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Estimating abundance and habitat suitability in a micro-endemic snake: the Walser viper

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Abstract. Recently described species suffer lack of information that hampers setting up appropriate conservation strategies. The situation is particularly complex with micro-endemic snakes, for which detection and monitoring are particularly challenging. The Walser viper *Vipera walser* is a recently described snake inhabiting a small area of the SW Italian alps. We combined information on species distribution with repeated monitoring to identify the areas most suitable for the species, and to obtain estimates of species abundance. Species distribution models were used to identify the topographical, climatic, and land-cover features related to the occurrence of vipers. Furthermore, repeated transects and *N*-mixture models were used to estimate abundance and to identify factors related to the variation of abundance. The available data suggested that the species has a disjunct range, with a Northern range of ~45 km^2, and a southern range of ~225 km^2. Distribution models suggested that vipers are associated with areas with open vegetation, altitude between 1300 and 2300 m, high precipitation, low forest cover, low slope, and southern aspect. *N*-mixture models confirmed very low detection probability of these vipers, and suggested that the species has a low abundance, with the highest abundance in south-facing plots. We provide the first quantitative information on habitats and abundance variation for Walser vipers. The broad confidence intervals of abundance estimates exemplify the complexity of providing range-wide measures of abundance for secretive species. Given the narrow range of these vipers, continuous monitoring is required to understand how they respond to ongoing environmental changes in mountainous areas.

Keywords. Alpha-hulls, detectability, endemism, habitat suitability models, land cover, *Vipera walser*.

INTRODUCTION

Italy is among the European countries with the highest endemism of amphibians and reptiles (Sillero et al., 2014). In the last decades, the integration of new genetic and morphological data has greatly expanded our knowledge of Italian biodiversity, with the identification of several reproductively isolated lineages, that have been pro-

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posed as new candidate species (e.g., Cornetti et al., 2015; Dufresnes et al., 2018; Senczuk et al., 2019).

The Walser viper has been a striking addition to the Italian herpetofauna. In NE Piedmont, the occurrence of vipers morphologically assumed to be adders (*Vipera berus*) has long been recognized (Andreone and Sindaco, 1998; Sindaco et al., 2006). However, recent genetic data revealed strong genetic differences between these "adder-like" vipers and true adders; the closest relatives of these vipers belong to the cluster regrouping *V. dinniki*, *V. kaznakovi* and *V. darevskii*, in the Caucasus area. As a consequence, these adder-like vipers have been described as a new species, the Walser viper (*Vipera walser*, Ghielmi et al., 2016), a species endemic of a small area from the mountains between Biella and the Ossola valley, with a possible range < 500 km^2 (Fig. 1).

Recently described, micro-endemic species often suffer a tremendous lack of biological data. This is particularly problematic, because the small range inherently exposes these species to a high risk of extinction. Therefore, sound biological and ecological information is required to set up appropriate monitoring and conservation programs. Habitat suitability and spatial variation of abundance represent critical information to assess the conservation status of species. In the last years, advances in analytical tools have greatly improved our ability to provide sound biological information even in species for which only a limited amount of data is available (e.g., Raxworthy et al., 2003; Peterson et al., 2011; Mazerolle, 2015).

In this paper, we combine modelling and field data to improve our knowledge of the ecology of the Walser viper. First, we used habitat suitability models to evaluate the relationships between viper distribution, land cover, and topographical features at a fine spatial scale. Correlative species distribution models (SDM) allow to identify relationships between localities of presences and spatial variation of environmental variables, and can provide multiple key information (Peterson et al., 2011). SDM can reveal how the species respond to broad-scale variation of environmental features, thus providing first information on the habitats that are more suitable for the target species (Guisan and Thuiller, 2005; Peterson et al., 2011). These models often have a coarse spatial scale and sometimes lack accurate information on species microhabitat or on habitat features that can only be measured in the field (Beck et al., 2012; Potter et al., 2013; Ficetola et al., 2018b). Still, if reliable maps of habitats or land cover are available, it is possible to obtain relatively detailed information on species responses to major land cover categories. Furthermore, SDM can produce spatially-explicit maps, that can refine information on species range. For instance, Raxworthy et al. (2003) developed SDM for chameleons in Madagascar, and then performed targeted surveys in areas suggested to be suitable by models. In several cases, these targeted surveys revealed new localities of occurrence, suggesting that SDM can greatly improve our knowledge on species living in remote areas, or for which information on the distribution is limited.

Second, we performed repeated visits in a large number of patches within the species range, to assess spatial variation of abundance. Variation of abundance is a key parameter to assess the threat status of species, still, accurate measurement of abundance can require extremely extensive workload (Pollock et al., 2002; Dodd, 2010). In the last years, approaches have been proposed to obtain estimates of abundance from repeated counts, without marking or capturing individuals (Royle and Nichols, 2003; Royle, 2004). The number of individuals counted at fixed sites on multiple occasions can be used to estimate the detection probability, and the size of populations can be estimated on the basis of *N*-mixture models (Royle and Nichols, 2003; Royle, 2004; Kéry et al., 2009; Dail and Madsen, 2011). Despite several limitations (Barker et al., 2018; Link et al., 2018), such models can provide cost-effective estimates of abundance while accounting for imperfect detection (Ficetola et al., 2018a; Kéry, 2018), as detection probability is typically low in snakes. In addition, *N*-mixture models can provide insights on the factors determining spatial variation of population abundance and, if monitoring is repeated through time, they can provide reliable estimates of population trends (Ficetola et al., 2018c). These data are particularly critical for recently described species, for which information on abundance is nearly absent.

