually associated with higher levels of intra/inter-sexual selection (Wells, 2007). Further, in populations from Rio Grande do Sul state, SSD has been found, where females attain larger SVL than males, and they also classified the pattern of reproduction as prolonged, although highly seasonal (Kaefer et al., 2009).

Its geographic distribution includes the south of Brazil (from Paraná to Rio Grande do Sul), the northeast of Argentina (Misiones and Entre Ríos), the southeast of Paraguay (Alto Paraná), and almost the entire Uruguayan territory (Frost, 2020). However, despite being a relatively common species, geographical variation in morphology has not been investigated in *L. macroglossa* overall distribution. This circumstance is relevant since most of the distribution range occurs in Uruguay, and thorough evaluation of the morphological variation across such distribution is necessary given its latitudinal, environmental gradient. Lastly, in reference to its conservation status, is categorized nationally and globally as Least Concern according to the IUCN criteria (Silvano et al., 2004; Maneyro et al., 2019).

The aim of this work was to evaluate the occurrence of sexual (size and shape) dimorphism in *Limnomedusa macroglossa* based on morphometrics and secondary sexual characteristics across populations from Uruguay.

We hypothesized that:

(1) Sexual dimorphism and minimum size at sexual maturity (MSSM) are important life history traits due of their value in reproductive output of a species. Besides, most anuran females have larger body sizes than males (female biased SSD) and, thus, females usually reach sexual maturity at larger sizes.

(2) Isolation pattern by distance triggered by hydrographic basins favor geographical differences in morphology.

From which the following predictions emerge:

(1.1) we expect that *L. macroglossa* present sexual size dimorphism with females larger than males, and in fact, with females reaching MSSM at bigger sizes than males.

(2.1) Finally, hydrographic basins, due to environmental differences, will favor a greater morphological differentiation in *L. macroglossa* between than within basins.

MATERIALS AND METHODS

Field data collection

We hand-captured 180 individuals of *Limnomedusa macroglossa* between January 2012 and March 2015, of which 102 were juveniles, 34 mature females and 44 mature males. The individuals were collected along a latitudinal gradient of six hydrographic basins from Uruguay (based on Achkar et al., 2013): Río Uruguay (7 females and 8 males), Río Santa Lucía (3 females and 4 males), Océano Atlántico (2 males), Laguna Merín (3 females and 1 male), Río de la Plata (6 females and 6 males), and Río Negro (11 females and 14 males) (Fig. 1) (see Appendices 1, 2 and 3). Latitude and longitude location data of these individuals were obtained from a GPS (Garmin, eTrex 20). In addition, 13 individuals not georeferenced (4 females and 9 males) were also used for SSD and SMA analyses. Lastly, juvenile individuals were used in another investigation (Fernández, 2016).

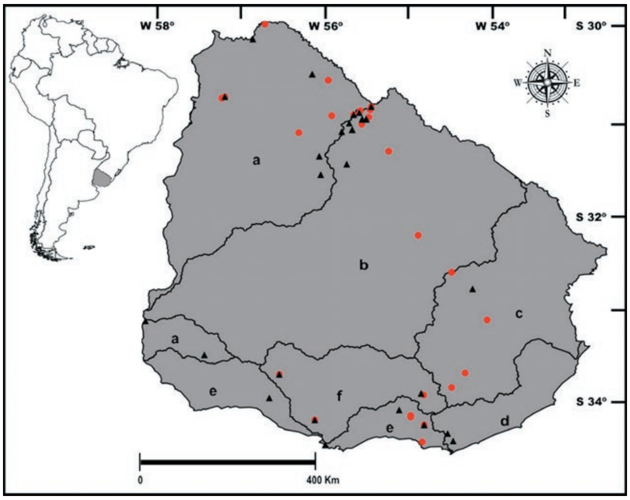

Fig. 1. Maps of South America and Uruguay showing basins where *Limnomedusa macroglossa* was sampled for analyses of geographic variation in sexual dimorphism and morphometric differentiation. Names of sampling basins are as follows: a = Río Uruguay, b = Río Negro, c = Laguna Merín, d = Océano Atlántico, e = Río de la Plata and f = Río Santa Lucía. Black triangles are males and red circles are females (based on Achkar et al., 2013).

All collected individuals were euthanized using topic lidocaine and intraperitoneal injection of sodium pentobarbital (0.5 ml of a 0.2 g/ml solution), fixed with 10% formaline, and preserved in 70% ethanol, following the experimental protocol "Euthanasia method for amphibians and reptiles in the field" approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Science, University of the Republic. Individuals were euthanized with the purpose of being genetically studied by Fernández (2016), therefore in this work, those individuals were reused. All the specimens are stored in the Vertebrate Zoology Collection (ZVC-B) of the Faculty of Sciences, University of the Republic.

We measured eleven morphometric variables using a digital calliper (0.01 mm precision) by a single observer for consistency (Grenat et al., 2012): snout-vent length (SVL), mandibular width (MW), head length (HL), inter-orbital distance (IOD), eye diameter (ED), inter-narial distance (IND), eye–nostril distance (END), arm length (ARML), tibia length (TiL), tarsus length (TaL) and metatarsus length (MtL). We followed the methodology of Duellman (1970) to obtain the measurements of SVL, IOD, ED, IND, TiL, and MtL, as well as Napoli (2005) for END, and Greene and Funk (2009) for ARML. Finally, we measured TaL as the straight length of the tarsus, MW as the straight line between oral commissures, and HL as the straight line distance from the posterior edge of the skull to the tip of the snout. All individuals were measured twice to ensure accuracy and all measurements were taken on the right side of the body (Fig. 2).

For each individual, sex and maturation status (juvenile/adult) were determined by gonadal analysis. Additionally, males were considered mature by the presence of nuptial pads in their fingers. Finally, to infer MSSM on each sex, we pooled all individuals from all basins and register the size of the adult male/female with the lowest SVL.

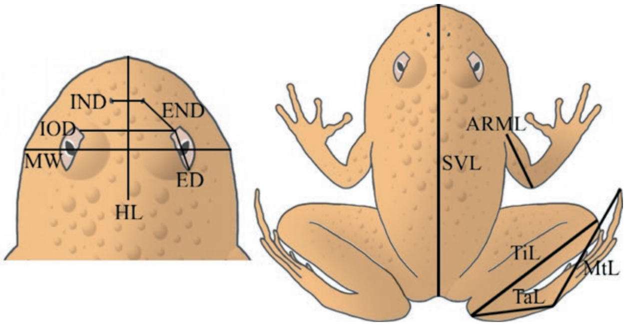

Fig. 2. Morphometric measurements used for the analysis of sexual dimorphism in *Limnomedusa macroglossa* (Anura: Alsodidae) from Uruguay: SVL = snout–vent length; MW = mandibular width; HL = head length; IOD = inter-orbital distance; ED = eye diameter; IND = inter-narial distance; END = eye–nostril distance; ARML = arm length; TiL = tibia length; TaL = tarsus length and MtL = metatarsus length.

Data analysis

Using the morphometric variables, we tested for sexual dimorphism and quantified morphometric variation through univariate and multivariate analyses while taking into account geographic distribution.

To remove allometric effects of body size in the sexual dimorphism analyzes we applied the transformation proposed by Lleonart et al. (2000), which scales all individuals to same size and adjust their shape to that they would have in the new size.

For all the variables we tested the normality (Lilliefors' test) and homogeneity of the variance (Levene's test) of raw and transformed data. A priori, the raw data did not reject the hypotheses of normality neither homogeneity of variances. Although, with the transformed data, there were rejected. No outlier individuals were found in the analyzed sample.

We performed a t-test to evaluate for a significant difference in SVL between males and females. Sexes were also compared through one-way perMANOVA using Euclidean similarity index. As differences in body size between sexes are not always related to SVL and can involve body parts used in various behavioral contexts (Kupfer, 2007), we conducted multivariate analyses. Differences in shape between males and females were examined through a Principal Component Analysis (PCA) using the Variance-Covariance matrix, and a Hierarchical Cluster with Unweighted Pair-Group Average algorithm and Euclidean similarity index with 9999 pseudoreplicates.

SVL, MtL and TiL variables were log-transformed to estimate standardized mayor axis (SMA) regression slopes. This method estimates the line of best fit (slope) when both variables are measured with error (Falster et al., 2006; Warton et al., 2006). We estimated the SMA relationship between SVL and MtL/TiL. We tested for significant allometry assuming the null hypothesis that the slope was equal to 1 (isometry), performed slope comparisons between sexes, tested for shifts along the common SMA slope and in elevation of slope between sexes using Wald test, with 1000 iterations and critical P-value to 0.05.

To evaluate morphometric variation, we analyzed differences among basins by one-way perMANOVA (using Bonferroni correction for P-values) and PCA, based on raw measured variables, because our goal was also to evaluate the effect of body size, and we box plot SVL, TiL and MtL variables according to basins. In addition, we calculated the average leg length (= TiL + TaL + MtL) among individuals belonging to each basin. Finally, we tested for significant differences in the leg length and SVL among basins through t-test. The latitude vs. SVL relationship was evaluated through regression analysis (using Reduced Major Axis algorithm). In these analyses we used 35 mature males and 30 mature females because coordinate data were not available for all individuals.

We used the freely available online programs PAST 3.21 (Hammer et al., 2001), GNUMERIC 1.12 (The Gnome Project, 2018), SMATR 2.0 (Falster et al., 2006) and QGIS 18.24 (QGIS Development Team, 2016) for all statistical analyses performed in this work.

RESULTS

Sexual dimorphism

In total, we examined 180 specimens of which 102 were juveniles, 34 mature females and 44 mature males. We found dark nuptial pads in the first, second, and sometimes, the third fingers of all mature males (Fig. 3). We found that females longer than 49.82 mm and males longer than 41.29 mm in SVL were sexually mature (i.e., nuptial pads in males and fully-developed oocytes in females). Taking this into account, we set the MSSM in females at 49.82 ± 0.01 mm and in males at 41.29 ± 0.01 mm.

Significant differences in mature body size were found between sexes. Mature females had an average SVL (56.99 ± 4.27 mm) significantly higher than that of mature males (49.69 ± 4.73 mm) ($t = 7.04$, $P < 0.001$).

Fig. 3. Male displaying dark nuptial pads above fingers of the foreleg (ZVC-B 23281).

Table 1. Descriptive statistics of each morphological variables measured in *Limnomedusa macroglossa* (Anura: Alsodidae) from Uruguay. Morphological differences between sexes were tested for each variable through t test. Sex: ♂ = male, ♀ = female; n: sample size; Min: minimum value; Max: maximum value; x: mean; SE: standard error; SD: standard deviation; Vc: variance coefficient. Variables for which significant differences were obtained are in bold. All measurements are shown in millimeters.

	Sex	n	Min.	Max.	x	SE	SD	Vc.	t test	P value
SVL	♂	44	41.29	60.92	49.69	0.71	4.73	9.51	7.04	**<0.001**
	♀	34	49.82	64.25	56.98	0.73	4.27	7.5		**<0.001**
MW	♂	44	16.61	24.28	19.66	0.29	1.94	9.87	6.56	**<0.001**
	♀	34	19.7	24.47	22.32	0.26	1.54	6.92		**<0.001**
HL	♂	44	14.47	20.98	17.13	0.23	1.53	8.96	6.78	**<0.001**
	♀	34	16.95	22.02	19.46	0.25	1.46	7.51		**<0.001**
IOD	♂	44	7.05	9.82	8.38	0.12	0.79	9.44	5.71	**<0.001**
	♀	34	8.2	10.93	9.4	0.13	0.77	8.15		**<0.001**
ED	♂	44	4.17	7.2	5.45	0.1	0.69	12.59	4.27	**<0.001**
	♀	34	5.07	7	6.05	0.09	0.51	8.51		**<0.001**
IND	♂	44	3.26	5.48	4.22	0.08	0.56	13.22	5.34	**<0.001**
	♀	34	3.69	5.76	4.85	0.08	0.46	9.48		**<0.001**
NED	♂	44	4.01	6.33	5.02	0.08	0.56	11.16	7.32	**<0.001**
	♀	34	4.92	7.05	5.88	0.08	0.45	7.73		**<0.001**
ARML	♂	44	9.93	15.62	12.46	0.19	1.25	10.06	6.1	**<0.001**
	♀	34	11.85	16.73	14.2	0.21	1.25	8.78		**<0.001**
TiL	♂	44	24.7	37.72	30.53	0.49	3.26	10.68	6.43	**<0.001**
	♀	34	30.21	39.86	35.02	0.47	2.76	7.89		**<0.001**
TaL	♂	44	13.57	18.72	16.05	0.22	1.49	9.29	6.93	**<0.001**
	♀	34	15.66	20.65	18.34	0.24	1.39	7.57		**<0.001**
MtL	♂	44	21.82	31.93	26.46	0.41	2.72	10.28	5.59	**<0.001**
	♀	34	25.1	32.85	29.63	0.37	2.15	7.24		**<0.001**

Furthermore, significant differences were found in means of all other variables, with females reaching larger measurements than males (Table 1). When all the morphometric variables were introduced in a nonparametric perMANOVA test, the comparison of sexes was not significant (F = 0.349, P = 0.865).

The PCA of size-adjusted measurements showed a total of ten components, with 69.09 % of the variance comprised by the first two components, with PC1 accounting for 54.14% and PC2 14.95% of the total variation. The bi-dimensional projection of the first two components exhibited a substantial overlap of sexes (Fig. 4A). The loadings indicate that PC1 has a strong positive correlation with TiL (0.61) and MtL (0.58), and the lowest with ED, IND, END and IOD (Fig. 4B), whereas PC2 is moderately correlated with TaL (0.35) and ARML (0.33), while MtL stood out with a very strong negative correlation of - 0.76 (Fig. 4C).

The dendrogram obtained, with hierarchical clustering, was a good representation of the data matrix given the obtained coefficient of cophenetic correlation (CCC = 0.74) and showed a topology of females and males in congruence with PCA and perMANOVA analyses, determining the absence of morphometric, sexual shape dimorphism. However, the low bootstrap percentages (<50%) do not indicate high support for most of the similarity relationships.

Morphometric differentiation

Since multivariate analyses performed previously did not reveal significant differences between sexes, we pooled males and females within each basin in subsequent analyses. When all the morphometric variables were analyzed through perMANOVA test, significant differences were found among the six hydrographic basins evaluated in this work (F = 2.553, P < 0.05). The pairwise comparisons showed significant differences between Río Negro and Río de la Plata basins (Table 2).

The PCA of original measured variables (including SVL variable), showed a total of eleven components, with 96.4% of the variance comprised by the first two components, with PC1 accounting for 93.96% and PC2 2.47% of the total variation. The bi-dimensional projection of PC1 vs. PC2 showed the convex polygons grouping indi-

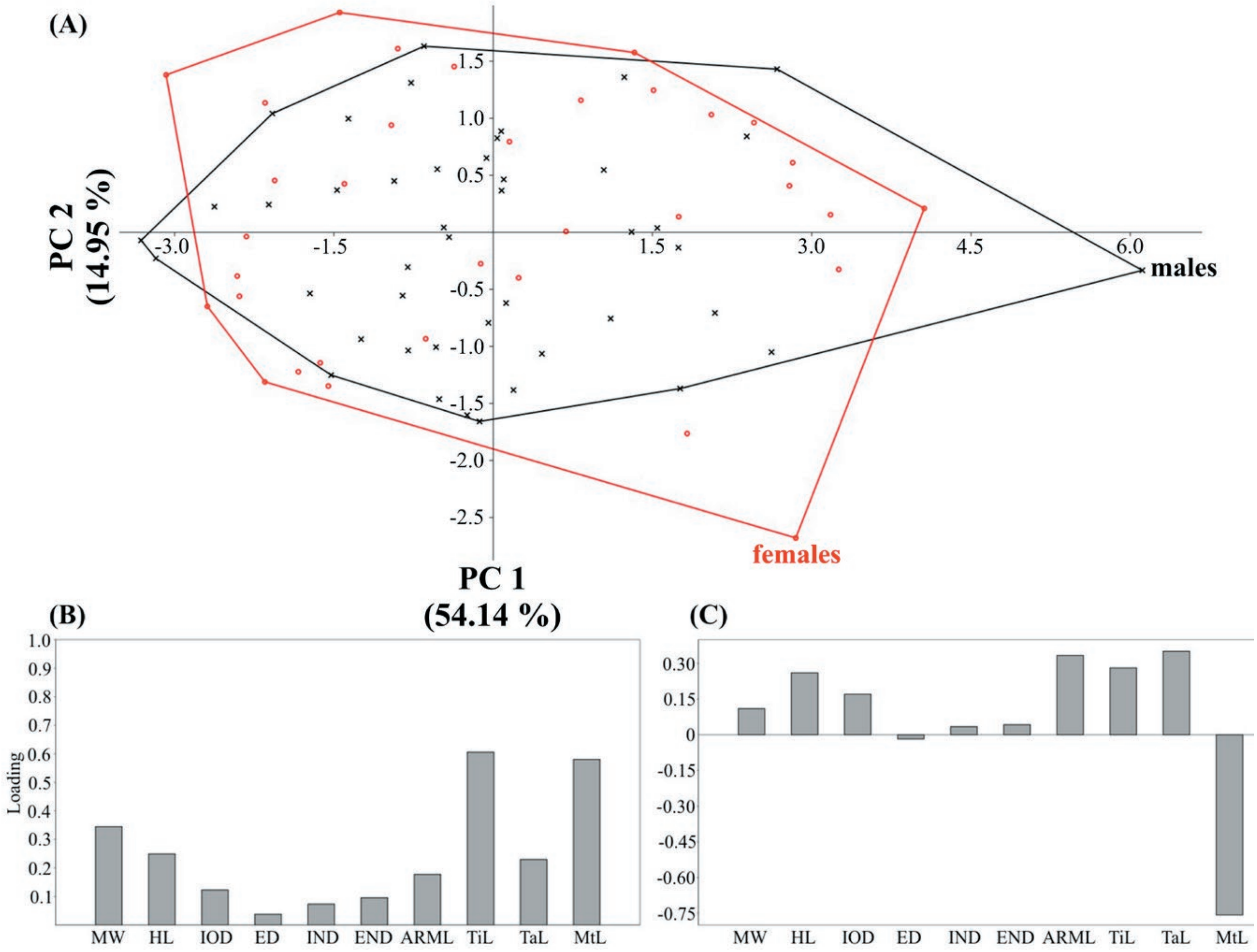

Fig. 4. (A) Scatter plot for the first two principal components obtained from a principal component analysis of eleven morphological variables measured in *Limnomedusa macroglossa* (Anura: Alsodidae) from Uruguay, including convex polygons grouping individuals according to their sex. Red circles represent females and black crosses are males. Coefficients of association of each morphometric variable with the first principal component (PC1) (B) and with the second principal component (PC2) (C). SVL = snout-vent length, MW = mandibular width; HL = head length; IOD = inter-orbital distance; ED = eye diameter; IND = inter-narial distance; END = eye–nostril distance; ARML = arm length; TiL = tibia length; TaL = tarsus length and MtL = metatarsus length.

viduals from different basins with an elevated degree of overlap (Fig. 5A). According to Greene and Funk (2009), in PCA of morphological data, the first axis (PC1) is usually associated with size, and the remaining axes describe orthogonal axes of variation in shape. Indeed, we found that, the first axis has a strong positive correlation with body size and a moderate correlation with a few hind leg measurements: SVL (0.69), TiL (0.45) and MtL (0.33). Meanwhile, head measurements (IOD, ED, IND and END) showed the weakest correlation (Fig. 5B). The second axis (shape axis) has a strong positive correlation with MtL (0.56), and a moderate correlation with MW (0.32) and TiL (0.31), while SVL stood out with a strong negative correlation -0.67 (Fig. 5C).

Given the considerable contribution of SVL, TiL and MtL variables in size and shape axis of PCA, we did a box plot according to the hydrographic basins in order to show the differences between them. Significant differences were found between hind leg length of individuals from Río de la Plata and Río Negro basins ($t = 3.533$, $P < 0.001$), being those of Río de la Plata basin the longest hind leg (82.12 ± 2.21 mm, $n = 12$), while those of Río Negro basin were the shortest legs (72.55 ± 1.55 mm, $n = 25$) (Fig. 6A,B), reaching a difference of 11,65%. Regarding SVL, we found a similar pattern, reaching higher values in Río de la Plata basin (56.31 ± 1.57 mm, $n = 12$) and lower ones in Río Negro basin (50.12 ± 1.06 mm, $n = 25$; $t = 3.302$, $P < 0.002$; Fig. 7A), reaching a difference

Table 2. One-way perMANOVA results for morphometric data of *Limnomedusa macroglossa* from Uruguay taking into account the six hydrographic basins evaluated in this work: Río Uruguay, Río Santa Lucía, Océano Atlántico, Laguna Merín, Río de la Plata and Río Negro. Bonferroni corrected P values are displayed. Basins for which significant differences were obtained are in bold.

	Río Santa Lucía	Río Negro	Laguna Merín	Río de la Plata	Océano Atlántico
Río Uruguay	1	0.462	1	1	1
Río Santa Lucía		1	1	1	1
Río Negro			1	**0.024**	1
Laguna Merín				1	1
Río de la Plata					1

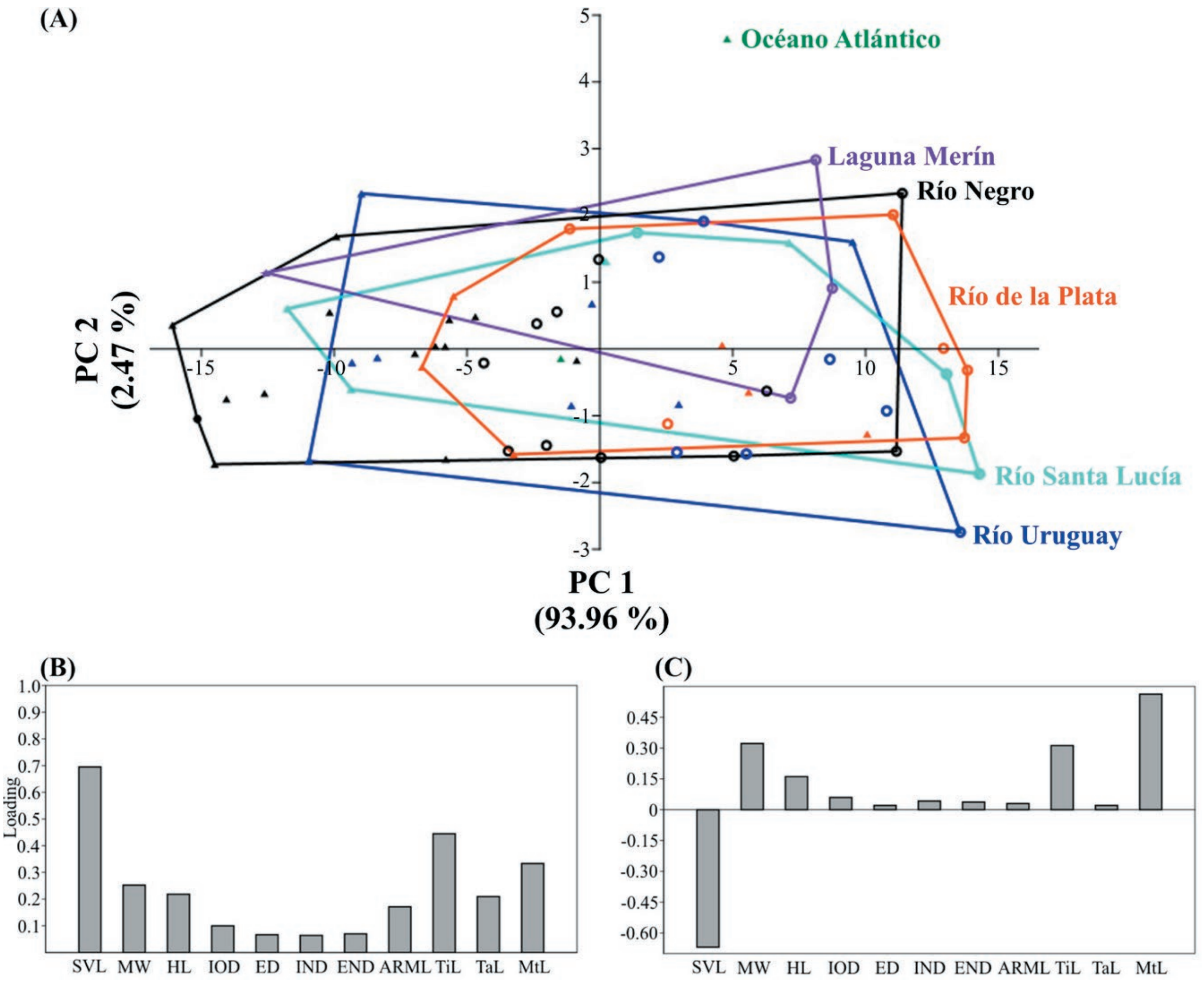

Fig. 5. (A) Scatter plot for the first two principal components obtained from a principal component analysis of eleven morphological variables measured in *Limnomedusa macroglossa* (Anura: Alsodidae) taking into account hydrographic basins from Uruguay, including convex polygons grouping individuals according to basins. Fill triangles represent males and circles are females. Basins: blue = Río Uruguay; sky blue = Río Santa Lucía; green = Océano Atlántico; violet = Laguna Merín; orange = Río de la Plata; black = Río Negro. Coefficients of association of each morphometric variable with the first principal component (PC1) (B) and with the second principal component (PC2) (C) taking into account hydrographic basins from Uruguay. SVL = snout-vent length, MW = mandibular width; HL = head length; IOD = interorbital distance; ED = eye diameter; IND = inter-narial distance; END = eye–nostril distance; ARML = arm length; TiL = tibia length; TaL = tarsus length and MtL = metatarsus length.

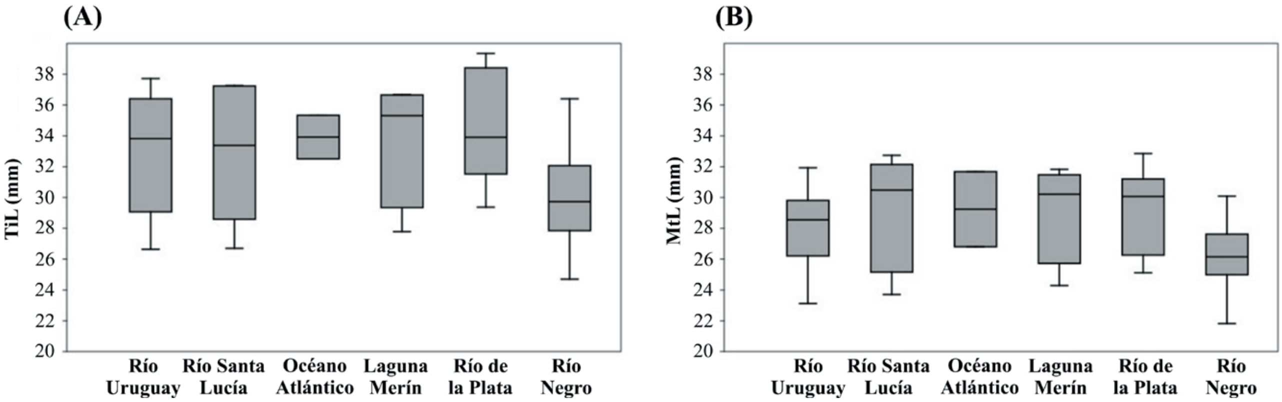

Fig. 6. Box plots of TiL (A) and MtL (B) variables of *Limnomedusa macroglossa* (Anura: Alsodidae) according to hydrographic basins from Uruguay, considering males and females grouped. TiL = tibia length and MtL = metatarsus length. The line inside de boxes represents the median. All measurements are in millimeters.

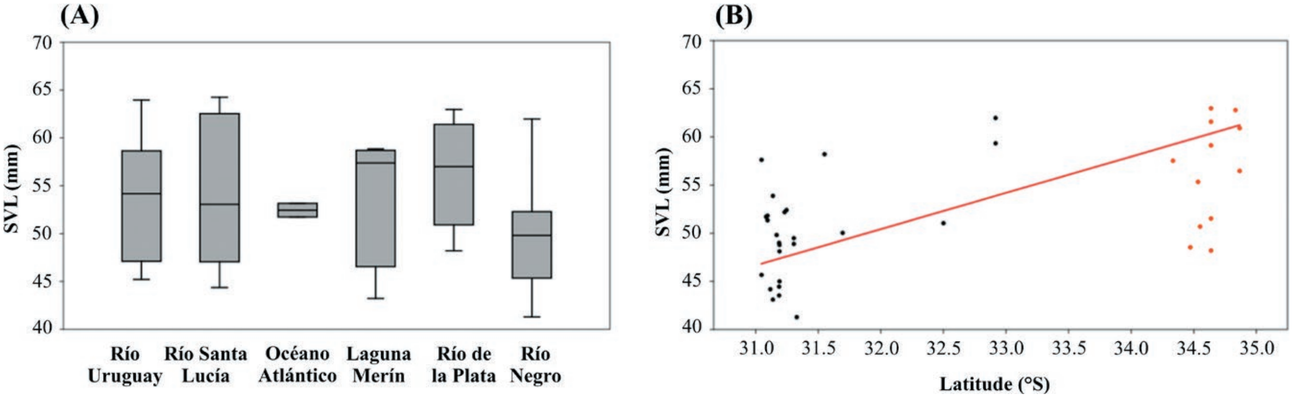

Fig. 7. (A) Box plot of body size (SVL) of *Limnomedusa macroglossa* (Anura: Alsodidae) according to hydrographic basins from Uruguay, considering males and females grouped. (B) Latitude-SVL relationship for *L. macroglossa*. The line represents the regression model. Red circles represent individuals of Río de la Plata basin and black circles are individuals of Río Negro basin. The line inside de boxes represents the median. SVL measurements are in millimeters.

of 11% between groups. Additionally, a significant correlation between latitude and SVL was found (r = 0.60, F = 4.35, $P < 0.001$; Fig. 7B).

Allometric regressions

We performed a SMA analysis with the variables that showed the highest correlations with PC1 and PC2 in PC analyzes. We found a significant SMA relationship between SVL and MtL in females [b=0.98, 95% confidence interval (CI) = 0.78-1.23] and males [b=1.09, 95% CI = 0.94-1.25]. It was also significant between SVL and TiL in females [b=1.06, 95% CI = 0.88-1.27] and males [b=1.13, 95% CI = 1.02-1.25] and between SVL and MW in females [b=0.93, 95% CI = 0.75-1.16] and males [b=1.03, 95% CI = 0.90-1.18]. In males, the SVL vs. TiL relationship showed a significant positive allometry (b=1.128). On the other hand, in all the other cases, there were no significant differences from isometry (Table 3).

In all cases, the test for common slope across sexes indicated that there are no significant differences in common slope between males and females. When testing for shifts along the common slope, we found significant shifts in all relationships with higher values in females (SVL-MtL relationship: W = 42.952, $P < 0.01$; SVL-TiL relationship: W = 47.729, $p<0.01$). The test for shift in elevation was only significant in the SVL vs. MtL relationship in favor of males (W = 4.411, $P < 0.05$), but the difference in elevation was rather small and close to our resolution limit (0.01 mm).

Table 3. Standarized major axis (SMA) regression results and test of isometry for *Limnomedusa macroglossa*. Variables used in analyses were: SVL = snout–vent length; TiL = tibia length and MtL = metatarsus length. Abbreviations: *a* = intercept. Significant regressions are in bold.

Variables	SMA regression			Test of isometry	
	a	r^2	*p*	F	*p*
log MtL vs. log SVL					
Females	-0.2471	0.575	**< 0.01**	0.034	0.855
Males	-0.4193	0.800	**< 0.01**	1.422	0.240
log TiL vs. log SVL					
Females	-0.3117	0.733	**< 0.01**	0.370	0.548
Males	-0.4295	0.891	**< 0.01**	5.620	**0.02**

DISCUSSION

In this study we determined the minimum size at sexual maturity (MSSM) and described morphometric and intersexual differences in *Limnomedusa macroglossa*. We showed that females and males differ in MSSM, presence of dark nuptial pads in males (a sexually dimorphic characteristic) and body size, while no differences were found in body shape. Nuptial pads can be observed during the breeding season in response to increases in circulating levels of androgens, but later regress during the non-breeding period, although without resembling to a female-like morphology (Wells, 2007). Some authors argued that well-developed nuptial pads are associated with breeding in water to prevent the female´s escape during amplexus (Duellman and Trueb, 1986). However, according to Savage (1961), nuptial pads also allow the male to hold the female while defending her against rival males.

The MSSM is the size at which an individual has all the morphological and physiological conditions to begin to breed, and along with sexual dimorphism, are important life history traits to understand population changes through time. Life history theory explains the variation in MSSM between sexes through natural selection mechanisms, mainly related with adult mortality rates (Tolosa et al., 2014). We found that females of *Limnomedusa macroglossa* reach sexual maturity around 49.82 mm, while males reach it at a smaller size of about 41.29 mm. This difference between the sexes can be explained by sexual selection: selection for mating effort in males to defend territories, in detriment of larger males due to the high energetic expenditures and risks of mortality, and parental effort in females to produce more eggs to maximize their reproductive output, which favors females with a larger size; both processes have been pointed out as potential explanations for sexual maturation at different ages (Howard, 1981).

There was sexual dimorphism in size in *Limnomedusa macroglossa* with females being larger than males, as it occurs in more than 90% of anurans species (Shine, 1979). These results agree with those found in a population of *L. macroglossa* in southern Brazil based on SVL only (Kaefer et al., 2009). Taking into account the main hypotheses regarding the causes of sexual dimorphism in anurans, natural and/or sexual selection might adequately explain the differences in body sizes between females and males found in this work. Given the available data until date (Kaefer et al., 2009; de Olivera et al., 2018) and our results, it seems that the preference for larger females evolved because they produce more oocytes per clutch (Bionda et al., 2011) or bigger eggs (the fecundity advantage hypothesis), whereas in males, natural selection works against of bigger body sizes due to the existence of possible differential predation, since the long reproductive period exposes and makes them more vulnerable to predators (Camargo et al., 2008). Furthermore, intra/intersexual selection could be playing an important role in the differentiation between males and females, through male-male competition and/or female choice (Darwin, 1871; Shine, 1979; Woolbright, 1983; Arak, 1988). Although, in our field work, we did not observe such behaviors, we cannot rule out their existence, since it has been reported that its a species with a prolonged reproduction pattern (Kaefer et al., 2009; de Olivera et al., 2018) which is usually associated with more territorial males, choosy females, and overall higher levels of sexual selection (Wells, 2007). Finally, the age structure in the reproductive populations may also be operating between sexes (Halliday and Verrell, 1986; Monnet and Cherry, 2002). Thus, the sexual dimorphism in size found in *L. macroglossa* could be the result of distinct, possibly opposing, selective forces that trade-off differently in each sex.

In addition to size, anurans exhibit other forms of sexual dimorphism, including: the proportions and muscular development of the forelimbs (related with clasping behavior), skin color, texture and glands (visual, tactile and chemical cues for sex recognition), fangs and tusks (related with combat), abdominal and laryngeal muscles, and lung capacities (calling behavior) (Wells, 2007; Bell and Zamudio, 2012) and head morphology (feeding strategies) (Khoshnamvand et al., 2018).

No differences were found in shape between sexes, but significant differences were found among basins. Some variables related with the hind legs showed the highest contributions to overall shape differentiation. A functional interpretation of the differentiation in the hindlimb length found in *L. macroglossa* could be that leg proportions may influence locomotor performance. Several experimental studies have shown how longer hindlimbs may improved locomotor performance (Orizaola and Laurila, 2009; Drakulic et al., 2016; Zamora-Camacho, 2018; Zamora-Camacho and Aragón, 2019), as well as jumping distance increases as the individual grows larger (Zug, 1978). Meanwhile, other studies revealed that locomotor performance is negatively affected at larger sizes (Moreno-Rueda et al., 2020), relatively large differences (>10%) in leg length can affect the jumping efficiency (Emerson, 1978; Babik and Rafinski, 2000). Differences in jumping ability could be occurring in *L. macroglossa* because our results showed differences greater than 10% in body size and leg length in individuals from Río de la Plata basin compared to those from the Río Negro basin.