METHODS

Species range and distribution models

In March 2019, we combined bibliographic data (Ghielmi et al., 2006; Ghielmi et al., 2016) with new field observations performed in 2016-2018 by the authors, and personal communications by local naturalists, to gather an exhaustive dataset of *V. walser* occurrences. We used the alpha-hull approach for an accurate definition of the species range. The alpha-hull approach is a procedure based on Delauney triangulation that uses presence points to estimate species ranges, and can allow for the exclusion of unoccupied areas within a species range (Burgman and Fox, 2003). Simulations showed that the alpha-hulls provide better approximations of species ranges compared to minimum convex polygons, particularly when the ranges have discontinuities (Burgman and Fox, 2003). Alpha-hulls were built using the alphahull package in R (Pateiro-Lopez and

Rodrjguez-Casal, 2010), following the procedure detailed in Ficetola et al. (2014).

Land cover data were obtained from the geoportal of the Piedmont region (http://www.geoportale.piemonte.it/) at the resolution of 2 m. We considered four land cover categories: agricultural, pasture, natural open vegetation, and forest. As a measure of land cover, we calculated the percentage of each land cover category in 100 × 100 m cells. Furthermore, we considered three topography variables: altitude (m a.s.l.), aspect (northness), and slope. As a climatic parameter, we considered summed annual precipitation, obtained through the Chelsaclim dataset (Karger et al., 2017). This dataset is available at a coarser resolution than the other predictors (30 arc-seconds, approx. 650 × 900 m in the study area). We therefore used the *B*-spline interpolation to downscale it at the 100-m resolution (see Karger et al., 2017). Temperature variables were not included because they were strongly collinear with altitude ($r > 0.9$). The pairwise correlation between the remaining environmental variables was always $|r| < 0.65$, suggesting limited collinearity issues (Dormann et al., 2013).

We used maximum entropy modelling (MaxEnt) (Phillips et al., 2006; Elith et al., 2011) to build species distribution models relating the occurrence of vipers to land cover and topographical data. MaxEnt is a presence-background approach that evaluates the suitability of a given cell on the basis of environmental features (habitat, climate, topography…) in that cell. Comparative studies showed that MaxEnt is among the most efficient approaches to build SDM (Elith et al., 2006; Elith et al., 2011). MaxEnt is well suited to evaluate complex or nonlinear relationships between species and environmental features, and produces an output representing the suitability of a specific area. We run models with linear, quadratic, and hinge features. To identify the best regularization parameter (b), we build five models with increasing values of b: 1, 2, 5, and 10 (Warren and Seifert, 2011). The model with b = 2 showed the highest cross-validation performance (see below), and was selected as model with highest generality (Warren and Seifert, 2011).

We used two approaches for the validation of species distribution model. First, the model was tested using a 5-fold cross-validation. Presence records were split into five sub-sets; the model was built using 80% of data (calibration data), and we tested predictive performance on the remaining 20% (test data). We repeated this procedure five times, each time using a different test dataset (Nogués-Bravo, 2009). As a measure of model performance, we calculated the area under the curve of the receiver operator plot (AUC), averaged over the five runs. AUC is an imperfect measure of the performance of SDM (Lobo et al., 2008), thus we also used a binomial test to evaluate if our models predict presence records better than expected under randomness, comparing the observed frequencies of correct and incorrect predictions. In this test, we assumed that a cell is suitable for vipers if it has suitability larger than 10th percentile training test threshold (Pearson et al., 2007). Second, the model was developed in late winter 2019. Therefore, we used data collected during the 2019 field activities to confirm the reliability of model predictions. Specifically, we used a likelihood ratio test to assess whether viper observations are more frequent in areas with high SDM suitability, compared to areas with low suitability. Since we have a-priori expectation on the frequency of observations (i.e., we expect more observations in high-suitability areas), we used a one-tailed test (Warren and Seifert, 2011).

Field activities

In the period 2016-2019, we established 71 fixed plots using visual encounter surveys (Crump and Scott, 1994). Each plot was visited during one year only (range: 5-26 plots per year); plots were visited multiple times (average: 5.7 surveys per plot; range: 3-12 surveys) from late May to early October, i.e., during the period of highest activity of vipers. The size of plots ranged between 600 and 23,000 m^2 (mean: 7600 m^2), because of logistic and accessibility constraints. Visits were performed from 7 am to 17 pm (solar time), but most of them (69%) were performed in late morning (between 8.30 and 12.00 am). Plots were placed across the whole range of the species, mostly nearby areas with previous records of Walser vipers. In 2019, the location of plots was selected after the development of species distribution models, in order to validate the SDM and identify eventual new locations of the viper. Out of the 17 plots surveyed in 2019, three were in areas with low SDM suitability (suitability below the 10th percentile training presence threshold; range: 0.06-0.22) but nearby areas with high suitability. Ten were in areas with high SDM suitability (> 0.45) and nearby localities where the species is known to be present, and four were in areas with high suitability but out of the known species range (Mombarone; roughly 3 km SE of the southern limit of the species range, see Fig. 1b). During surveys, one to four observers carefully patrolled the plots, searching for active vipers. In other areas, the search under artificial shelters greatly improved the detection of reptiles (De Leo et al., 2006; Joppa et al., 2010; Sewell et al., 2012). Therefore, in 2019, visual encounters were integrated with the use of artificial cover objects (shelters). In each plot, we placed three corrugated bitumen 70 × 90 cm shelters that were checked during each survey.