Alternatively, differences in the hindlimb length may be the result of unequal growth and developmental rate during the larval and juvenile stages (Emerson et al., 1988; Babik and Rafinski, 2000). Because amphibians are ectotherms and depend on water, they show phenotypic responses to changes in environmental factors. In this sense, some phenotypic plasticity can be attributed to environmental factors such as the duration of the larval period and its relation to size as a function of temperature (Vences et al., 2002). A general Temperature-Size rule for ectotherms states that higher temperatures increase developmental rates, at the cost of smaller size (Drakulic et al., 2016) and conversely, at low temperatures develop more slowly, so they metamorphose at larger body sizes (Harkey and Semlitsch, 1988). Moreover, some studies replace the idea of temperature and relate body size to latitude, predicting that body size within species increases with latitude (Lindsey, 1966; Schäuble, 2004). In this study we found that individuals which had the longest legs were from Río de la Plata basin, which correlates with the colder climate in the studied distribution (InUMet, 2020). On the other hand, the individuals which had shortest legs were found in Río Negro basin, where the temperature is significantly higher (InUMet, 2020). So, we can expect that differences in environmental temperature during the larval period may have been responsible for the variation in the hindlimbs length in *L. macroglossa*. This trend has already been reported in other studies (Atkinson, 1994, 1995; Angilletta et al., 2004). Furthermore, our results are consistent with the intraspecific version of Bergmann's rule. It relates to geographic variation in the body sizes of animals (Blackburn et al., 1999) which has been briefly stated by Mayr as: The smaller-sized geographic races of a species are found in the warmer parts of the range, the larger-sized races in the cooler districts (Ray, 1960).

In this study we report a clinal variation in the relative leg length and body size of *Limnomedusa macroglossa* along a latitudinal gradient in Uruguay. The body size dimorphism likely reflects differences in growth rates of males and females. In organisms with indeterminate growth, body size is a result of a trade-off between growth and reproduction (Camargo et al., 2008). Therefore, females of *Limnomedusa macroglossa* appear to delay sexual maturity, while maintaining the same body shape and proportions as the males, reaching larger sizes at maturity, based on the combined evidence of distinct MSSM and the body size shift along the common isometric slopes of males and females. This difference in size could be adaptive for females, since a larger body size would increase the volume of the abdominal cavity, being able to accommodate larger ovaries (de Olivera et al., 2018) and consequently, increasing their reproductive output [the so called fecundity advantage hypothesis (Darwin, 1871)]. Therefore, sexual dimorphism in *L. macroglossa* could be determined by differential growth rate between the sexes, since the growth rates are usually asymptotic after maturation and sexes generally mature at different ages (sexual bimaturity) (Monnet and Cherry, 2002; Kupfer, 2007; Wells, 2007), or it may be the result of difference in the age distributions of males and females (Howard, 1981). Therefore, the sexual dimorphism found in body size is probably the consequence of higher growth rates and/or late sexual maturity in females of *Limnomedusa macroglossa*, which favors a larger body size and larger ovaries, and consequently, higher reproductive output.

CONCLUSIONS

In conclusion, our data on MSSM and SSD of *Limnomedusa macroglossa* from Uruguay may contribute to the knowledge of the life history traits of this species.

Our results show that females attained sexual maturity at larger sizes than males with a marked female biased sexual size dimorphism. These traits are driven by a trade-off between natural and sexual selection on each sex: parental effort in females does favor larger sizes to maximize their reproductive output, because bigger females can accommodate more eggs in their abdominal cavity. Meanwhile, mating effort in males does not favor large sizes due to energetic expenditures and risk of mortality during the long breading season, because bigger males invest most of their energy in search and calling behavior and have high mortality rates due to predation risk.

We also report a clinal variation in the relative leg length and body size of *Limnomedusa macroglossa* along a latitudinal gradient in Uruguay. Individuals with longest legs and bigger body sizes were from Río de la Plata basin, meanwhile individuals with shortest legs and smaller body sizes were those found in Río Negro basin. These differences could be explained by phenotypic plasticity in age and size at metamorphosis when separate populations are exposed to different environmental conditions (Ruthsatz et al., 2018). Studies demonstrated a plastic response of metamorphic traits in anuran larvae to changes in environmental conditions such as temperature. With increasing temperature time to metamorphosis may be reduced and metamorphosis occurs at smaller body sizes (Vences et al., 2002). Then, this may be occurring in *L. macroglossa*, since Río de la Plata basin is correlated with the colder climate in the studied distribution, meanwhile Río Negro basin is correlated with a warmer one.

All the evidence gathered in this work and its interpretations show that sexual dimorphism found in body size is likely the consequence of higher growth rates and/or late sexual maturity in females of *Limnomedusa macroglossa*, which favors a larger body size and bigger ovaries, and consequently, higher reproductive output. Examination of adult females and males, already in progress, will soon allow a more in depth understanding of *L. macroglossa* reproductive biology in Uruguay.

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APPENDIX 1

Six hydrographic basins of Uruguay and its geographic location (based on Achkar et al., 2013).

Basins	Latitude (S)	Longitude (W)
Río Uruguay	30°5'10"-33°54'59"	55°48'45"-58°26'17"
Río Santa Lucía	33°42'1"-34°50'24"	54°59'24"-57°07'11"
Océano Atlántico	33°39'56"-34°58'26"	53°22'13"-55°10'8"
Laguna Merín	31°54'18"-34°24'51"	53°02'27"-55°22'10"
Río de la Plata	33°52'17"-34°58'26"	54°55'14"-58°24'47"
Río Negro	30°49'59"-33°57'37"	54°9'42"-58°25'7"

APPENDIX 2

Thirty four mature females (♀) of *Limnomedusa macroglossa* used in the analyses and their respective morphometric measurements, basin and latitude/longitude from Uruguay. ZVC-B: vertebrate collection of the Faculty of Sciences, University of the Republic. SVL: snout–vent length; MW: mandibular width; HL: head length; IOD: inter-orbital distance; ED: eye diameter; IND: inter-narial distance; END: eye-nostril distance; ARML: arm length; TiL: tibia length; TaL: tarsus length; MtL: metatarsus length.

ACRONYM (Adult ♀)	SVL	MW	HL	IOD	ED	IND	END	ARML	TiL	TaL	MtL	Basin	Latitude	Longitude
12	60.95	23.98	20.47	10.75	7	5.76	5.88	14.2	37.59	18.73	29.81	Río Uruguay	-30.1166667	-57.05
24	53.06	21.3	18.27	8.39	5.49	4.77	5.76	13.05	32.81	17.59	30.48	Río Santa Lucía	-34.0666667	-56.8833333
127	58.88	23.21	20.53	9.22	5.95	5.06	6.09	14.32	36.41	19.58	30	Río Uruguay	-30.75	-56.3333333
132	56.15	21.17	18.02	8.66	6	4.76	5.75	14.34	32.44	17.4	28.55	Río Uruguay	-30.75	-56.3333333
133	54.11	20.13	17.73	8.62	5.36	4.29	5.91	12.99	35.53	17.54	32.12	-	-	-
151	61.97	22.3	20.19	8.93	6.79	5.09	6.36	14.05	36.41	19.83	31.83	Río Negro	-32.9166667	-54.9333333
153	56.55	24.12	20.36	10.22	6.62	5.15	5.77	15.14	36.56	19.36	31.83	Laguna Merín	-34.2166667	-54.9333333
310	58.85	23.17	20.38	9.49	6.67	5.14	5.91	13.36	34.05	17.71	30.38	Laguna Merín	-33.45	-54.5333333
317	55.34	20.79	18.85	9.01	5.83	4.35	6.03	13.09	33.2	18.38	27.91	Río de la Plata	-34.5333333	-55.4
495	55.97	22.51	19.37	9.79	6.01	5.34	6.1	14.08	36.71	19.3	30.35	-	-	-
651	64.25	23.89	21.04	9.01	6.56	5.68	6.35	16.36	37.26	20.03	32.14	Río Santa Lucía	-34.5833333	-56.4833333
691	62.54	23.65	21.93	9.78	5.99	4.82	6.66	15.12	37.23	19.27	32.74	Río Santa Lucía	-34.3	-55.25
813	61.95	24.23	22.02	9.98	6.23	5.45	7.05	15.07	39.86	20.57	31.31	-	-	-
826	55.4	21.27	18.16	8.59	6.15	4.98	5.05	12.29	32.81	16.98	28.63	-	-	-
829	58.22	24.47	20.64	10.6	6.48	4.82	5.92	15.55	36.68	18.22	30.05	Laguna Merín	-34.05	-54.7833333
996	59.34	24.34	21.63	10.68	6.72	5.15	6.3	14.11	38.53	18.96	32.66	Río Negro	-32.9166667	-54.9333333
1189	50.7	21.48	19.06	9.36	6.24	4.29	5.43	13.72	32.48	16.63	27.55	Río de la Plata	-34.55	-55.4
1247	58.21	23.69	18.62	9.3	6.44	4.85	5.93	13.24	34.01	17.62	30.09	Río Negro	-31.55	-55.65
1324	62.79	23.58	21.14	10.05	5.96	5.12	6.57	16.46	37.97	20.08	32.85	Río de la Plata	-34.8333333	-55.2666667
1414	51.05	19.83	17.55	8.23	5.96	4.54	5.6	13.26	31.57	16.03	27.47	Río Negro	-32.5	-55.3166667
1511	53.55	22.15	19.26	8.99	5.44	4.95	6.18	13.66	33.94	16.96	28.9	Río Uruguay	-30.95	-57.5333333
1523	54.17	22.39	19.45	9.14	6.29	5.14	6.07	14.3	34.84	18.28	29.76	Río Uruguay	-30.9333333	-57.5
23088	51.71	21.63	18.21	9.03	5.44	4.39	5.56	13.14	33.35	18	27.64	Río Negro	-31.0825	-55.8555556
23106	57.63	22.3	18.44	9.15	5.95	4.48	5.61	13.8	34.05	17.88	28.54	Río Negro	-31.0466667	-55.8477778
23343	63.96	23.13	21.56	10.47	6.38	5.09	5.96	16.09	37.67	19.99	30.24	Río Uruguay	-31.34177	-56.66407
23586	52.41	19.76	17.69	8.38	5.58	4.35	5.45	13.23	30.85	16.87	26.24	Río Negro	-31.24676	-55.95104
23594	62.97	23.51	20.73	10.93	6.22	5.05	6.05	16.73	38.83	20.27	30.86	Río de la Plata	-34.63791	-55.24744
23597	59.13	24.32	19.78	9.62	5.53	4.97	6.18	16.01	38.56	19.73	32.41	Río de la Plata	-34.63791	-55.24744
23598	61.58	24.06	20.24	10.03	6.26	4.83	5.61	15.66	39.35	20.65	31.31	Río de la Plata	-34.63791	-55.24744
23601	57.31	21.11	20.28	9.98	6.98	5.54	5.91	14.67	34.53	19.02	27.79	Río Uruguay	-31.15043	-56.29138
23608	51.35	19.98	17.87	8.53	5.27	4.2	5.51	13.75	31.87	16.84	27.77	Río Negro	-31.09436	-55.96907
23609	53.88	21	17.86	9.19	5.64	4.65	5.38	13.48	31.98	17.19	26.15	Río Negro	-31.13739	-56.04582
23610	49.82	20.95	17.37	9.41	5.07	4.27	5.1	12.68	30.21	16.54	25.1	Río Negro	-31.16724	-55.87382
23611	51.82	19.7	16.95	8.2	5.33	3.69	4.92	11.85	30.39	15.66	26.12	Río Negro	-31.09436	-55.96907

APPENDIX 3

Forty four mature males (♂) of *Limnomedusa macroglossa* used in the analyses and their respective morphometric measurements, basin and latitude/longitude form Uruguay. ZVC-B: vertebrate collection of the Faculty of Sciences, University of the Republic. SVL: snout–vent length; MW: mandibular width; HL: head length; IOD: inter-orbital distance; ED: eye diameter; IND: inter-narial distance; END: eye-nostril distance; ARML: arm length; TiL: tibia length; TaL: tarsus length; MtL: metatarsus length.

ACRONYM (Adult ♂)	SVL	MW	HL	IOD	ED	IND	END	ARML	TiL	TaL	MtL	Basin	Latitude	Longitude
90	46.51	17.73	16.14	7.78	4.83	3.92	4.41	11.49	28.95	14.71	23.95	-	-	-
140	46.71	17.01	15.67	7.22	4.17	3.39	4.38	11.56	28.44	15.38	25.62	Río Uruguay	-30.2833333	-57.1833333
329	57.13	22.88	20.3	9.41	6.11	5.22	5.57	13.81	35.57	17.97	31.82	Río Santa Lucía	-34.0666667	-56.8833333
357	52.61	21.27	17.88	8.58	6.31	4.96	4.98	13.78	31.25	16.25	29.04	Río Uruguay	-33.4666667	-58.4
549	51.73	19.68	18.28	7.85	5.04	3.7	5.52	13.38	32.51	17.24	26.8	Océano Atlántico	-34.8166667	-54.9166667
588	42.8	17.04	15.5	7.35	4.68	4.23	4.93	11.34	26.87	15.47	23.72	-	-	-
908	44.37	19	16.85	7.36	5.35	3.86	4.54	11.68	26.7	13.91	23.7	Río Santa Lucía	-34.5833333	-56.4833333
1121	50.87	19.84	17.73	9.15	5.65	4.28	5.3	12.6	31.87	14.64	27.81	-	-	-
1156	53.16	24.28	20.98	9.15	6.57	5.14	5.6	13.37	35.33	18.7	31.68	Océano Atlántico	-34.7333333	-54.9833333
1195	58.65	23.76	19.77	9.82	6.07	5.48	6.33	15.62	37.72	17.12	31.93	Río Uruguay	-33.85	-57.7333333
1245	43.22	16.99	14.73	7.99	5.56	4.25	5.07	11.38	27.78	14.87	24.29	Laguna Merín	-33.1	-54.7
1342	45.21	18.21	16.38	7.88	4.88	4.01	5.1	11.89	29.07	15.27	26.89	Río Uruguay	-30.9333333	-57.5
2120	60.92	22.81	19.61	9.82	7.2	5.24	5.86	13.81	36.88	18.72	30.62	Río de la Plata	-34.8666667	-56.3666667
2124	56.47	22.36	18.85	9.45	6.55	5.14	5.58	13.17	33.2	18.02	30.36	Río de la Plata	-34.8666667	-56.3666667
2853	44.19	17.68	14.71	7.48	5.03	3.47	4.04	10.51	24.7	13.57	21.96	Río Negro	-31.1166667	-55.9833333
3013	47.1	18.95	16.45	7.9	5.27	3.84	4.11	12.36	28.96	14.88	24.44	Río Uruguay	-31.8166667	-56.4166667
3444	52.17	20.43	17.24	9	6.06	4.66	5.19	12.08	31.68	16.82	27.14	-	-	-
3456	57.54	22.25	18.64	9.25	6.38	4.66	5.49	13.94	34.61	17.21	29.78	Río de la Plata	-34.3333333	-57
4902	49.53	20.28	17.77	8.62	5.22	4.42	5.21	13.91	32.35	17.93	27.04	-	-	-
10254	51.5	21.19	17.31	8.98	5.5	4.29	5.55	12.59	32.01	16.81	28.36	-	-	-
10278	54.01	21.24	18.81	9.02	5.61	5.09	6.08	13.8	33.59	16.78	27.94	-	-	-
10279	53.21	20.67	18.29	9.78	5.9	5.05	5.99	13.94	34.98	18.38	28.82	-	-	-
10845	55.79	21.99	17.15	8.96	5.71	4.39	4.97	13.66	34.23	17.17	30.09	-	-	-
23105	45.68	18.31	16.37	7.78	5	3.56	4.18	10.8	27.58	15.04	25.23	Río Negro	-31.0466667	-55.8477778
23341	46.66	18.33	15.81	7.43	5.27	4.06	4.52	10.82	26.64	13.76	23.12	Río Uruguay	-31.60659	-56.43186
23358	48.55	18.87	15.91	7.86	5.54	3.81	4.64	12.15	29.37	15.63	25.83	Río de la Plata	-34.471741	-55.529168
23583	49	18.48	16.49	8.44	5.11	4.07	5.08	12.6	29.33	15.42	27.6	Río Negro	-31.18738	-55.9483
23587	52.19	19.95	18.14	8.46	5.89	3.93	4.99	13.95	32.14	17.52	27.27	Río Negro	-31.23137	-56.09116
23588	49.49	19.95	16.74	8.94	5.38	4.16	5.03	12.56	29.73	16.2	27.03	Río Negro	-31.3044	-56.05855
23589	48.9	19.61	17.14	8.4	5.69	3.83	4.64	12.12	29.64	15.61	25.81	Río Negro	-31.30467	-56.05854
23590	41.29	17.28	14.47	7.43	4.26	3.26	4.16	11.2	25.46	14.29	22.07	Río Negro	-31.32815	-56.17757
23591	50.04	18.75	17.04	8.95	4.64	4.11	5.11	11.92	28.91	15.88	25.02	Río Negro	-31.69442	-56.12402
23592	47.05	17.24	15.46	7.54	5.48	3.57	4.01	10.48	28.59	14.85	25.16	Río Santa Lucía	-34.28159	-55.27949
23593	52.11	20.85	17.79	9.52	6.91	4.52	4.83	13.22	33.38	17.23	28.57	Río Santa Lucía	-34.28159	-55.27949
23595	48.21	20.16	16.79	8.05	4.98	4.21	5.17	12.63	30.95	16.81	25.31	Río de la Plata	-34.63791	-55.24744
23596	51.53	19.58	17.17	8.37	4.94	4.25	5.19	12.33	31.21	16.74	25.11	Río de la Plata	-34.63791	-55.24744
23599	52.37	20	17.48	8.41	5.65	4.56	5.41	13.77	32.47	17.47	26.21	Río Uruguay	-30.67911	-56.51333
23600	55.14	21.15	19.55	9.06	6.29	4.45	5.37	14.37	33.82	18.33	27.39	Río Uruguay	-30.67911	-56.51333
23602	45	19.81	17.11	8.35	4.91	3.84	4.94	10.97	28.12	14.49	24.95	Río Negro	-31.19047	-55.90129
23603	48.79	18.62	16.56	8.13	5	3.75	5.12	12.3	29.26	15.55	26.65	Río Negro	-31.19068	-55.90163
23604	48.13	19.05	16.68	8.06	4.94	3.96	5.08	11.45	29.42	15.87	25.33	Río Negro	-31.19068	-55.90163
23605	44.46	17.86	16.14	7.79	4.96	3.74	4.52	11.45	26.73	13.78	22.39	Río Negro	-31.18738	-55.9483
23606	43.54	16.61	15.16	7.1	4.72	3.63	4.31	11.53	25.93	14.24	22.67	Río Negro	-31.18738	-55.9483
23607	43.11	17.04	14.79	7.05	4.66	3.81	4.6	9.93	25.46	13.86	21.82	Río Negro	-31.13739	-56.04582

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Reptile diversity in a Mediterranean wetlands landscape (Alto Guadalquivir region, southeastern Spain): are they affected by human impacts?