Assessment of species abundance

We used *N*-mixture models to estimate the abundance of vipers on the basis of repeated counts at plots, and to identify environmental variables related to variation of abundance. *N*-mixture models allow the joint estimation of animal abundance and detection probability on the basis of repeated surveys at fixed sites, without the need of capturing and marking individuals for identification (Royle, 2004). Some analyses highlighted that jointly estimating detection probability and abundance could be problematic, and these models are sensitive to violations of their assumptions (Barker et al., 2018; Duarte et al., 2018), still analyses of real-world data showed that *N*-mixture models can provide reliable estimates of the abundance of wild vertebrates (Ficetola et al., 2018a; Kéry, 2018; Costa et al., 2019). We assumed that each plot, sampled during one single season, represented a closed population. We thus used a static (i.e., non-dynamic) *N*-mixture model formulation. The average distance between each plot and the closest one was 170 m (SE

= 19.6), a distance much longer than the typical movements of related species (e.g., Zuffi et al., 1999; Nash and Griffiths, 2018). *N*-mixture models were fitted using a Poisson error distribution, as in preliminary analyses Poisson models showed a lower Akaike's Information Criterion (AIC) (Burnham and Anderson, 2002) than the corresponding zero-inflated Poisson models. In *N*-mixture models, we set the upper bound to approximate the infinite summation of the likelihood (K) at the maximum observed species abundance +100, since simulations suggested that this value provides stable and robust estimates (Ficetola et al., 2018c).

We considered four variables potentially influencing the detection of vipers: date (day of the year), hour, length of the survey (in minutes, log transformed), and the number of people participating in the survey. For date and hour, we also tested quadratic terms to take into account potential non-linear relationships. To identify the model best explaining variation of viper detection, we built models including all the potential combinations of independent variables, and ranked them on the basis of their AIC values. The model with lowest AIC value explains more variation with a limited number of parameters, and was selected as the minimum adequate model (Burnham and Anderson, 2002). First, we performed model selection on variables affecting detectability, then we performed model selection on variables affecting abundance.

We considered six variables potentially affecting the abundance of vipers at plots: plot surface, aspect, altitude, slope, and suitability predicted by the species distribution model. SDM suitability was included because previous studies suggested that environmental suitability models also help to predict parameters such as population density and fitness-related traits (Brambilla and Ficetola, 2012; Weber et al., 2017; Lunghi et al., 2018). Variables representing land cover were not included because 85% of plots were in areas with open natural vegetation, which is the most important land cover variables determining viper distribution (see results). We built models with all the possible combination of variables potentially affecting abundance, and ranked them using AIC, while keeping constant the observational component of the model (i.e., including the variables best explaining variation in detection probability). The correlation coefficient between site covariates was weak ($r < 0.4$ for all the considered variables).

N-mixture models were run with the package Unmarked in R (Fiske and Chandler, 2011). Before analyses, independent variables were scaled at mean = 0 and SD = 1 to improve model convergence. A goodness-of-fit test showed limited overdispersion and confirmed that the model was appropriate to the data ($\chi^2 = 432.6$, permutation $P = 0.12$, c-hat = 1.1).

RESULTS

Distribution data, species range, and distribution model

Overall, we gathered 117 distribution records (Fig. 1a). Localities from the literature (Ghielmi et al., 2006; Ghielmi et al., 2016) accounted for 50% of records, while 47% of records were obtained through recent surveys by the authors; we also obtained some personal communications by local naturalists (3% of records). Species records were clustered in two main areas: a northern group between the Strona and the upper Sesia Valley, and a southern group between the lower Sesia Valley and the Biella Alps. The lack of known records between these two areas suggests the possibility of a disjunct distribution, with a northern range of approx. 45 km^2, and a southern range of approx. 225 km^2.

The species distribution model suggested that vipers were associated to areas with open natural vegetation, altitude between 1300 and 2300 m, high annual precipitation, low forest cover, low slope, and with a southern aspect (Fig. 2). The presence of agricultural land cover and pastures showed very limited importance (relative importance < 1%). The cross-validation procedure suggested excellent performance, with average AUC on test data = 0.924 (SD = 0.038). Using the 10th percentile training presence threshold (suitability threshold = 0.36), the model correctly predicted occurrence at 86.3% of test data, a performance significantly better than expected by chance (binomial test: $P << 0.001$; success rate expected under randomness = 13.5%).

The model identified several patches of highly suitable habitats through the whole species range, in mountainous areas at altitude of 1300-2200 m, with gentle, south-facing slopes and covered by open vegetation. Within the species range, the total suitable surface (i.e., with suitability higher than the 10th percentile threshold) was 81.9 km^2. Several areas with good suitability were detected outside the known range of the species, for instance, in the SW of the study area (e.g., the Mombarone area) (Fig. 1b). Potentially suitable areas were also detected between the northern and southern populations.

Model validation with the 2019 data

Four plots surveyed in 2019 were outside the known range of *V. walser*, but in areas showing high suitability according to the SDM (Mombarone area; Fig. 1b). Each of them received six surveys; we never detected vipers at these plots. Thirteen plots surveyed in 2019 were inside the known range of *V. walser*; four were in areas with low suitability (suitability ≤ 0.2) and nine in areas with high suitability (> 0.45). Vipers were never detected in the plots with low suitability, while they were detected in 56% of plots with high suitability. The possibility to obtain at least one detection was significantly higher in plots with high suitability (likelihood ratio test: $\chi^2_1 = 4.96$, $P = 0.013$), confirming that the SDM can successfully predict viper occurrences.

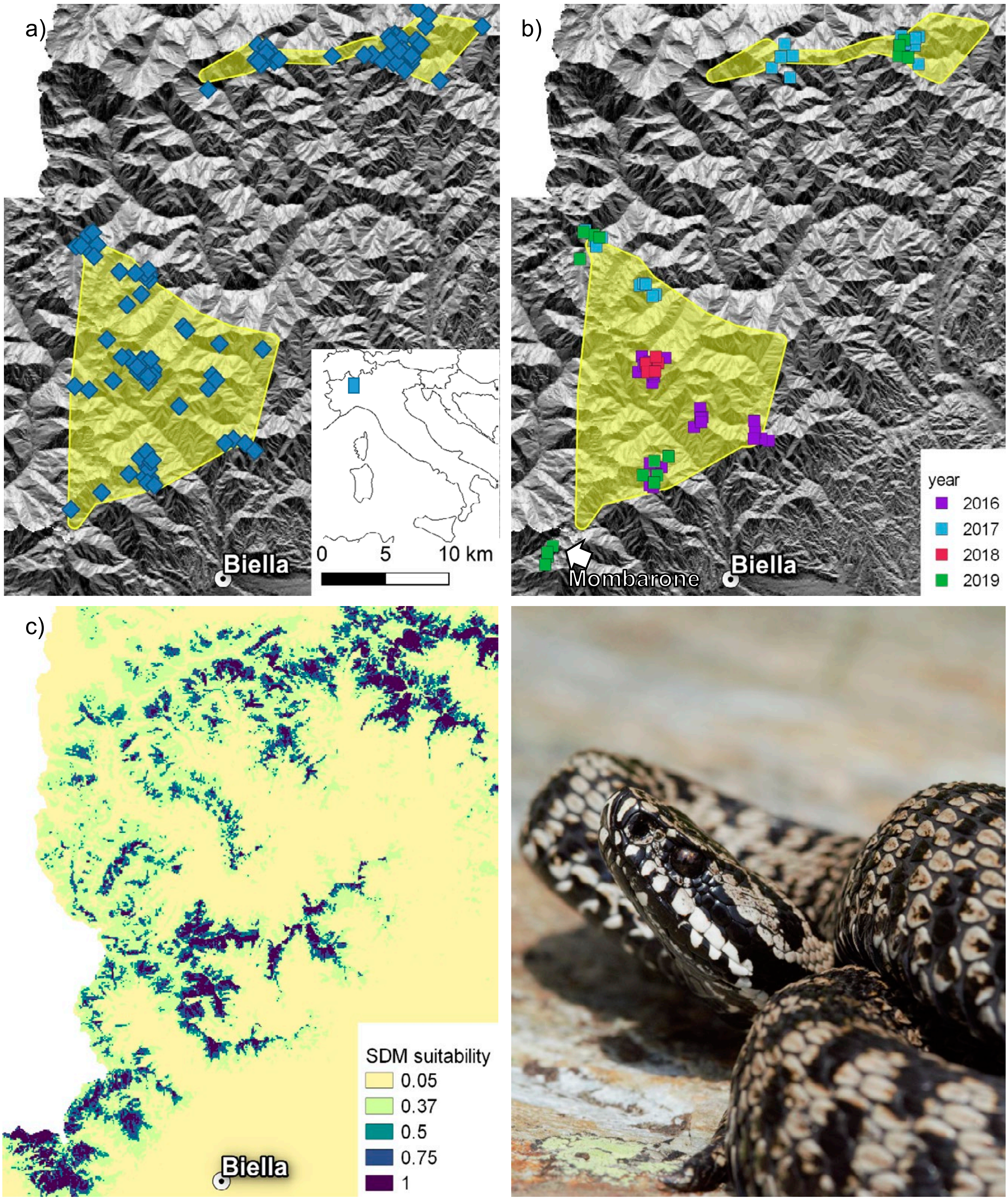

Fig. 1. a) Distribution records used to build species distribution models, and species range estimated using alpha-hulls. b) Location of fixed plots where we performed repeated counts. The arrow indicates the plots located in possibly suitable sites outside the known range of the species (Mombarone). c) Suitability map, estimated using species distribution models. 0.37 is the 10th training presence threshold. To limit the risk of poaching, points plotted on the maps have a random spatial error of up to 2 km (Lunghi et al., 2019). d) An adult male of *V. walser* (photo by GFF).

Repeated counts and abundance estimations

Overall, we obtained 39 viper detections from 24 out of the 67 plots within the species range. Vipers were detected across the whole study period (from June to early October). The best-AIC model suggested that the number of detection was only affected by the length of surveys, longer surveys allowing a higher probability of detecting vipers ($B \pm SE = 0.73 \pm 0.16$, $z = 4.45$, $P < 0.001$). Conversely, the detection rate was unrelated to date or hour of the survey, nor to the number of observers (Table 1). The detection probability of vipers was approx. 0.036 in 35-min. surveys (35 min. was the average length of surveys), but the incertitude of these estimates was large (95% CI of detection probability after 35 min: 0.007-0.16).

The abundance of vipers was negatively related to the northness of plots, with higher abundances in south-facing plots ($B = -0.70 \pm 0.30$, $z = -2.35$, $P = 0.019$). We did not detect any relationship between the remaining variables and the abundance of vipers (Table 1). The estimated abundance per plot ranged between zero and five individuals; the best estimate of total abundance across the 67 plots was 175 vipers, but confidence intervals were wide (95% CI: 38-383 vipers). Therefore, at the surveyed plots, the average density was 3.4 individuals / ha.