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Abstract. This study was carried out to evaluate the diversity in reptile communities in wetland landscapes located in the Mediterranean region. For this, the status of the reptile populations linked to different Mediterranean wetlands in relation to the different types of land use established in the nearby drainage basins (500 m around the wetlands perimeter) was determined. The different types of land use were determined together with the presence/absence, abundance and size class of the different reptile species. The results showed that areas with high anthropic pressure had a lower diversity of species, as well as a less balanced community structure, that could put at risk the effective recruitment and hence the maintenance of the reptile populations in these areas. The reasons behind the decline in the reptile community are similar to those put forward for explaining the decline in amphibians in the same area.

Keywords. Aquatic ecosystems, biodiversity crisis, Mediterranean landscapes, reptiles decline.

INTRODUCTION

Species extinction is a natural process, but in recent times there has been a more general decline in biodiversity with a species extinction rate that is estimated to be higher than it might be expected (Rockström et al., 2009; De Vos et al., 2014). The decline in biodiversity has been reported by many authors, and different causes have been considered in order to understand the complexity of joining cause and effect, being human activities identified as a crucial threat (Wake and Vredenburg, 2008; Pimm et al., 2014; Ripple et al., 2019). In this process of loss of diversity, herpetological fauna is one of the groups with the highest risk of extinction (Böhm et al., 2013; Alroy, 2015). The report of the International Union for Conservation of Nature (IUCN, 2010) reflects this critical situation, indicating that 41% of the amphibians, and 28% of the reptiles evaluated are critically threatened. As with other vertebrate groups, the main reasons behind decline are climate change (Foufopoulos et al., 2011), destruction and alteration of their habitats (Lizana and Barbadillo, 1997; Wake and Vredenburg, 2008), ecosystem pollution (Sparling, 2003; Sparling et al., 2015) or the introduction of allochthonous species (Pleguezuelos, 2002), among others.

In the Iberian Peninsula there are 56 species of reptiles (Márquez and Lizana, 2002). One of the most important threats to these species is the destruction of habitats, as a consequence of the mechanization of the agroecosystem since the Industrial Revolution (Ceacero et al., 2007). The establishment of monocultures, due to intensive agriculture, has led to a homogenization of the ecosystem, characterized today by simplistic mosaic models, which has caused a reduction in biodiversity

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and to the loss of natural habitats (Guerrero et al., 2006; García-Muñoz et al., 2010, 2016). This simplification of the agroecosystems has been widely linked to the excessive use of agrochemicals that are endangering many species (Sparling et al., 2000; Mann et al., 2009; Böll et al., 2013; García-Muñoz et al., 2019). In fact, many species of reptiles live in highly specific environments, have very restricted distributions and population growth to very slow rates (Pleguezuelos et al., 2002). Accordingly, the presence of some species of reptiles or the state of their populations will depend, among other factors, on the characteristics of the species and to what extent the habitats have been disturbed (Pleguezuelos, 2002).

Therefore, the aim of this research was to determine the status of the reptile populations in different wetlands of the Alto Guadalquivir region (Andalusia, south of Spain), in connection to the different types of land use established in the surrounding drainage basins. The hypothesis in this study was that the wetlands in which a greater number of habitats were conserved in their drainage basins would have reptile populations with higher diversity and abundance compared to those in which intensive monoculture had been established. In order to test this hypothesis, we proceeded to (i) determine the different types of land use in the drainage basins of each wetland; (ii) determine the presence/absence, abundance and size structures of the different reptile species in each wetland under study; and (iii) determine how the conservation status of the wetland drainage basin influenced reptile communities. The results from this research could enable us to propose conservation measures to be implemented for the reptile species present in the wetlands.

MATERIALS AND METHODS

Study area

The study was conducted in the Alto Guadalquivir (Andalusia), a region located in the southeast of the Iberian Peninsula. This area has a complex geological history and is ideal scenario for herpetological studies since it has both Iberian-North African and Baetic endemisms and species of Atlantic origin (Ceacero et al., 2007). The study area is characterized by three geological units: the depression of the Guadalquivir river with its tributaries, which has had a long history of anthropization, as well as two mountainous units, Sierra Morena to the north and Baetic mountains ranges to the south and east of the province (Vera, 1994). Due in large part to this complex geological history, this last region displayed the highest species richness in terms of reptiles, with a total of 39 species, which is higher than the surrounding regions (Salvador and Pleguezuelos, 2002; Ceacero et al., 2007).

Three types of wetlands can be classified based on the extent of the alteration of their drainage basins (García Muñoz et al., 2010, 2016; Gilbert et al., 2014, 2017): (i) those that have been greatly altered and have a very high level of human activities, situated in the valley of the Guadalquivir river; (ii) those that have been partially conserved in the areas of the Sierra Morena and there is a low level of human activities; and (iii) those that have been conserved, such as the wetlands belonging to the Baetic mountain ranges. In this study, nine wetlands representative of the three zones in the study area in the Alto Guadalquivir region have been investigated (Fig. 1): Sierra Morena (Ardal, Tobaruela and Santisteban), the Guadalquivir Valley (Honda, Brujuelo-Cirueña and Hituelo) and the Baetic mountain ranges (Castillo, Siles and Orcera). Although we are aware that the number of wetlands per area is limited, the value of this study is precisely to address three different areas and to be able to make comparisons between them. Notwithstanding, since the selected wetlands are the most representative of each zone (García-Muñoz et al., 2010; Gilbert et al., 2014, 2015, 2017), in the future this study should be expanded to a greater number of wetlands.

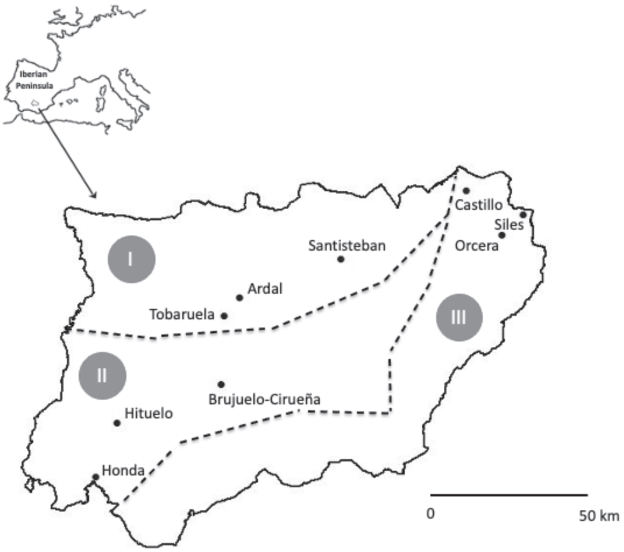

Fig. 1. Geographical location of the study area in southern Spain and the nine wetlands in the Alto Guadalquivir region. These wetlands are representative of the three zones presented in the study area: (I) Sierra Morena; (II) the Guadalquivir valley; and (III) Baetic mountain ranges.

Data collection

For each area, a sampling buffer zone of 500 m from the shoreline of the wetland included in the drainage basin was established. We characterized the different land uses in the area according to the habitat classifications of the Spanish Society of Ornithology (SEO, 2008). For each habitat category, the surface (in hectares) was obtained within QGIS 2.14 software environment (see Table 1). A bibliographic review on the reptile species present in each wetland was also carried out (S_{Ref}; Pleguezuelos et al., 2002; Ceacero et al., 2007; AHE database, 2016).

Table 1. Percentage land use in the study wetlands following the habitat proposed by SEO (2008).

Habitat	Ardal	Tobaruela	Santisteban	Honda	Brujuelo-Cirueña	Hituelo	Castillo	Siles	Orcera
Wetland surface area	4.32	1.20	3.37	10.49	4.46	3.86	0.15	0.32	0.03
Olive crops area	1.128	40.77	12.03	131.36	122.26	102.30	24.67	--	--
Rainfed crops area	19.57	27.10	69.86	--	12.21	--	37.91	--	--
Roads and tracks length (m)	4821.1	5967.98	6745.08	4021.97	1785.43	2650.54	2584.13	1029.82	1937.59
Urbanization area	22.58	17.33	8.92	--	--	0.44	--	--	--
Scrubland area	--	10.85	--	6.87	3.33	--	11.14	--	--
Holm oak and cork oak forest area	68.86	--	--	--	--	--	2.58	--	--
Pines forest and grassland area	--	--	--	--	--	--	--	76.69	70.13
Altitude (m a.s.l.)	400	363	637	446	458	476	780	1280	1270

For field data collection (S_{Obs}), the same procedure was replicated (three times regarding each wetland) for all samplings from April to July 2016 (concerning the season in which there is the highest reptile activity on this area). Different time-constrained searches were used (see Hutchens and DePerno, 2009): (i) linear transects, covering all the land uses present in each drainage basin of each wetland, within the delimited area of study; (ii) standardized road searches during twilight hours; and (iii) sampling based on fixed points for 30 minutes in each one, with a more intensive search of possible refuges, such as stones, logs or abandoned farmhouses, in all the habitats that were in each wetland. All the individuals registered were identified, their abundance was counted, and each individual was classified into the three size ranges: juvenile, sub-adults and adults. The authors are aware that the detection of adults, sub-adults and juveniles is not the same. However, as the sampling strategy is the same and the detection level is similar between wetlands, we consider that the data obtained are totally valid at a comparative level between wetlands. For aquatic reptiles (*Mauremys leprosa*, *Natrix maura* and *N. natrix*) we chose to use a visual detection method and fixed points sampling (three fixed points on the shores of each wetland). Attending to surface area of the ponds, we chose three points because the entire wetland could be observed as a whole and thus avoiding an underestimation of the aquatic species. This was made to avoid possible translocations of diseases among study wetlands, because there have been verified cases of chytridiomycosis in the wetlands of the Baetic mountain ranges (Bosch and González-Miras, 2012), and that reptiles may be potential vectors of this disease (Kilburn et al., 2011).

Statistical analysis

To evaluate the relationship between land uses and the reptile species in each wetland, multivariate analyses were carried out with the CANOCO program (v 5.03). First, a Segmented Correspondence Analysis (DCA: Detrended Correspondence Analysis) was performed to calculate the gradient length. When the value of the gradient length is less than 1.5, as was our case, an RDA is recommended (Iturrondobeitia et al., 2004). The RDA (Redundancy Analysis) is based on a constrainer that explains the variation in species composition by environmental variables (Ramírez-González, 2005). Similarity Percentage Analysis (SIMPER, PRIMER v. 5.2) was subsequently used to identify the features of the species of each geographic group of wetlands (Clarke and Warwick, 2001): group 1 (Sierra Morena wetlands), group 2 (Guadalquivir valley wetlands), and group 3 (Baetic mountain ranges wetlands). Both statistical analyses were carried out with all the species registered in the wetlands under study ($S_{total} = S_{Obs} + S_{Ref}$).

Finally, the Simpson's dominance index (Simpson, 1949) was calculated just using the observed abundance data (S_{Obs}) in the three types of wetlands previously mentioned. Simpson's index measures the probability that two individuals randomly selected from a sample will belong to the same species. Diversity values can also be obtained after subtracting by one the value given in Simpson's dominance.

RESULTS

Table 2 shows the fifteen species of reptiles that have been observed in the wetlands under study (S_{Obs}) and five more species that were within the study area following the bibliographic review (S_{Ref}). The DCA results showed that the three first axes explained 72.65% of the variation. The results of the multivariate RDA analysis showed the relationship between land use and species (Fig. 2). Moreover, in the centre of the graph a group of more generalist species, such as *Malpolon monspessulanus*, *Timon lepidus*, *Psammodromus algirus* or *Rhinechis scalaris* could be seen; while other groups with more specialist species, such as *Vipera latastei*, *Coronella girondica*, *Natrix natrix* or *Chalcides bedriagai*, were located far from the centre of the graph. Siles and Orcera were locations in the plot that were farthest from rainfed crops, roads, olive groves and built-up land. Therefore, they appeared as less degraded and more inaccessible. Honda, Santisteban, Hituelo, Tobaruela and Brujuelo-Cirueña wetlands were closely linked

Table 2. Presence registered in the field sampling (+, S_{obs}), presence registered by bibliographic data (+*, S_{ref}) and absence (-) of the reptile species in the study wetlands.

Species	Ardal	Tobaruela	Santisteban	Honda	Brujuelo-Cirueña	Hituelo	Castillo	Siles	Orcera
Acanthodactylus erythrurus	$+^{*}$	$+^{*}$	-	-	-	-	-	-	+
Podarcis vaucheri	-	$+^{*}$	-	$+^{*}$	-	+	$+^{*}$	$+^{*}$	+
Psammodromus algirus	+	+	+	+	+	+	+	+	+
Psammodromus hispanicus	$+^{*}$	-	-	-	+	-	-	+	$+^{*}$
Tarentola mauritanica	+	$+^{*}$	+	+	+	+	+	+	+
Timon lepidus	+	+	$+^{*}$	+	+	$+^{*}$	+	+	+
Blanus cinereus	$+^{*}$	+	-	$+^{*}$	-	-	-	$+^{*}$	$+^{*}$
Coronella girondica	-	-	-	-	-	-	-	+	-
Hemorrhois hippocrepis	$+^{*}$	$+^{*}$	-	+	-	+	$+^{*}$	-	$+^{*}$
Malpolon monspessulanus	$+^{*}$	+	$+^{*}$	+	+	+	+	$+^{*}$	$+^{*}$
Rhinechis scalaris	$+^{*}$	+	$+^{*}$	$+^{*}$	+	$+^{*}$	$+^{*}$	$+^{*}$	$+^{*}$
Chalcides striatus	$+^{*}$	-	+	-	$+^{*}$	-	$+^{*}$	+	+
Chalcides bedriagai	+	-	-	-	-	-	-	+	-
Mauremys leprosa	+	+	$+^{*}$	$+^{*}$	$+^{*}$	+	+	-	-
Natrix maura	$+^{*}$	$+^{*}$	$+^{*}$	$+^{*}$	-	$+^{*}$	$+^{*}$	$+^{*}$	$+^{*}$
Vipera latastei	-	-	-	-	-	-	$+^{*}$	$+^{*}$	$+^{*}$
Macroprotodon brevis	$+^{*}$	-	-	-	-	-	$+^{*}$	$+^{*}$	-
Hemidactylus turcicus	$+^{*}$	-	$+^{*}$	$+^{*}$	-	-	-	-	-
Algyroides marchi	-	-	-	-	-	-	-	$+^{*}$	$+^{*}$
Natrix natrix	-	-	-	-	-	-	-	$+^{*}$	-
Richness	15	11	9	11	8	9	12	16	14

to the olive groves and dry land crops, and a multitude of roads have been created in order to provide better accessibility for agricultural purposes. Ardal wetland is mostly surrounded by oak forests but is also near built-up land because a few years ago a housing estate was built nearby.

The results of the SIMPER analysis (Fig. 3) show the average contribution of each species in the three groups analysed. It can be seen how *P. algirus, T. mauritanica, T. lepidus, R. scalaris, M. monspessulanus*, *N. maura* and *M. leprosa* are the species that most contributed to the percentage of similarity within each group and among the three study groups. Moreover, *M. leprosa* could be seen in 100% of the wetlands of groups 1 and 2, but in group 3 it could only be observed in Castillo. *Vipera latastei* is the species that most contributed to the dissimilarity in group 3 compared to the other ones (Group 1-3: 13.24%, Group 2-3: 10.64%). *A. erythrurus* is the species that most contributed to dissimilarity in group 1 (Group 1-2: 13.41%; Group 1-3: 7.2%) compared to the other ones, although a juvenile was also found in Orcera.

Table 3 shows the results of the size structures in each wetland under study. In all of them an inverted pyramid has been observed, which showed there to be more adults than juveniles or sub-adults. In addition, in some wetlands, such as Tobaruela, Honda and Brujuelo-Cirueña, there did not appear to be any recruitment; while in other ones, for example, Ardal, Castillo, Orcera and Siles, the highest number of juveniles and sub-adults were found in comparison with the other wetlands under study (see Table 3). The results of Simpson's dominance index (D) showed a gradient for group 1 (Sierra Morena; D = 0.501), with maximum dominance for group 2 (Guadalquivir valley; D = 0.323) and group 3 (Baetic mountain ranges; D = 0.250) with the maximum diversity value.

DISCUSSION

Reptiles play important ecological roles contributing to the maintenance of environmental heterogeneity (Kaczor and Hartnett, 1990) having a keystone functions in the ecosystem structure (Ashton, 2010). In fact, they tend to be strongly associated with local habitat quality and could be excellent bioindicators of the ecosystem conservation state (Pianka, 1967; Overmann and Krajicek, 1995; Crain and Guillette, 1998; Amaral et al., 2012).

Although extinction marks the end of any form of life, the speed at which a group reaches this point could

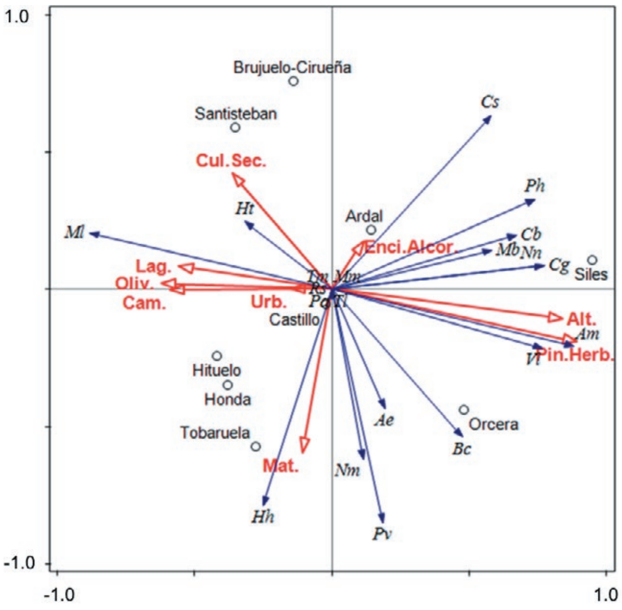

Fig. 2. Relationship of wetlands land uses and reptile species by RDA. *Acanthodactylus erythrurus* (Ae), *Podarcis vaucheri* (Pv), *Psammodromus algirus* (Pa), *Psammodromus hispanicus* (Ph), *Tarentola mauritanica* (Tm), *Timon lepidus* (Tl), *Blanus cinereus* (Bc), *Coronella girondica* (Cg), *Hemorrhois hippocrepis* (Hh), *Malpolon monspessulanus* (Mm), *Rhinechis scalaris* (Rs), *Chalcides striatus* (Cs), *Chalcides bedriagai* (Cb), *Mauremys leprosa* (Ml), *Natrix maura* (Nm), *Vipera latastei* (Vl), *Macroprotodon brevis* (Mb), *Hemidactylus turcicus* (Ht), *Algyroides marchi* (Am), *Natrix natrix* (Nn).

increase due to the combination of causes and effects derived from the activity of other species. As expressed in the title of this paper, the current situation of reptiles in the wetlands of the south of Iberia is reminiscent of the path taken by amphibians in the same study area (García-Muñoz et al., 2010, 2016). The simplification of agroecosystems with extensive and intensive monocultures that is ever present nowadays, that impoverishes the natural ecosystem are leading to the extinction of species worldwide.