Table 1. a) Relationships between detections of *Vipera walser* and variables potentially influencing detection. The table reports the outcome of univariable *N*-mixture models, in which only each of these variables was related to the viper detections. All the models with more than one variable showed higher AICc values than the one with length of survey only. b) Relationships between detections of *Vipera walser*, and variables potentially influencing abundance. The table reports the outcome of *N*-mixture models, in which viper detection probability was related to length of survey, and viper abundance was related to each of these variables. All the models with more than one variable showed higher AICc values than the model with aspect only (Appendix 1).

	AICc	*z*	*P*
a) variables potentially influencing detection			
Date*	262.9	-0.321	0.749
Hour*	261.6	-1.14	0.256
N observers	262.8	0.51	0.610
Length of survey	244.4	4.48	**<0.001**
b) variables potentially influencing abundance			
Altitude*	246.7	0.13	0.895
Aspect	238.6	-2.35	**0.019**
Slope	245.2	1.24	0.217
Plot area	246.7	-0.22	0.830
Precipitation	245.7	1.03	0.302
SDM suitability	243.5	1.66	0.097

* Preliminary tests did not show any effect also for quadratic terms.

DISCUSSION

For recently described species, rapid biological and ecological studies are pivotal for a prompt assessment of the species status. By combining distribution records with ecological modelling and repeated surveys we provide key ecological data on the endemic, poorly known, Walser viper.

The application of alpha-hulls to species distribution data suggested that this viper has a disjunct range, with a northern range comprising the Strona and the upper Sesia Valley, and a southern range between the Biella Alps and the lower Sesia Valley. Alpha-hulls have an excellent capacity to reveal discontinuities in the specie range (Burgman and Fox, 2003), still they can be affected by the lack of biological records caused, for instance, by limited surveys. The available genetic data did not detect strong differences among Walser vipers (Ghielmi et al., 2016), still additional genetic or genomic studies, using highly variable or fast-evolving markers, are needed to understand the fine-scale genetic variation among the different populations.

Habitat suitability

Species distribution models showed that viper distribution is related to the interplay between topographic, climatic, and habitat parameters. First, vipers are associated with gentle, south-exposed slopes at altitudes between 1500 and 2300 m. This study was performed on a rather small geographical extent, and at this scale, the correlation between altitude and the available temperature data is almost perfect ($r > 0.9$), therefore altitude can be considered as a proxy of temperature. The narrow altitudinal range suggests a very narrow thermal niche, which is typical of many micro-endemic species (Quintero and Wiens, 2013; Slatyer et al., 2013; Cunningham et al., 2016). This inherently exposes micro-endemic species to a high risk of extinction, and is particularly alarming under scenarios of climate change (Botts et al., 2013; Slatyer et al., 2013; Böhm et al., 2016). Until recently, Walsers vipers were assumed to be adders *Vipera berus* (Andreone and Sindaco, 1998; Sindaco et al., 2006). However, it should be remarked that the altitudinal range of Walser's vipers does not match the one observed for adders in the Southern Alps. Present-day adder populations living in Lombardy (approx. 80 km E of the study area) have a broad altitudinal range (550-2500 m) and 26% of records are above 2000 m (Bernini et al., 2004). Conversely, all available Walser viper data are restricted at altitudes of 1300-2300 m, with just 4% of records above 2000 m. This suggests that Walser vipers have a

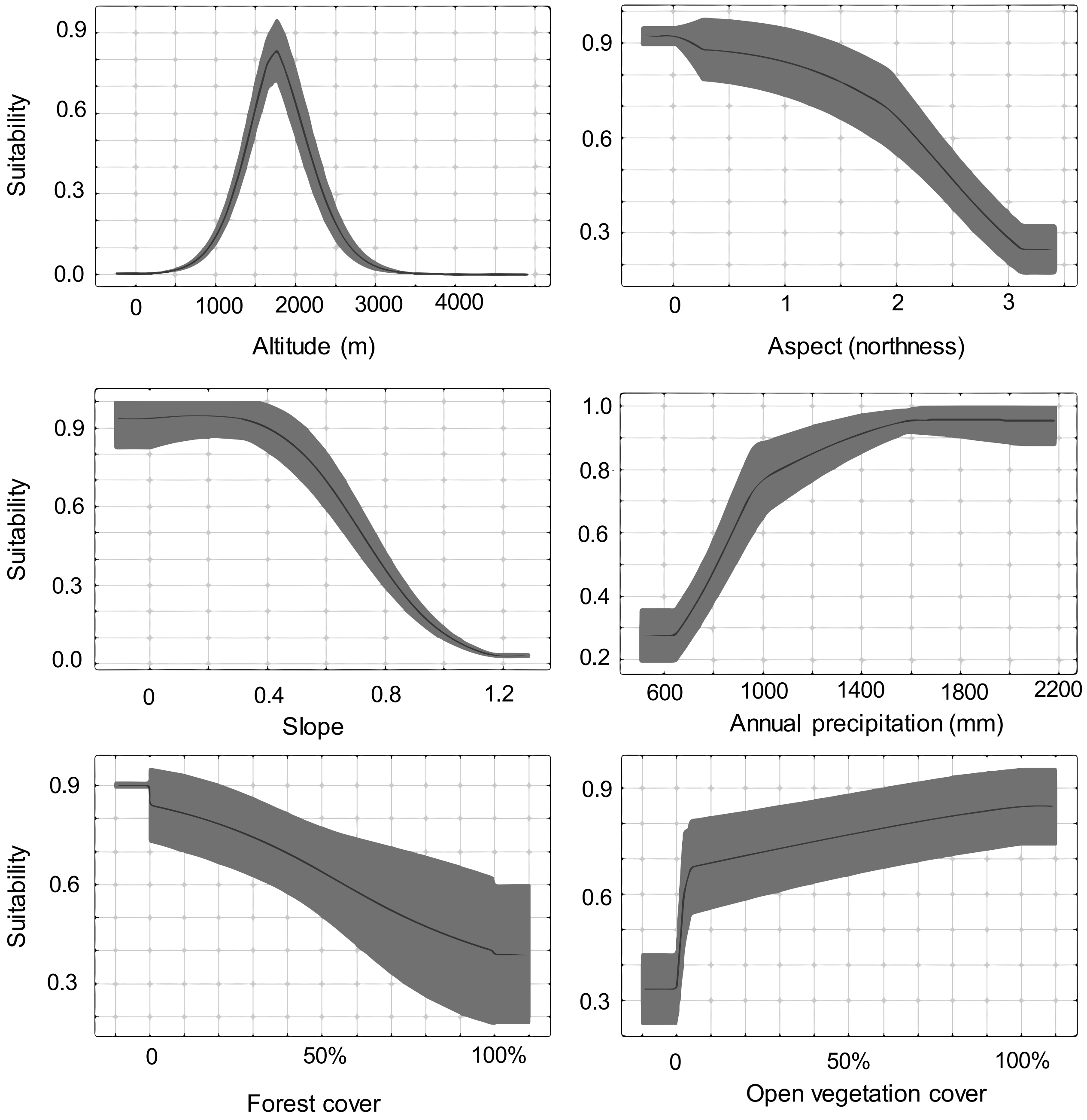

Fig. 2. Relationship between environmental variables and suitability, as estimated by species distribution models. The dark lines are the average response across the cross-validated runs; the confidence bands indicate ± one standard deviation.

narrower niche than adders, still, additional studies are required to compare the niches of the two species.

Second, vipers were associated to areas with relatively high precipitation levels and high cover of natural, open vegetation. The Walser viper range includes some of the valleys with the highest rainfall in the Alps (Mercalli et al., 2008). High precipitation levels can allow the existence of a relatively humid environment with open vegetation, which is the main habitat of this species (Ghielmi et al., 2016). Given the very small surface of suitable habitats, ensuring the long-term persistence of these environments will be essential for the long-term survival of Walser vipers.

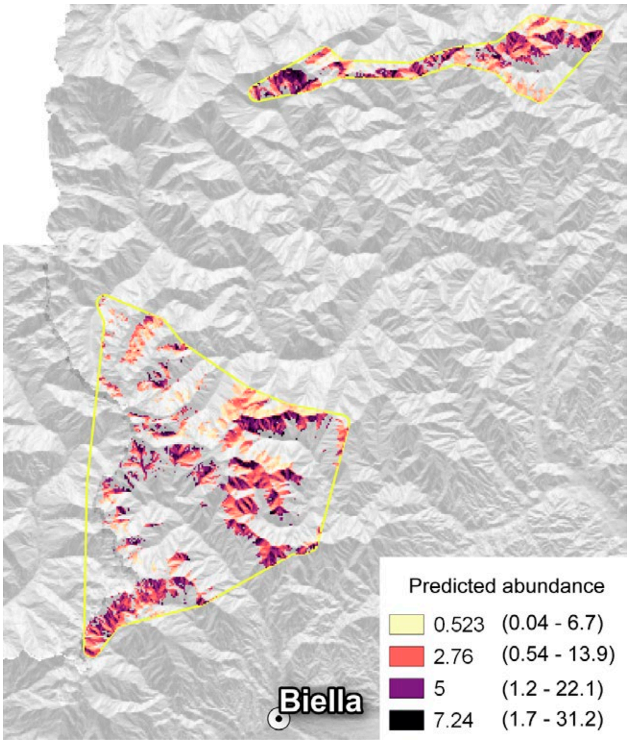

Fig. 3. Spatial variation of the density of *Vipera walser*: best predictions of the *N*-mixture model, assuming that abundance is affected by slope. The map indicates the averaged density (individuals / ha); 95% confidence intervals of the estimates are available in the legend. Projections have only been performed for the most suitable areas within the species range, according to the results of the species distribution model.

Variation in abundance

Repeated counts and *N*-mixture models can provide estimates of the absolute abundance of individuals, and are particularly useful when no data from other sources are available. Our models showed that, for individuals of Walser viper, detection probability is low (around 4% in standard surveys), and the species generally has a limited abundance (average: 3.4 individuals / ha). A low detection probability has been often observed in snakes (Luiselli et al., 2011; Rodda, 2012), and density estimates are comparable to the values observed in other studies (reviewed in Santini et al., 2018a). For instance, Neumeyer (1987) used capture-mark-recapture to assess the abundance of *Vipera berus* in subalpine environments, and found a density of approx. 3 individuals / ha. Translating modelled abundance in density estimates is often complex. Nevertheless, the home range size of the study species is likely small compared to plot size, thus the derived density probably does not suffer by temporary emigration (Kery and Royle, 2016).