In general terms, the species richness obtained is inversely related to the rise in monocultures (olive and cereal crops), both in terms of surface expansion and intensive production. As a result, the wetlands showed greater species richness in those areas (Sierra Morena and Betic mountains) where there are fewer crops and greater inaccessibility. It can therefore be seen that wetlands related to olive groves, cereal crops, and built-up land are more accessible for humans, and, consequently, show a lower richness in reptiles. However, and eliminating the option of the existence of an intrinsic geographical gradient in the distribution of species in the study area, wetlands located in mountainous areas (Baetic mountain ranges) showed greater species richness. As regards the different presence of species in the wetlands investigated by the study (Table 2), five of them were found in all ecosystems. These species could be used in future research to develop different biomarkers (oxidative stress, bioaccumulation, etc.) to compare the state of health of the ecosystems. However, these species could be resistance to disturbance and, hence, only indicators of severe stress. Therefore, the effects on the species most sensitive to disturbances (stenoic species), which have been shown to present a negative spatial response to fragmentation and disturbance of the landscape (Devictor, 2008), must also be taken into account to allow early detection of degradation processes.

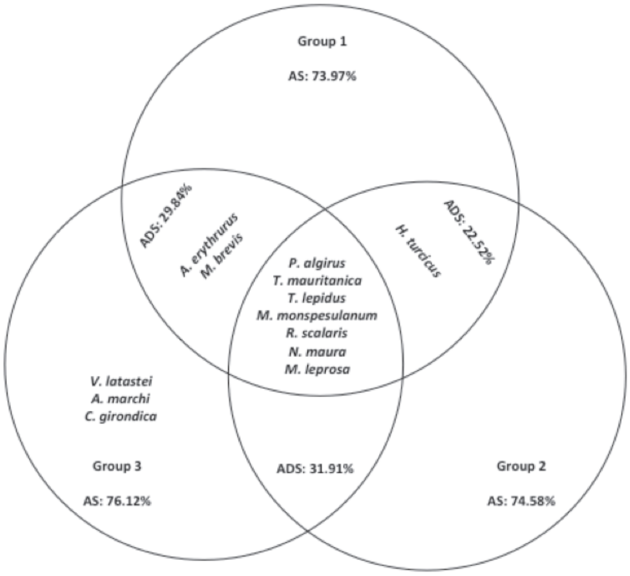

Fig. 3. Main species organization according to values of exclusivity. Typical species to each group is represented in the exclusive zone of each circle, while species shared between two groups are represented with the union of two circles. The species that appear in all three zones are in the intersection of the three circles (AS: average similitude; ADS average dissimilitude from SIMPER analysis).

Other research carried out in the same study area showed how complex the interaction between human activity and nature was. Specifically, García-Muñoz and Carretero (2013) studied the rate of water loss in two species of lizards (*Podarcis vaucheri* and *Algyroides marchi*) that coexisted in the same habitat but had different physiological rates. Minimal variations on a micro-ecosystems level could have been related to their physiological capacities (such as their ability to lose water) that could have explained specific (local) extinctions (Santos and Cheylan, 2013; Ferreira et al., 2016). The establishment of a monoculture and the consequent ecological oversimplification (e.g., loss of micro-habitats with dif-

Table 3. Species observed (S_{obs}) and number of individuals in the three size ranges in the studied wetlands.

Species		Ardal	Tobaruela	Santisteban	Honda	Brujuelo-Cirueña	Hituelo	Castillo	Siles	Orcera	Number
Acanthodactylus erythrurus	Adults	--	--	--	--	--	--	--	--	--	--
	Subadults	--	--	--	--	--	--	--	--	--	--
	Juveniles	--	--	--	--	--	--	--	--	1	1
Podarcis vaucheri	Adults	--	--	--	--	--	1	--	--	--	1
	Subadults	--	--	--	--	--	--	--	--	1	1
	Juveniles	--	--	--	--	--	--	--	--	--	--
Psammodromus algirus	Adults	7	21	--	14	26	4	16	14	52	154
	Subadults	--	--	--	--	--	--	--	--	1	1
	Juveniles	2	--	--	--	--	--	--	--	3	5
Psammodromus hispanicus	Adults	--	--	--	--	1	--	--	37	--	38
	Subadults	--	--	--	--	--	--	--	--	--	--
	Juveniles	--	--	--	--	--	--	--	--	--	--
Tarentola mauritanica	Adults	4	--	1	--	5	3	2	1	1	17
	Subadults	--	--	--	8	--	--	--	--	--	8
	Juveniles	1	--	--	--	--	--	--	--	--	1
Timon lepidus	Adults	4	--	--	--	4	--	4	5	6	23
	Subadults	--	1	--	1	--	--	--	2	--	4
	Juveniles	--	--	--	--	--	--	1	2	2	5
Blanus cinereus	Adults	--	1	--	--	--	--	--	--	--	1
	Subadults	--	--	--	--	--	--	--	--	--	--
	Juveniles	--	--	--	--	--	--	--	--	--	--
Coronella girondica	Adults	--	--	--	--	--	--	--	--	--	--
	Subadults	--	--	--	--	--	--	--	1	--	1
	Juveniles	--	--	--	--	--	--	--	--	--	--
Hemorrhois hippocrepis	Adults	--	--	--	--	--	--	--	--	--	--
	Subadults	--	--	--	1	--	--	--	--	--	--
	Juveniles	--	--	--	--	--	1	--	--	--	1
Malpolon monspessulanus	Adults	--	3	--	1	2	1	2	--	--	9
	Subadults	--	--	--	--	--	--	2	--	--	2
	Juveniles	--	--	--	--	--	--	1	--	--	1
Rhinechis scalaris	Adults	--	1	--	--	2	--	--	--	--	3
	Subadults	--	2	--	--	--	--	--	--	--	2
	Juveniles	--	--	--	--	--	--	--	--	--	--
Chalcides striatus	Adults	--	--	11	--	--	--	--	7	5	23
	Subadults	--	--	--	--	--	--	--	1	1	2
	Juveniles	--	--	1	--	--	--	--	3	--	4
Chalcides bedriagai	Adults	1	--	--	--	--	--	--	5	--	6
	Subadults	--	--	--	--	--	--	--	--	--	--
	Juveniles	1	--	--	--	--	--	--	--	--	1
Mauremys leprosa	Adults	--	--	--	--	--	--	20	--	--	20
	Subadults	--	1	--	--	--	1	--	--	--	2
	Juveniles	1	--	--	--	--	--	1	--	--	2
Natrix maura	Adults	1	--	12	1	2	9	2	--	--	27
	Subadults	--	1	--	--	--	--	2	--	--	3
	Juveniles	--	--	1	--	--	--	1	--	--	2
Total	Adults	17	26	24	15	40	15	44	69	64	314
	Subadults	--	5	--	10	--	1	2	4	2	24
	Juveniles	5	--	2	--	--	1	5	5	6	24

ferent humidity ranges, as has occurred in the Guadalquivir valley) could have brought about changes that gave rise to more complex extinction processes. This concept could have been linked with the facilitation theory, with abiotic facilitation to be specific (facilitation that is mediated through changes in the abiotic environment; Wright et al., 2017), so that oversimplifications in the agroecosystem could lead to an insufficient number of habitats, which, in turn, would bring about an alteration in the species richness. In this respect, other studies on the state of conservation of the Alto Guadalquivir wetlands (Gilbert et al., 2017) showed how intensive cultivation activities affected conservation of the diversity of wetlands. Furthermore, oversimplification in ecosystems brought about by monocultures, not only hinders the number of species and individuals, but also reduces reptile recruitment. The disappearance of natural refuges as a consequence of the management methods inherent to monocultures (whose aim is to remove any objects from the agricultural area), the indiscriminate use of herbicides and insecticides (which eradicate natural refuges and food) and the bioaccumulation and bioamplification process (Daley et al., 2014), could explain the lack of recruitment in these areas.

This study was designed in order to find out what the current status of reptiles in the Alto Guadalquivir region was. However, more studies are necessary to determine what has led to this process of decline. In order to anticipate moves in the game of extinction, we need to consider a range of environmental strategies. The first measure proposed is linked to the theory of conservation biology (Soulé, 1985; Sagoff, 2013), by maintaining biodiversity, the structure and function of ecosystems, expanding protected areas in the Sierra Morena and the Baetic mountain ranges. The second one lies within the context of the ecosystem management theory; that in the study area is related to integrated crop management to make monoculture more beneficial by recovering their biodiversity (Rey-Zamora et al., 2017). The latter measure increases heritage values, environmental sustainability and landscape values. Moreover, it enables traditional landscape planning to be recovered. These changes in agricultural strategy have been more specifically linked to a reduction in erosion, a decrease in the use of toxic substances and to a rise in land-use heterogeneity. Moreover, these actions could also promote conservation of the biodiversity of other related groups, such as amphibians (García-Muñoz et al., 2010). In addition, the third measure for consideration could be to use biomarkers developed with the species found in all the wetlands, such as *P. algirus*, *T. mauritanica*, *T. lepidus*, *M. monspessulanus* and *R. scalaris*.

On a final note, authors would like to indicate an issue of concern, which is the increasing popularity of leisure pursuits in areas of natural beauty where access is limited for most people. The rise in this phenomenon, without taking into account the negative effects they have on wildlife, could increase the likelihood of encountering reptiles that, apart from the aversion that these organisms arouse in some people, could lead to the re-emergence of threats to this group that have been forgotten and also give rise to new ones that have not yet been created. In the former we are referring to direct persecution of reptiles by humans (Ceríaco, 2012) and in the latter the translocation of diseases such as chytriodiomycosis, which has been detected in Baetic mountains (Bosch and González-Miras, 2012).

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Sex chromosome diversification in the smooth snake *Coronella austriaca* (Reptilia, Serpentes)

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Abstract. The smooth snake *Coronella austriaca* is a widespread Palearctic colubrid species. The species has been the subject of several molecular and phylogeographic studies which highlighted the occurrence of distinct genetic lineages in different areas of the species distribution, but scarce cytogenetic data are currently available on the species. In this paper we present a molecular and karyological study performed with several banding, staining methods and NOR-FISH on samples of *C. austriaca* from different geographical areas (Italy and Greece) of the species distribution. The molecular and phylogenetic analysis unambiguously placed the studied samples in different clades with a clear geographical pattern. The karyotype of the two female samples studied was composed of 2n = 36 chromosomes with 16 macro- and 20 microchromosomes and a mix of plesiomorphic and derivate chromosome features. All macrochromosomes were biarmed with the exception of pair 5 that was telocentric. NORs were detected on a microchromosome pair. In both females, the pair 4 was heteromorphic (and completely heterochromatic after C-banding in the Italian female), representing the first report of a ZZ/ZW sex chromosome system with female heterogamety in *C. austriaca*. In addition, the W chromosome showed a different morphology between the two female studied (submetacentric and subtelocentric), highlighting the occurrence of a chromosomal diversification among distinct geographical areas of the species distribution and further supporting that the species contains different diverging evolutionary clades.

Keywords. Karyotype, heterochromatin, FISH, sex chromosomes, squamates, snakes.

INTRODUCTION

The smooth snake *Coronella austriaca* Laurenti, 1768, is a small sized ovoviviparous colubrid with a widespread distribution across the western Palearctic (Strijbosch, 1997). The species occurs in western, central and southern Europe reaching as far as the Ural Mountains and the Caspian Sea, up to northern Iran and western Kazakhstan. *Coronella austriaca* is absent from European and Mediterranean islands, except for Southern England, Sicily, the Island of Elba and the Island of Krk (Engelmann, 1993; Strijbosch, 1997; 2006). In its large distribution range, the species have a relatively uniform morphology and only two subspecies are currently recognised: *C. a. acutirostris* Malkmus, 1995, from the north-western Iberian Peninsula and the nominal subspecies *C. a. austriaca* which occupies the rest of the geographical range of the species. A third subspecies, *C. a. fitzingeri* (Bonaparte, 1840) was formerly described from southern Italy and Sicily, but it has been recently synonymised with the nominal subspecies (Speybroeck et al., 2016).

The species has been the object of several molecular and phylogeographic studies (Santos et al., 2008; Llorente et al., 2012; Galarza et al., 2015; Jablonsky, 2019) and rep-

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resents an ideal candidate to assess patterns of intraspecific genetic diversity among different populations as well as the occurrence of different refugial areas across its distribution range. These studies evidenced that *C. austriaca* comprises several distinct clades showing a complex haplotype structure and deep genetic divergence. These evidences do not reflect the current taxonomy of the species and a revision is probably required to better describe its molecular and geographic intraspecific diversity.

On the other hand, the available chromosome data on *C. austriaca* are dated and refer to the studies by Matthey (1931) and Kobel (1967) that described the karyotype of two males from Switzerland, but no information is currently available from other, geographically and genetically distinct clades of the species. The karyotype described by Matthey (1931) and Kobel (1967) was composed of 2n = 36, with 16 macrochromosomes and 20 microchromosomes. All the macrochromosomes were biarmed, excluding the elements of 5th pair that were telocentric. Furthermore, these studies were performed only with standard staining methods. No information is currently available on the sex chromosome system, location of nucleolar organizer regions (NORs) or other chromosome markers which would be useful for karyotype comparison among different population of the species or with other phylogenetically related colubrid species. In fact, cytogenetic inferences, especially when linked to molecular data in a phylogenetic perspective, are useful tools to detect plesio- and apomorphic states and to reconstruct evolutionary trends in the studied species (Odierna et al., 1987; Mezzasalma et al., 2013, 2017 a, 2017b; Fuller et al., 2018).

In this work we present the results of a karyological study performed on smooth snake samples belonging to different populations and geographical areas of the species distribution (Italy and Greece) using several standard staining and banding methods and molecular cytogenetics. The aim of this study was to evidence the possible presence of a chromosome sex determination system, the occurrence and location of heterochromatic regions and of NORs. We also performed a molecular and a phylogenetic analysis on the studied samples, adding our data to those available from the literature, in order to evaluate their genetic diversity and place our chromosome data in a phylogenetic context.

MATERIAL AND METHODS

Sampling

We studied preserved tissue and cell suspensions of two samples of *C. austriaca* obtained from existing collections and no aminal was collected during the realization of this study. Studied samples include one female from Italy (Picentini Mountains, Avellino), and one female from Greece (Peloponnese) both hosted at the Dipartimento di Biologia Evolutiva e Comparata, Università degli Studi di Napoli Federico II, since 1972 (sample numbers CA0201- CA0202). These two samples were both used in a preliminary molecular analysis and in the karyolgical study as described below. The samples used in this study were already used in previous analyses (Mezzasalma et al., 2014, 2016a).

Molecular and phylogenetic analysis

In order to properly identify the studied samples, assess their genetic diversity and establish the taxonomic affinities with other available sequences on the species, a molecular analysis was performed using a fragment of the mitochondrial Cytochrome b (Cytb) gene. This mtDNA gene was chosen considering the number of available sequences for several populations of *C. austriaca* (Llorente et al., 2012; Santos et al., 2008, 2012; Galarza et al., 2015; Jablonsky et al., 2019).

Genomic DNA was extracted from both samples using the standard phenol-chloroform method by Sambrook et al. (1989) and then used in the PCR amplification of a fragment of about 600 bp of the Cytb. The primers used were FORWARD: 5' AACTTCGGATCCATACTACTAA 3' and REVERSE: 3' TAAAGATGTTAGGGGTGAATGA 5', and the PCR parameters those reported by Mezzasalama et al. (2015a).

PCR products were sequenced on an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA) using BIGDYE TERMINATOR v3.1 (ABI). Sequences were blasted in GenBank and chromatograms were checked and edited using CHROMAS LITE 2.1.1 and BIOEDIT 7.2.6.1 (Hall, 1999). The best-fitting substitution model (GTR+I+G) was chosen using JMODELTEST 2.1.7 (Darriba et al., 2012), under the corrected Akaike information criterion (AICc). In the phylogenetic analysis we used the two newly determined sequences (Accession Numbers: MW861682-MW861683) and available homologous sequences from Jablonski et al. (2019), downloaded from GenBank, choosing when possible, the longest available sequences for each major clade (see Fig. 1). As outgroup we used an available homologous sequence of *C. girondica* (AN: AF471088). Phylogenetic analysis with Bayesian inference (BI) was performed using MRBAYES 3.2.7 (Ronquist et al., 2012), with two parallel runs of 8 000 000 generations and four incrementally heated Markov chains (using default heating values), with a burn-in of 25% and sampling the chains every 500 generations. A 50% majority-rule consensus tree was retrieved from the post-burn-in samples and chain convergence was checked with convergence diagnostic values (average standard deviation of split frequencies >> 0.01, potential scale reduction factor).