Our plots covered well the whole range of the species (Fig. 1b), still performing extrapolations of abundance across a species range is extremely complex (Santini et al., 2018b). If we assume that the relationship between viper density and plot aspect is constant across the whole range, we can perform projections of the spatial variation of potential density (Fig. 3) that, in turn, could be used to obtain rough estimates of abundance across the species range. Such a projection would lead to a best unbiased prediction of 26,000 vipers, but the associated confidence intervals are extremely wide (95% intervals: 5500-130,000 vipers). These figures can be useful to obtain a first approximation of abundance when no other data are available, but must be taken with extreme caution. First, model extrapolation outside the sampled areas is always challenging, because we cannot be sure that the relationship between habitat and abundance is constant across the range. Importantly, species distribution models often overestimate species distribution (Guisan and Rahbek, 2011). This occurs because additional factors can influence species abundance, for instance, when a given patch is unsuitable because of the presence of a limiting factor not considered in this analysis. Second, the reliability of estimates obtained through *N*-mixture heavily depends on the verification of model assumptions (Barker et al., 2018; Link et al., 2018). For instance, unmodelled heterogeneity of detection probability can heavily bias total estimates of abundance (Link et al., 2018), and it is unlikely that our model took into account all the potential factors affecting viper detections. Third, the reliability of abundance estimates is sensitive to variation of detection probability, and low values of detection probability always challenge models that rely on unmarked individuals (Ficetola et al., 2018c). Unfortunately, the detection probability of vipers was extremely low.

Many conservation agencies require quantitative measures of abundance (e.g., IUCN, 2001; Stoch and Genovesi, 2016), but obtaining reliable estimates, with high accuracy and limited uncertainty, can be challenging. Our study showed that uncertainty can be large even for micro-endemic species for which a very large number of surveys is performed. Given the low detectability, an alternative approach for the monitoring of Walser vipers could rely on occupancy modelling (MacKenzie et al., 2017). Modelling co-occurrence in an occupancy framework or in a joint SDM framework may help improving inference on species occupancy when data for other species are available (e.g., common lizard). In addition, distribution may be better inferred using spatially and temporally replicated data in an occupancy framework, instead of presence-only data alone in a SDM framework.

One solution would be integrating opportunistic information (i.e., presence-only data, probably the most abundant and widely distributed source of information for the species) with detection-non detection data from an occupancy design (Dorazio, 2014).

Recommendation for monitoring and conservation

For data deficient species, a key goal for the near future is certainly improving knowledge on distribution, abundance, and threats. Walser vipers are rare and elusive, thus improving information on total abundance and population trends will be essential. Repeated counts at fixed sites are an excellent strategy for a consistent assessment of temporal variation in abundance, and have been recommended for the monitoring of many reptiles (Stoch and Genovesi, 2016). However, a large number of sites and surveys are required for a correct estimate of trends in elusive species. For instance, several tens of sites, each surveyed multiple times per year, are required if we want to obtain reliable estimates of species trends with dynamic *N*-mixture models (Ficetola et al., 2018c). The plots surveyed in this study can provide the needed baseline for the development of a long-term monitoring program. Alternatively, approaches involving the capture of individuals can be used to collect individual longitudinal data simultaneously to occupancy data. Capture-mark-recapture information would allow estimating population growth rate, the factors affecting it and its temporal variability. This can be done without the need to estimate population size and with the freedom to simultaneously test effects on drivers of demographic rates (e.g., survival, recruitment, and fecundity) (Tenan et al., 2014).

Despite the importance of future monitoring, conservation biologists must identify priorities and plan management actions even if information is incomplete (Soulé, 1985). Our data can already be useful for the conservation planning of Walser vipers. Distribution data and species distribution models suggest a very restricted distribution, with a total extent of occurrence < 300 km^2, and an estimated area of occupancy = 86.5 km^2. A limited geographic range is a key criterion for redlist assessment. For instance, the IUCN (2001) classifies as Threatened under the criterion B species that have small geographic range (extent of occurrence < 5000 km^2 and / or area of occupancy < 500 km^2), and also satisfy at least two of these three conditions: a) severely fragmented or restricted number of locations; b) continuing decline of the species or his habitat; c) extreme demographic fluctuations. The Walser viper has a very small range and is known from a limited number of locations. Until now, no information is available on the decline of his habitat, and unrecorded habitat loss is possible. Remote sensing data are an efficient approach to assess habitat trends through time, when ground information is lacking (Tracewski et al., 2016). Data from Corine land cover suggest that the cover of suitable habitats within the species range has remained stable during the last decade, with approx. 124.6 km^2 of natural open vegetation in 2006, and 125.6 km^2 in 2018. However, during the last decades, the cover of open vegetation has decreased in several areas of the Alps because of the abandonment of traditional livestock farming (Falcucci et al., 2007), and this could cause habitat loss for Walser vipers in the future. If future monitoring will identify declines of abundance, occupancy, or of suitable habitat, Walser vipers can be classified as Endangered according to the IUCN redlist criteria.

Climatic change and the loss of open natural vegetation are the strongest threats to the biodiversity of montane environments of Europe (Brambilla et al., 2010; Brambilla et al., 2017), and will likely also affect Walser vipers in the next future. Despite only recently described, these vipers already are one of the most iconic animals of the Alps, and could serve as a flagship species for the prioritization of conservation management in these increasingly threatened environments.

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APPENDIX

Appendix S1. N-mixture models relating the detections of *Vipera walser* to variables potentially influencing abundance. The table reports the regression coefficient of each variable included in each model; models are ranked on the basis of the corrected Akaike's information criterion. Only models with weight >0.001 are shown. k: *N* of parameters in the model.