Chromosome analysis

Chromosomes were obtained from tissue samples and cell suspensions using the air-drying method, as described in Mez-

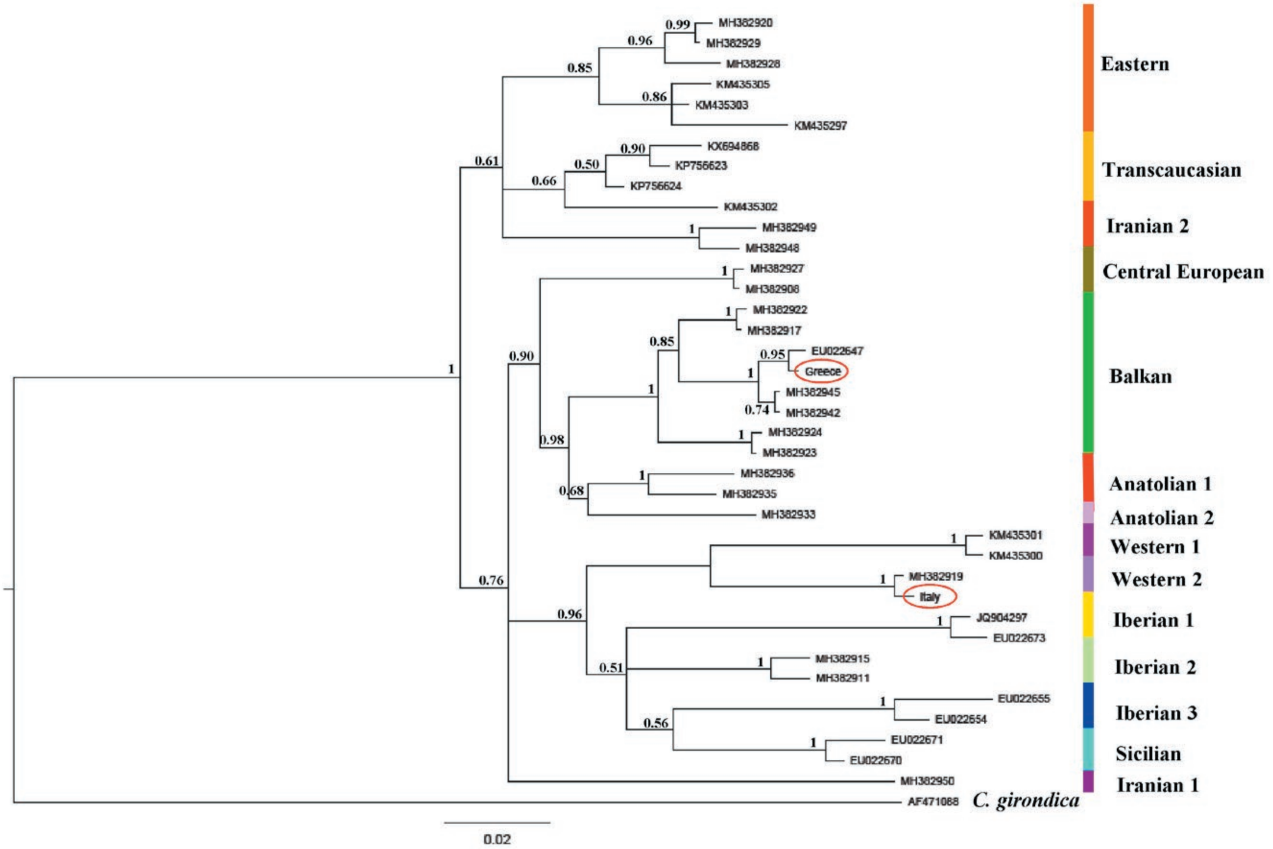

Fig. 1. Phylogenetic analysis with Bayesian Inference (BI) of Cytb sequences (up to 1031 bp) of *C. austriaca*. Numbers at nodes represent posterior support values. Clades denomination follows Jabloski et al. (2019). Red circles = original samples used in the cytogenetic analysis.

zasalma et al. (2019). The chromosome analysis was performed with traditional staining (5% Giemsa solution at pH 7 for 10 min) and several chromosome staining and banding techniques: Quinacrine staining, Chromomycin A_3-methyl green staining (CMA/MG) (Mezzasalma et al., 2015b), C-banding (Sumner, 1972), and sequential C-banding + CMA + DAPI (Sidhom et al. 2020a, b). Karyotype reconstruction was performed after scoring at least five metaphase plate from each sample studied. Nucleolus organizing regions (NORs) were located following the Ag-NOR banding protocol reported by Howell and Black (1980) and fluorescence *in situ* hybridization (NOR-FISH) as described in Sidhom et al. (2020a), using as probe the PCR-amplified and biotinylated 18S rRNA gene of the gekkonid *Tarentola mauritanica*. The detection of FISH signals was carried out using ExtrAvidin FITC (Sigma Aldrich), counterstained with propidium iodide (PI).

Metaphase plates were detected and recorded using an epifluorescent microscope (Axioscope Zeiss) equipped with an image analysis system.

RESULTS

Molecular and phylogenetic analysis

A a fragment of about 500 bp of the Cytb was successfully amplified and sequenced from the two original samples, respectively from Italy and Greece (see Sampling), used in the cytogenetic analysis. No interruptions of the reading frame were detected in either sequences. Nucleotide identity samples was about 99% between the newly sequenced sample from Italy (Monti Picentini) and an available sequence from Italy (AN: MH382919), and 100% between the newly determined sequence from Greece and an available sequence from Greece (AN: EU022647).

In the phylogenetic analysis, the final Cytb alignment contained 39 sequences and 1031 nucleotide positions. The resulting tree (Fig. 1) retrieved all the main clades and a topology similar to that reported by Jablonski et al. (2019). The relative evolutionary relationships among the main clades mostly reflect an East-West

Fig. 2. Giemsa stained karyotypes of *C. austriaca* from Italy (Picentini Mountains) (A) and Greece (Peleponnese) (B). The frame includes the ZW sex chromosome pair.

scenario of geographical diversification by distance. Our tree retrieved, four major clades, comprising several smaller subclades: Iranian 1, Sicilian + Iberian 1-3 + Western 1-2, Anatolian 1-2 +Balkan + Central European, and Iranian 2 + Transcaucasian + Eastern, respectively (Fig. 1). In our tree, the Easternmost major clade (excluding the only sample of the Iranian 1 clade), comprising the Iranian 2 + Transcaucasian + Eastern subclades, is the outgroup of all the remaining clades. The two original samples from Italy and Greece clustered unambiguously within the Western 2 and Balkan clades, respectively (Fig. 1). Statistical support values were generally high at terminal nodes while varied from high to low at deeper nodes (Fig. 1).

Chromosomal analysis

The Italian and Greek samples of C. *austriaca* showed a very similar karyotype composed of 2n = 36 chromosomes, with 16 macrochromosomes and 20 microchromosomes (Fig. 2). Among macrochromosomes, pairs 1, 3, 6 and 8 were metacentric, pairs 2 and 7 were submetacentric, and pair 5 was telocentric. The pair 4 was heteromorphic, carrying the female sex chromosomes, ZW. In particular, the chromosome Z was always metacentric while the W resulted subtelocentric in the female from Picentini Mountains (Italy) (Fig. 2A) and submetacentric in the female from Peloponnese (Greece) (Fig. 2B).

Other chromosome stainings and banding techniques were performed only on the Italian sample of *C. austriaca*, as quality and quantity of metaphase plates of the Greek individual were adequate just for its karyotype description.

Quinacrine stained evenly both macro- and microchromosomes (Fig. 3A), while CMA/MG evidenced two microchromosomes and the telomeric regions of all macrochromosomes and the telomeric regions almost all microchromosomes (Fig. 3B). A microchromosome pair resulted specifically marked by both Ag-NOR staining and NOR-FISH, (Fig. 3C and D).

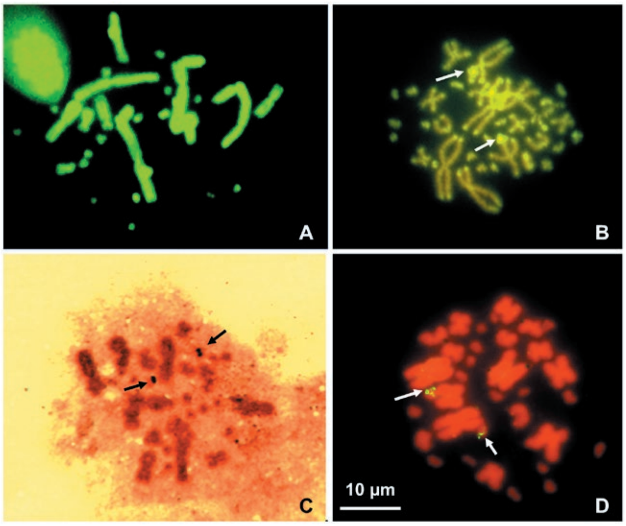

Fig. 3. Metaphase plates of *C. austriaca* stained with Quinacrine (A), CMA/MG (B), Ag-NOR staining (C) and NOR-FISH banding (D). Arrows point at a microchrosome pair evidenced with CMA/MG, Ag-NOR staining- and NOR-FISH. Scale bar applies all images.

After C-banding, heterochromatic bands were hardly visible, if not absent, on most macro- and microchromosomes, with the exclusion of the W chromosome, which was completely heterochromatic (Fig. 4A), but not evidenced with CMA or DAPI (Fig. 4B, C).

DISCUSSION

Our phylogenetic inference produced similar results to those obtained by Jablonski et al. (2019), retrieving all the 14 clades previously described. The main differences concern the position of one of the major clades (comprising Iranian 2 + Transcaucasian + Eastern sub-

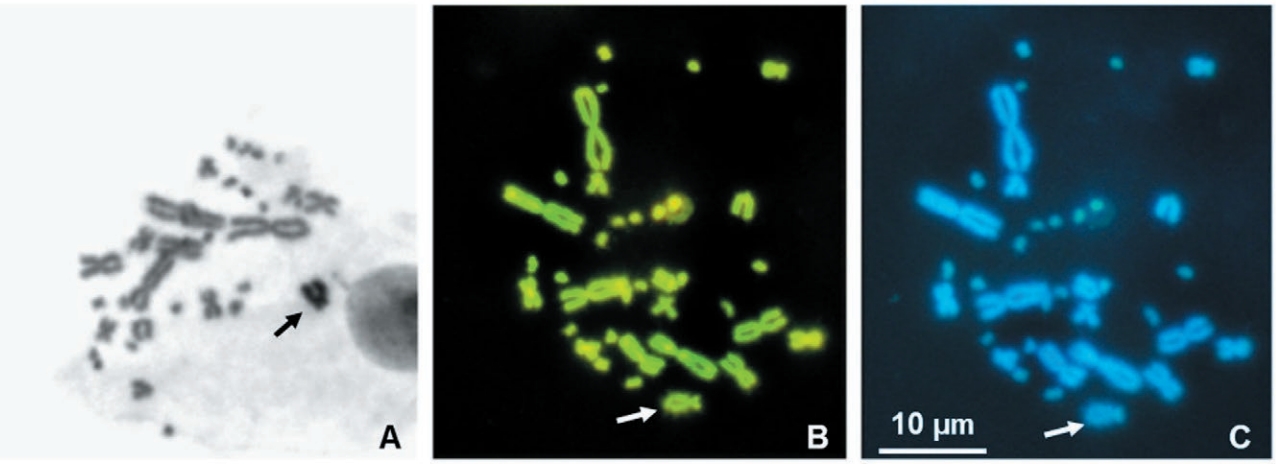

Fig. 4. Metaphase plates of *C. austriaca* sequentially stained with C-banding + Giemsa (A),+CMA (B), and +DAPI (C). Arrows point at the W sex chromosome. Scale bar applies to all images.

clades) which is not involved in a basal polytomy with the other major clades but appear to be the outgroup of all the remaining clades. This results further supports the hypothesis of an East-West geographical diversification process in *C. austriaca*. Similar East-West differentiation processes are also shared by different European and Palearctic reptile taxa, including distinct major snake lineages of Asiatic origin (see, e.g., Utiger et al., 2002; Nagy et al., 2004; Mezzasalma et al. 2015a, 2018). However, the phylogenetic position and the evolutionary relationships of some clades (such as the Iranian 1 clade) remain to be better determined and this hypothesis should be further tested. In fact, as already highlighted by Jablonski et al. (2019), the uncertain phylogenetic position of some subclades as well as the low statistical support values at some nodes is probably due to a number of missing haplotypes and a more inclusive sampling with the addition of more molecular markers is probably required to better assess the evolutionary relationship of some subclades.

The karyological formula of the two females of *C. austriaca* here studied is consistent with that previously described by Mattey (1931) and Kobel (1967) for two males from Switzerland. However, the comparison among the pair 4 of the different karyotypes allowed us to detect for the first time the ZZ/ZW sex chromosome system in *C. austriaca*. In particular, the metacentric element of the 4th pair was identified as the Z sex chromosome and its heteromorphic counterpart as the W chromosome. In addition, the W chromosome showed a different morphology among the two studied females, suggesting the occurrence of a chromosomal diversification among distinct molecular clades. Overall, the karyotype of *C. austriaca* shows a mixture of plesiomorphic and derivate chromosomal characters. In particular, the chromosomal characters that can be considered plesiomorphic in colubrids include the karyological formula, the microchromosomal localization of the NORs, and the pair 4 representing the ZZ/ZW sex chromosomes. A chromosome complement composed of 2n = 36 chromosomes with 16 macro- and 20 microchromosomes is displayed by both primitive (Henophidia) and advanced (Caenophidia) snake lineages and is supposed to represent the ancestral snake karyotype (Gorman and Gress, 1970; Singh, 1972;, 1986, Oguiura et al., 2009; Mezzasalma et al., 2014, 2016b, 2019).

Concerning the localization of NORs, the methods here used show their presence on two microchromosomes. Ag-NOR banding suggests that both loci of NORs are active. In fact, Ag binds to proteins essential to nucleolar structure and therefore to the transcriptional activity of ribosomal cistrons during the previous interphase (Howell, 1977; Jiménez et al., 1988). The occurrence of NORs on a microchromosome pair is not unusual among snakes, being exhibited in representatives of various families, including Colubridae (Olmo and Signorino, 2006), and it is considered a primitive condition in Squamata (Porter et al., 1991; Aprea et al., 2006). Derivate chromosome characters in the karyotype of *C. austriaca* can be considered the morphology of the pair 5 (telocentric in all the studied samples), and the occurrence of an heteromorphic, completely heterochromatic W sex chromosome, which is also morphologically differentiated betwenn the two studied samples from different geographic regions. In fact, in the putative ancestral snake karyotype of 2n = 36 all the macrochromosome pairs are biarmed (meta- or submetacentric), though a telocentric morphology of the pair 5 has been observed in different colubrid species belonging to independent evolutionary lineages, such as different species of the genera *Elaphe* and *Hierophis* (see also Singh, 1972; Kobel 1967; Mezzasalma et al., 2015b). According to Singh (1972), because the fifth pair is biarmed in most of the other colubrids, a simple pericentric inversions can be assumed to explain the different morphology of this pair. The ZZ/ZW sex determination system was supposed to be a plesiomorphic state in snakes (Mengden, 1981; Matsubara et al., 2016), however recent evidences suggest that different sex chromosome systems evolved multiple times, independently, in different snake lineages, including species with either female (ZZ/ZW) or male (XX/XY) heterogamety along with a discrete number of species with undifferentiated sex chromosomes (Gamble at al., 2017; Mezzasalma et al., 2019). This makes the suborder Serpentes, and more in general the whole order Squamata, which also includes various taxa with temperature-dependent sex determination (Gamble, 2010; Gamble et al., 2017; Pallotta et al. 2017; Alam et al., 2018), a unique study system to analyze the evolution and diversification of different mechanisms of sex determination. Nevertheless, in the family Colubridae, the fourth macrochromosome pair is usually composed of the ZW elements: the Z is metacentric and conserved in most species, while the W is often heteromorphic compared to the Z and largely heterochromatic (see e.g., Mengden, 1981; Mezzasalma et al., 2015a; Rovatsos et al., 2015; Matsubara et al., 2016). In *C. austriaca*, the studied Greek and Italian samples display a different morphology of the W chromosome, resulting submetacentric and subtelocentric, respectively. In the studied Italian female, the W chromosome is completely heterochromatic but not evidenced with fluorochromes (DAPI and CMA_3). The lack of data on the chromatin composition and distribution of the W chromosome from the Greek female here studied, does not allow us to establish if the differences between the W chromosomes of the Italian and the Greek sample also concern the heterochromatin pattern. Nevertheless, the different W morphology here found among Greek and Italian samples of *C. austriaca* highlight the occurrence of a karyological diversification among different clades of the species (Balkan and Western 2, see Fig. 1 and Jablonski et al. 2019), further supporting that the species contains different diverging evolutionary lineages. From a biogeographic point of view, as already documented for other Palearctic reptiles, the Quaternary climatic oscillations had an important role in shaping the current diversity of extant species, mainly through the contraction and re-expansion of their distribution ranges and the isolation of populations in different “refugia within refugia” (see, e.g., Ursenbacher et al., 2008; Gvoždík et al., 2010; Kindler et al., 2013; Mezzasalma et al., 2018). Unstable climatic conditions, fragmentation of the distribution range, small population size and isolation in distinct glacial refugia are also particularly favorable conditions for the fixation of chromosome mutations (see also Mezzasalma et al., 2015a, 2017a for similar examples in different amphibian and reptile species), which may happen in different populations independently.

In conclusion, this paper provides the first record of a ZZ/ZW sex chromosome system in *C. austriaca*, with the occurrence of different morphologies of the W chromosome in different clades (Western and Balkan). More inclusive molecular and cytogenetic data from other areas of the wide distribution of *C. austriaca* would be useful to characterize the chromosome variability of different molecular clades the European smooth snake, helping to better assess their taxonomy.

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Rana temporaria on Monti della Laga (Central Italy): isolated population or wide distribution? First record in Abruzzo and Marche

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Abstract. In Central Italy *Rana temporaria* is only known to occur as a glacial relict on the eastern side of Monti della Laga (Lazio). In this study we report the presence of the species in other areas of the mountain chain, with documented sightings in five distinct localities in Marche and Abruzzo. We use these new records, together with other occurrence data from the Apennine chain, to generate a species distribution model and perform an analysis of the geological preference of the species in Central Italy. Although the model indicates a wide area of Marche and Abruzzo as suitable for *R. temporaria*, the actual distribution of the species in northern and central Apennine appears strongly associated with sandstones. Therefore, we argue that the presence of this geological substrate on Monti della Laga, but not in surrounding karst uplands, could be among the factors explaining its isolation. Our study aims at paving the way for future surveys and measures to protect these isolated populations from the threat posed by climate change.

Keywords. *Rana temporaria*, relict species, species distribution model, MaxEnt, Central Italy.

The common frog (*Rana temporaria* Linnaeus, 1758) is a monotypic Eurasian species particularly widespread in northern Europe (Gasc et al., 1997; Dufresnes et al., 2020). Adapted to cold climates, in its southern distribution the species is restricted to upland areas, reaching an altitude limit of c.a. 2800 m on the Alps (Tiberti and von Hardenberg, 2012). The southernmost limits of its range are located on the Pyrenees, in the Balkan Region and on the central Apennines (Gasc et al., 1997), where the species occurs mostly in fragmented mountain populations (Bernini and Razzetti, 2006). Indeed, in Italy *R. temporaria* shows a continuous distribution throughout the Alps and the north-eastern Apennines, becoming more localized on the mountainous areas toward Central Italy, where it is only known to survive as a single isolated population on the uplands of Monti della Laga (Bernini and Razzetti, 2006; Stefani et al., 2012). This population is located in Lazio (Rieti Province), on the western side of the mountain chain, about 160 km from the last known occurrence on the northern Apennines (Berni-

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ni and Razzetti, 2006; Razzetti et al., 2007). The site lies between 1400 and 1600 m a.s.l. in the so called "Agro Nero", an area consistent with the ecological requirements of the species, including a mosaic of meadows and beech forests with streams, small lakes (Lago Secco and Lago Selva) and seasonal ponds and puddles that are used as breeding sites (Authors, pers. obs.). The population was reported in 1982 together with a sympatric population of another cold-adapted amphibian, the alpine newt *Ichthyosaura alpestris apuana* (Capula and Bagnoli, 1982). Therefore, *R. temporaria* on Monti della Laga is recognised as a glacial relict, which survived thanks to the favourable environmental conditions of the area (Stefani et al., 2012; Bartolini et al., 2014). However, when compared to the orographically continuous upland karst areas of Monti Sibillini (northward) and Gran Sasso massif (southward), Monti della Laga show a very distinct geology, characterized by sandstones and marls (Pellegrini, 2007). This compact substrate allows rainwater and meltwater to retain longer on the surface, thus favouring the formation of perennial springs and permanent or seasonal small wetlands, both in high-altitude grasslands and in beech forests at lower altitudes. Even though these geological features are common to the whole Monti della Laga chain, *R. temporaria* was not detected in other areas such as the eastern side, which belongs to Marche and Abruzzo regions (Posillico et al., 2017; Cameli et al., 2014). Recently, new observations for the species were made between 980 and 1130 m a.s.l. in the western side of Lazio (Bruni et al., 2016). In the present study we report the first record of *Rana temporaria* in Abruzzo and Marche, providing an habitat suitability analysis to evaluate its potential distribution in Central Italy and facilitate future research activities.

The new observations were made by the authors or collected through Citizen Science.

The individuals were photographed in situ and the coordinates and habitat information were recorded and integrated using the Habitat Map of the Gran Sasso - Laga National Park (Bagnaia et al., 2015). The coordinates were projected on the 10-km^2 grid used in the Italian Atlas of Amphibians and Reptiles (Bernini and Razzetti, 2006) to detect the occurrence of the species in new squares.

Metamorphosed individuals were visually distinguished from congener species (*R. dalmatina* and *R. italica*) according to a combination of morphological features (i.e. size, body proportions, shape of the snout, warts, dorsal, ventral and upper lip colouration), whereas larval stages were identified based on body morphology, col-

Table 1. Relevant data about the five new localities for Marche and Abruzzo; observation date is given as dd/mm/yyyy; locality names are followed by Municipality, Province and Region.

Site	Date	Locality	Coordinates (Latitude / Longitude)	Altitude (m a.s.l.)	UTM square	Developmental stage; habitat, CORINE biotopes code (Bagnaia et al., 2015)	Observer
1	04/06/2015	Monte Comunitore (Arquata del Tronto, AP, Marche)	42.731922N 13.342084E	1607	33T UH63	tadpoles; seasonal pond in compact grasslands of the Mediterranean mountains to *Nardus stricta* and related communities (code 35.72)	Paolo Laghi and Dino Scaravelli
2	22/08/2016	Fosso Rio della Volpara (Arquata del Tronto, AP, Marche)	42.704861N 13.369226E	1238	33T UH62	1 adult female; stream in beech forests of southern and central Europe (code 41.17)	Giovanni Rossi
3	07/07/2017	Valle del Castellano (Valle Castellana, TE, Abruzzo)	42.683516N 13.361997E	1772	33T UH62	1 adult (sex undetermined); stream in compact grasslands of the Mediterranean mountains to *Nardus stricta* and related communities (code 35.72)	Matteo De Albentiis
4	01/05/2019	Fosso della Morricana (Rocca Santa Maria, TE, Abruzzo)	42.656455N 13.397107E	1814	33T UH62	1 adult (sex undetermined); stream in mid-European montane siliceous cliffs (code 62.21), compact grasslands of the Mediterranean mountains to *Nardus stricta* and related communities (code 35.72)	Giancarlo Tondi
5	17/07/2020 04/08/2020	Sorgente "Pane e Cacio" (Campotosto, AQ, Abruzzo)	42.573577N 13.396734E	1796	33T UH61	1 subadult and 1 adult female; stream in mid-European montane siliceous cliffs (code 62.21), Blueberry heaths of the Apennines (code 31.4A)	Francesco Di Toro and Valerio Ricciardi

ouration and mouthparts (Razzetti et al., 2007; Ambrogio and Mezzadri, 2018).

In order to predict and prioritize locations for future search of *R. temporaria* in the area of interest, a habitat suitability map of the species was generated via maximum entropy modelling (MaxEnt 3.4.0; Phillips et al., 2006). The model focused on central and northern Apennines in order to include only the Apennine lineage of *R. temporaria* (Stefani et al., 2012). The presence-only data (49 occurrence points from Abruzzo, Marche, Lazio, Toscana, Emilia-Romagna, Piemonte and Liguria) used for building the model were gathered from personal records and public databases (GBIF, 2020). The complete dataset used for the analysis can be requested to the corresponding author. The environmental variables were selected among bioclimatic, topographic and ecological layers. Altitude, as well as 19 bioclimatic layers averaging the period 1970-2000, were downloaded from the WorldClim 2.1 database (https://www.worldclim.org). Aspect, slope, and distance from water sources were calculated in QGIS 3.12 (http://www.qgis.org/). Ecological layers included vegetation (percent tree cover) and land cover class (https://globalmaps.github.io). All layers featured a 30 arc seconds spatial resolution and were clipped to the extent of the study area (41.5-45.0N; 8.5-14.5E). To eliminate spatial collinearity among predictors, a Pearson's correlation matrix was calculated in R 3.6.1 (R Core Team, 2019). For each pair of correlated variables ($|r|>0.7$), the one believed to be more relevant (according to the biology of *R. temporaria*) was retained. This resulted in the selection of the following variables: Bio8 (mean temperature of the wettest quarter); Bio10 (mean temperature of the warmest quarter); Bio16 (precipitation of the wettest quarter); Bio18 (precipitation of the warmest quarter); aspect; slope; distance from water sources; vegetation and land cover (20 classes). A total of 30 replicates were computed in MaxEnt (default settings), each with 70% of data points randomly used for training and 30% for

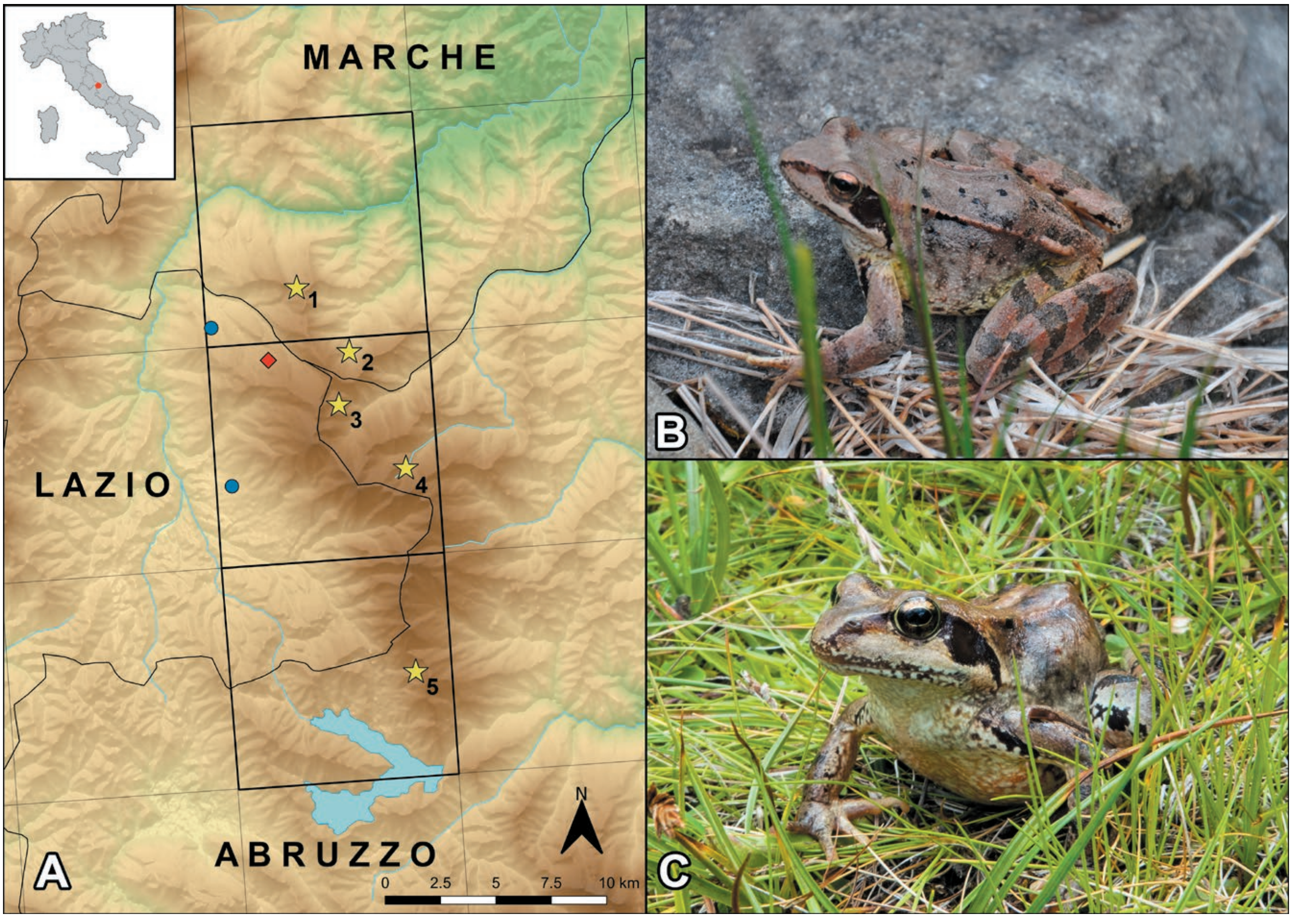

Fig. 1. A) Updated distribution of *Rana temporaria* in Central Italy: Agro Nero (red diamond), reports from Bruni et al. (2016) (blue circles), new data from the present study (yellow stars, numbers refer to Table 1). B) Adult individual from Valle Castellana (TE). C) Adult individual from Campotosto (AQ).

model validation. Jackknife analysis was applied for estimating relative contribution of each predictor to the final model. Model performance was evaluated based on average omission rate and area under curve (AUC) statistics. AUC is a measure of the model's discriminatory ability between presence and background points. A model with low detectability will have AUC values closer to 0.5 (indicating no greater fit than expected by chance), whereas a model with high detectability will have values closer to 1.0 (indicating perfect model fit) (Elith et al., 2006). The average model prediction was used to produce the habitat suitability map for *R. temporaria*. The occurrence data were also used to perform a geological analysis of the substrates, intersecting the coordinates with the Italian Geo-Lithologic Map layer (http://wms.pcn.minambiente.it) in QGIS.

Rana temporaria was found in 5 new localities (Table 1), which represent the first observations of the species for Marche and Abruzzo (Table 1; Fig. 1).

The species distribution model indicates that around 25% of the studied area is considered suitable for the species (Fig. 2). This is mostly concentrated around the Apennines, with suitability values increasing at higher altitudes. Mean temperature of the warmest quarter (Bio10) was by far the most important predictor (57.8% contribution), followed by mean temperature of the wettest quarter (Bio8; 17.2%), precipitation of the wettest quarter (Bio16; 8.0%) and precipitation of the warmest quarter (Bio18; 5.8%). Land cover was the most relevant ecological variable (4.7%), whether vegetation (2.6%), aspect (1.6%), distance from water (1.2%) and slope (1.1%) showed low contribution to the model. The average AUC for 30 replicated runs was 0.975 ± 0.005, indicating high model performance in predicting the species occurring pattern. Accordingly, test omission rate was consistently lower than what expected by chance ($P < 0.001$ for all replicates).

Results from geo-lithological analysis (Fig. 3) show that 42 of 49 points (86%; Tosco-Emiliano and central

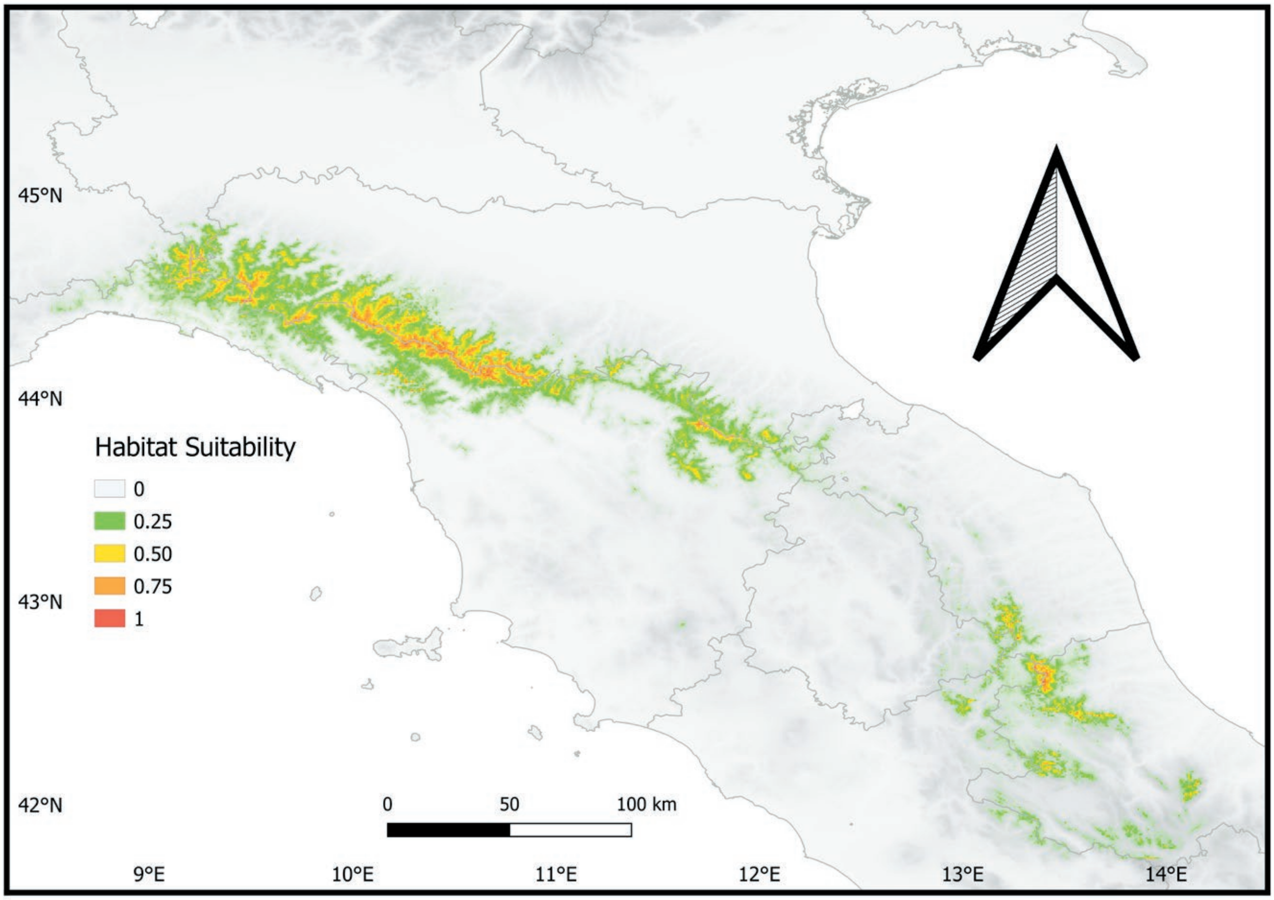

Fig. 2. Species distribution modelling for *Rana temporaria* in the northern and central Apennine region based on maximum entropy algorithm (MaxEnt 3.4.0; Philips et al., 2006). Warmer colours indicate higher habitat suitability.

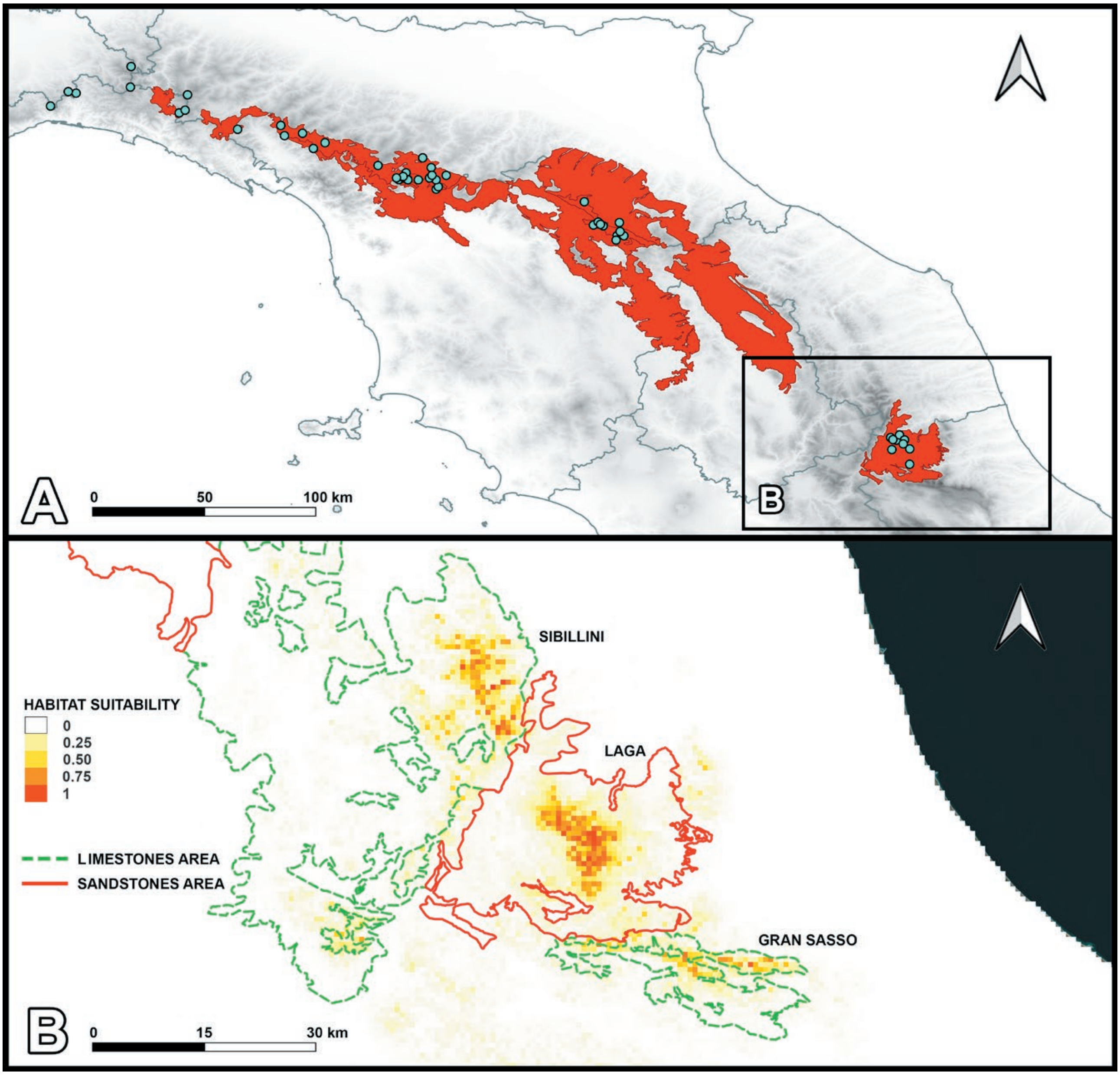

Fig. 3. Graphical results of geo-lithological analysis. A) Presence of sandstones areas (red) in northern and central Apennines and observations used for the analysis (dots). B) sandstones areas (continuous red lines) and limestones areas (dashed green lines) in central Apennines.

Apennines) are located on substrates mainly composed of sandstones, such as pelitic-arenaceous hills and mountains with parental material defined by undifferentiated tertiary sedimentary rocks. The other 7 points (14%; Liguria and Tosco-Emiliano Apennines) are located on more heterogeneous substrates, mostly on calcareous-marly reliefs (limestones) and metamorphic reliefs of basic and ultrabasic rocks.

The new records reveal that *R. temporaria* is far more widespread on Monti della Laga than previously known. The old and new occurrences for the species in Central Italy are located inside the Gran Sasso and Monti della Laga National Park. Besides the confirmation of the presence of *R. temporaria* in the 10-km square 33T UH63 (Bruni et al., 2016), the record near Campotosto (AQ) in the 10-km square 33T UH61 represents a new national atlas square for the species (Bernini and Razzetti, 2006), and the new southern latitudinal limit in the Italian Peninsula (Fig. 1).

The habitat suitability map (Fig. 2) shows that the uplands of the Apennines harbour suitable environmental conditions for *R. temporaria*. Interestingly, a suitability gap is present between the northern and central Apennines, reflecting the fragmented distribution of the species (Bernini and Razzetti, 2006). The contribution of mean temperature and precipitation of the warmest and wettest quarter on the species distribution model can be explained by the fact that these parameters influence the reproductive success *R. temporaria*, which breeding sites frequently consist of sun-exposed seasonal pools, especially in the study area (Cammerini, 2020; Authors, pers. obs.). However, according to the model, suitable environmental conditions are also present within the Monti Sibillini and Gran Sasso massif, areas where *R. temporaria* has never been observed. When considering geological features, it is noticeable that 86% of the occurrence records of *R. temporaria* on the Apennines are situated on sandstone substrates, the principal rock type of Monti della Laga, whereas only 14% occurs on limestones (Fig. 3). Monti Sibillini and Gran Sasso massif featuring mainly the latter, the formation of breeding pools and their hydroperiod might be amongst the factors explaining the absence of *R. temporaria* from these karst areas. Pleistocene fossils of *R. temporaria* were found on Apuan Alps (Bartolini et al., 2014), a karst mountain chain adjacent to the northern Apennines and composed mainly of limestones, where the species does not occur nowadays (Vanni and Nistri, 2006). Since in our model the Apuan Alps resulted currently suitable for the species, it is possible that *R. temporaria* was not able to survive warm periods at higher altitudes due to the unfavourable conditions determined by the type of substrate.

Since *R. temporaria* can be quite cryptic (e.g., Marino et al., 2020), the present study highlights the need for further investigations aimed at assessing its actual distribution in Central Italy. In-depth research about size and structure of the (meta-) populations, gene flow among them and local environmental preferences would be pivotal to guide proper conservation measures in view of future climate change, since frigophilous species limited to isolated mountain ranges are among the most threatened of extinction (Blank et al., 2013).

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Flight initiation distance of *Urosaurus ornatus* from the Sierra de Samalayuca, Mexico

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Abstract. In lizards, flight initiation distance (FID), the distance between a prey individual and a predator when escape begins, can be affected by numerous intrinsic and extrinsic factors, including sex, temperature, and level of conspicuousness. Here we report on a study of FID in a population of Ornate Tree Lizards, *Urosaurus ornatus*, from the Sierra de Samalyuca, Chihuahua, Mexico which are cryptic due to their dorsal coloration blending into their background. *Urosaurus ornatus* in our study population allowed close approaches (mean FID = 65 cm). Mean FID did not differ between males and females. We also found no effect of body, air, or substrate temperature on FID. The short FID we observed may be related to the cryptic nature of *U. ornatus*.

Keywords. Approach distance, anti-predator response, cryptic, lizard, sex, temperature.

For many prey individuals one of the most important decisions that must be made when confronted with an approaching predator is when to begin their antipredator response, which for many prey is flight (Ydenberg and Dill, 1986). Theoretically, this decision should reflect a balance between fleeing too early and losing fitness due to lost opportunities (e.g., foraging, thermoregulation, mating) and fleeing too late and being caught by the predator (Ydenberg and Dill, 1986; Cooper and Frederick, 2007). The distance from the predator to the prey when the prey begins to flee is the flight initiation distance (FID) and is a readily measured aspect of a prey's behavior (Ydenberg and Dill, 1986; Cooper and Frederick, 2007).

For lizards, FID can be affected by numerous intrinsic and extrinsic factors, including body and environmental temperature (e.g., Smith and Lemos-Espinal, 2005; Cooper et al., 2009; Braun et al., 2010; Cooper, 2011a); perch, habitat, or microhabitat characteristics (Cooper, 2003b; Cooper et al., 2009; Morris and Lattanzio, 2020), and sex (Vanhooydonck et al., 2007; Majláth and Majláthova, 2009; Salido and Vicente, 2019). However, these factors do not always affect FID in lizards (e.g., temperature: Martin and López, 2000; Amo et al., 2005; Smith and Lemos-Espinal, 2005; Cooper, 2006; sex: Cooper, 2003a, 2011b; Cooper and Pérez-Mellado, 2011; Kopena et al., 2015). For example, because lizards are ectotherms, temperature, whether body or environmental, can influence their locomotor performance, including sprint speed (van Damme and Vanhooydonck, 2001; Herrel et al., 2007). Thus, when temperature does affect lizard FID, the FID typically decreases with body or environmental temperature since lizards at higher temperatures can usually run faster and therefore can allow a predator to approach closer and still escape compared to lizards at lower temperatures (e.g., Cooper, 2006, 2011b; Cooper et al., 2009; Braun et al., 2010).

Another aspect of a lizard that could affect its FID is the level of crypsis. In general, organisms with effective

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crypsis should remain still and allow close approaches (Cooper et al., 2008). Compared to other aspects of lizards that might affect FID, the effects of conspicuousness are relatively understudied. However, previous work related to the conspicuousness of lizards and FID has generally found that more cryptic or more concealed lizards allow closer approaches than less cryptic or less concealed lizards (Cooper, 2006; Vanhooydonck et al., 2007; Cooper et al., 2009; Cooper and Sherbrooke, 2010).

Here we report on a field study of FID in a population of Ornate Tree Lizards, *Urosaurus ornatus*, from the Sierra de Samalyuca, Chihuahua, Mexico. *Urosaurus ornatus* are distributed from Utah and Colorado to northern Mexico (Wiens, 1993; Haenel, 2007) and their populations often primarily use trees as perches (Baltosser and Best, 1990; Smith, 1996b; James et al., 2003) but some populations are more terrestrial, using rocks as perches (Herrel et al., 2001; Haenel, 2018; Taylor et al., 2018), including the population we studied (see also Gadsden et al., 2021). *Urosaurus ornatus* tend to be relatively small lizards (mean SVL = 50 cm; Smith, 1996b) that are sit-and-wait foragers (Cooper et al., 2001). We examined whether body temperature and sex affect flight initiation distance. In addition, *U. ornatus* can adjust their dorsal coloration and reflectance to match their background (Zucker, 1989; Hamilton et al., 2008), making them cryptic. Given their ability to blend into their background (see Fig. 1), we expected that *U. ornatus* would allow closer approaches compared to other species (i.e., have a relatively short FID). We also predicted that temperatures, both body and environmental, would have little effect on FID since they may rely more on being still and remaining cryptic rather than relying on locomotor performance which can be affected by temperature (e.g., Gilbert and Miles, 2016, 2017).

We studied the FID of *U. ornatus* on 10 and 11 November 1998 in a population at the Ojo de Enmedio, Sierra de Samalyuca, Chihuahua, Mexico ((31°22’48.2”N, 106°35´2.7”W, 1344 m elevation). Ojo de Enmedio is a small ranch located approximately 10 km northwest of the town of Samalayuca (municipality of Juárez, Chihuahua), in the foothills of the extreme northwest of the Sierra de Samalayuca. The vegetation is typical xerophyte scrub of the Chihuahuan Desert.

When we spotted a stationary and undisturbed lizard, one of us (JLE) slowly and directly approached it at a constant speed. The same person always made the approach to promote a more consistent appearance and approach among lizards. We measured FID as the distance between the location where the approaching “predator” was when the lizard first fled and where the lizard was first observed (to nearest cm using a meter tape). Since we walked through the study area and did not return to a specific site it is highly unlikely we repeated measuring FID on any individual. We captured all lizards and recorded their sex. We also measured body temperature (T_b), air temperature (T_a: 1 cm above surface at location lizard first observed), and substrate temperature (T_s: on surface at location lizard first observed) to the nearest 0.1 C using a quick-reading cloacal thermometer. All lizards were captured within 1 minute of determining FID. All lizards were in full sun when first observed.

Fig. 1. Photograph of a *Urosaurus ornatus* on a rock substrate demonstrating its cryptic dorsal coloration. Photograph was taken from a population at San José de las Piedras, municipality of Ocampo, Coahuila but the *U. ornatus* from our study site are very similar. Photograph by J.A. Lemos-Espinal.

We compared FID between males and females using an analysis of variance on log-transformed FIDs. We used linear regressions to analyze the relationships between FID and T_b, T_a, and T_s. We used JMP Pro 14 (SAS Institute, Cary, North Carolina, USA) to conduct all statistical analyses and used an a-value of 0.05 to determine statistical significance. Means are given ± 1 S.E.

Overall mean FID was 64.7 ± 6.7 cm (n = 50). Mean FID did not differ between males (68.9 ± 8.4 cm; n = 32) and females (57.4 ± 11.2 cm; n = 18; $F_{1,48} = 0.18$, P = 0.67). Flight initiation distance was not affected by T_b (n = 50, $r^2 = 0.057$, P = 0.10), T_a (n = 50, $r^2 = 0.02$, P = 0.34), or T_s (n = 50, $r^2 = 0.03$, P = 0.22).

Urosaurus ornatus in our study population allowed close approaches by the human simulated predator (i.e., mean FID = 65 cm). Indeed, in Samalayuca, it is possible to capture *U. ornatus* directly with one’s hands rather than needing to use a lasso (J.A. Lemos-Espinal, pers. observ.). *Urosaurus ornatus* in Arizona also allow similarly close approaches, with a mean FID in males of 90 cm and 64 cm in females (Morris and Lattanzio, 2020). The short FID we observed, and as has been observed in other *U. ornatus*, may be related to the cryptic nature of *U. ornatus* (Zucker, 1989; Hamilton et al., 2008). The mean FID of *Urosaurus bicarinatus*, a congener but not sister species of *U. ornatus* that occurs further south in Mexico (Wiens, 1993; Reeder and Wiens, 1996) and uses acacia trees as perches (Lemos-Espinal et al., 1997), in the Cañón del Zopilote in Guerrero was 200 cm (Smith and Lemos-Espinal, 2005), which is almost 3x greater than the FID we observed. However, in our experience, *U. bicarinatus* in the Cañón del Zopilote occurs mainly on trees, and although still cryptic, are more readily seen by humans than the *U. ornatus* in the Sierra del Samalayuca (J.A. Lemos-Espinal, pers. observ.). Other phrynosomatid lizards from the southwestern United States and Mexico have greater FIDs (e.g., *Sceloporus virgatus*, 160-310 cm, Smith, 1996a; Cooper and Avalos, 2010; *S. anahuacus*, 260 cm, Smith and Lemos-Espinal, 2005; *S. gadoviae*, 283 cm, Smith and Lemos-Espinal, 2005; *S. jarrovii*, 150-290 cm, Cooper and Avalos, 2010; *S. mucronatus*, 605 cm, Smith and Lemos-Espinal, 2005, *Uta stansburiana,* 210 cm, Keehn and Feldman, 2018). Our results are also consistent with other studied lizards that are cryptic. For example, when in situations in which they are more cryptic, *Phrynosoma modestum* allowed closer approaches than when they were less cryptic, especially at lower temperatures (Cooper and Sherbrooke, 2010), and cryptic species of *Anolis* allow a human to approach closer than other less cryptic species (Cooper, 2006; Vanhooydonck et al., 2007).

We found no effect of body, air, or substrate temperature on FID, perhaps because of the relatively low overall FID we observed. It may be that the FID of cryptic species is less affected by body and environmental temperatures than other species since predator avoidance is not predicated on locomotor performance. This is also consistent with the fact that air and substrate temperature did not affect FID in *U. bicarinatus* (Smith and Lemos-Espinal, 2005). However, the FID of *U. ornatus* in Arizona decreased with increased body and perch temperature (Morris and Lattanzio, 2020), suggesting the situation may be more complex. Indeed, whereas temperature did not affect FID, *U. bicarinatus* that were captured were using perches with lower T_as than those that escaped (Smith and Lemos-Espinal, 2005), indicating that the ability to escape, if not their predilection to flee, may still be related to temperature in these lizards. The lack of effect of temperature on FID has also been observed in non-cryptic lizards (e.g., Martin and López, 2000; Amo et al., 2005; Smith and Lemos-Espinal, 2005; Cooper, 2006). However, in other species of lizards, FID typically decreases with increased body or environmental temperatures (e.g., Cooper, 2006, 2011a; Cooper et al., 2009; Braun et al., 2010), and FID in *Hobrookia propinqua* increases with substrate temperature (Cooper, 2000). The variation in the effects of temperature on FID in lizards needs more study to understand why there is a relationship in some species but not in others.

The lack of a difference in approach distance between male and female *U. ornatus* in our population contrasts with results from another population of *U. ornatus* where male *U. ornatus* had greater FID than females (Morris and Lattanzio, 2020). However, our result is consistent with results from *U. bicarinatus* (Smith and Lemos-Espinal, 2005). In a review of the literature, Cooper (2011b) found that in general most species of lizards show no sexual dimorphism in FID. However, females in some lizards allow closer approaches than males (Majláth and Majláthová, 2009; Vanhooydonck et al., 2017; Salido and Vicente, 2019). Why some populations and species show sexual dimorphism in FID and others do not is not clear and warrants more direct investigation.

In conclusion, the cryptic nature of *U. ornatus* in our population may lead to a short FID since staying still is probably better than fleeing. This supposition is supported by the lack of an effect of temperature, both body and environmental, and sex on FID in this population. However, directly assessing the effect of crypsis on FID is needed to confirm this conclusion.

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Book Review:
Mariella Turrini – La tartaruga nella cultura dei popoli – Miti, leggende e Folklore

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The title of this book, written in Italian, may be translated as: "Turtles in people's Culture – Myths, Legends and Folklore". The author, Mariella Turrini, is an Italian literature teacher loving animals and travels and this book is clearly the outcome of the cross-over of these two beloved passions.

There are nearly no books entirely dedicated to turtles, tortoises and terrapins in culture, art and myth, and therefore this volume seems to fill a real cultural gap. Among the exceptions, worth mentioning is "Tortoise" by Peter Young (2003), an interesting small book with a very different structure and with a much narrower scope. Remarkable, but focused on a single topic, is also the booklet edited by Del Cimmuto (2013) that nicely illustrates a thematic collection of art objects and painting portraying turtles, tortoises and terrapins. Conversely, Mariella Turrini's book is a lengthy, comprehensive work composed by 337 pages of text, 14 pages of notes and 60 illustrations, many of which are original colour artworks and line drawings by the author herself. The long lists of acknowledgments and references testify for a genuine research effort and a solid bibliographic background.

This volume has a well-defined structure. There is a short "Presentation", written by Franco Andreone, herpetologist from the Regional Museum of Natural Sciences of Torino. Then the "Introduction" recalls that it was from the Latin term *Testudo* that the Italian title word "*testuggine*" was derived. In the Latin language, *testudo* was associated not only with the animal, but also to a stringed musical instrument, obtained from its shell (i.e., the *Lyra*). Moreover, the *testudo* described a Roman military tight formation, in which rectangular shields were interlocked around and over soldiers underneath, to protect them completely.

After this "Introduction", four short chapters "Symbolism", "Etnomedicine", "Christianity" and "Medieval Age to the Renaissance" are dedicated to the historical use, the popular tales, and the ancient legends associated to turtles, terrapins and tortoises, in the Mediterranean area. It is noteworthy, that in some of these historical societies the turtles, and their symbolic representations, were often associated with a negative belief, in the sense that these animal were often considered belonging to the "*Tartarus*" the infernal region created by the ancient Greek mythology. However, in more recent cultures these animals assumed a much more positive symbolic meaning and, as stated by Andreone in the "Presentation", are now the only reptiles generally appreciated by humans. Indeed, in many different societies all over the World, these animals are considered harmless, calm, and wise creatures. Moreover, they often became associated with personal longevity, character stability and in general with popular wisdom. Finally, in many societies turtles and tortoises became also the symbolic representation of human fertility.

The book "La tartaruga nella cultura dei popoli", continues with a complete cultural tour around the World representing the core of the volume both in terms of number of pages and amount of information. Indeed, the central and main chapters are dedicated to a well-docu-

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mented and in most cases first-hand description of turtles in art, literature, poetry, myth and cultural traditions of "Europe", "Africa", "Middle East", "Asia", "Oceania", North America", "Central America - Pre-Columbian Societies" and, in the final chapter "Southern America - Circum-Caribbean and sub-Andean Societies".

It is clear that this book is the result of many years of passionate work and to the many travels all around the globe, completed by the author Mirella Turrini over the years.

All persons attracted to the many humanistic and cultural aspects related to these strange and very peculiar reptiles could be interested in reading this book, although it is written in Italian and therefore could have a limited distribution. An English translation, maybe omitting or shortening the purely geographical introductory notes to each country in order to avoid a certain dilution of the turtle topics, would be therefore very welcome.

The book "La tartaruga nella cultura dei popoli – Miti, leggende e Folklore" is edited and available from Edizioni Belvedere (Latina, Italy) that with this new publication consolidates furthermore its position as a leading publisher of herpetological books.

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