Model	Length of survey	Aspect	Altitude	Plot area	Precipitation	Slope	k	AICc	weight
1	0.69	-0.70	-	-	-	-	4	238.6	0.312
2	0.69	-0.68	-	-	0.06	-	5	240.8	0.103
3	0.70	-0.70	-	-0.02	-	-	5	240.9	0.098
4	0.69	-0.70	-0.01	-	-	-	5	240.9	0.097
5	0.69	-0.70	-	-	-	0.00	5	240.9	0.097
6	0.70	-0.71	-	-	0.10	-0.07	6	243.2	0.032
7	0.70	-0.68	-	-0.02	0.06	-	6	243.2	0.031
8	0.69	-0.68	-0.01	-	0.06	-	6	243.2	0.031
9	0.70	-0.70	-0.01	-0.02	-	-	6	243.3	0.029
10	0.70	-0.70	-	-0.02	-	0.00	6	243.3	0.029
11	0.69	-0.70	-0.01	-	-	0.00	6	243.4	0.029
12	0.73	-	-	-	-	-	3	244.4	0.017
13	0.72	-	-	-	-	0.21	4	245.2	0.012
14	0.71	-0.71	-	-0.02	0.10	-0.07	7	245.7	0.009
15	0.74	-	-	-	0.17	-	4	245.7	0.009
16	0.70	-0.71	0.00	-	0.10	-0.07	7	245.7	0.009
17	0.70	-0.68	0.00	-0.02	0.06	-	7	245.7	0.009
18	0.70	-0.70	-0.01	-0.02	-	0.00	7	245.8	0.008
19	0.74	-	-	-0.04	-	-	4	246.7	0.006
20	0.72	-	0.03	-	-	-	4	246.7	0.006
21	0.72	-	-	-	0.06	0.17	5	247.4	0.004
22	0.73	-	-	-0.04	-	0.21	5	247.5	0.004
23	0.72	-	0.00	-	-	0.21	5	247.5	0.004
24	0.75	-	-	-0.03	0.16	-	5	248	0.003
25	0.74	-	0.02	-	0.17	-	5	248	0.003
26	0.71	-0.71	0.00	-0.02	0.10	-0.07	8	248.2	0.003
27	0.74	-	0.03	-0.04	-	-	5	249	0.002
28	0.73	-	-	-0.03	0.06	0.17	6	249.8	0.001
29	0.72	-	0.01	-	0.06	0.17	6	249.8	0.001
30	0.73	-	0.00	-0.04	-	0.21	6	249.9	0.001
31	0.75	-	0.03	-0.03	0.17	-	6	250.4	0.001

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Potential effects of climate change on the distribution of invasive bullfrogs *Lithobates catesbeianus* in China

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Abstract. Climate plays important roles in determining the geographical distribution of species, including the invasion area of alien species. Little is known, however, about the influence of climate change on the distribution area of invasive amphibian species in China. We adopted a maximum entropy model to predict the potential suitable invasive range of invasive bullfrogs *Lithobates catesbeianus* in China under two future climate scenarios in 2050 and 2070. Our results reveal that bullfrogs were mainly distributed in East and Central China at present, and the suitable area for the species may decrease in future. This suggests that climate change may negatively impact this alien-invasive species.

Keywords. Bullfrog, climate change, environmental limitations, invasive species, potential distribution, species distribution model.

INTRODUCTION

Biological invasion of alien invasive species is considered to be the second leading cause of global biodiversity loss and habitat degradation (Pimentel et al., 2000; Bellard et al., 2012; Runyon et al., 2012), seriously threatening the health of ecosystems (Hobbs and Huenneke, 1992; D'Antonio et al., 2004; Vilà et al., 2011; Espíndola et al., 2012; Sorte et al., 2013) and causing significant economic losses (Pimentel et al., 2000). The proliferation and outbreak of invasive species are becoming more and more serious (Pyšek and Hulme, 2010). The acceleration of globalization has affected the distribution of invasive species and almost no ecosystem is immune to the impact of alien species (Weber and Li, 2008; Catford et al., 2012). China is a large country encompassing many different climatic regions, where many invasive species can find suitable habitats where to establish. Investigating the potential distribution of invasive species could help to address the conservation efforts to eliminate or reduce the negative effects of biological invasions on local wildlife and ecosystems (Xie et al., 2001).

As in the rest of the world, climate change is affecting also China's ecosystems (Hu et al., 2012). Climate change has shown enormous influence on species distribution (Erasmus et al., 2002; Walther et al., 2002; Root et al., 2003; Hari et al., 2006; Guralnick, 2007). For example, climate change in the 20th century has changed the distribution of butterflies (Parmesan et al., 1999), birds (Thoms and Lennon, 1999), amphibians (Araújo et al., 2006) and mammals (Hersteinsson and Macdonald, 1992). Climate change has attracted wide attention of governments and scientists because of its enormous influences on ecosystem functions and global environmental quality (Thomas et al., 2004; Kiritani, 2011).

The bullfrog *Lithobates catesbeianus* is native to eastern North America, but has been introduced throughout the world during the past two centuries (Lever, 2003).

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The species is considered as one of the most harmful and threatening invasive species, since it is relatively large and negatively affects native amphibians through competition (Zhou et al., 2005), predation (Kiesecker and Blaustein, 1998; Lowe et al., 2000) and disease transmission (Hanselmann et al., 2004). Knowledge of the patterns of bullfrog invasion is, therefore, extremely important for planning conservation strategies aiming to understand and reduce the impacts of their invasion. Bullfrogs were introduced into China in 1959 via the aquaculture and aquarium trades (Han, 1991). The species successfully established wild populations, and it is spreading locally (Li and Xie, 2004; Wu et al., 2004). Once established it is extremely difficult to eradicate (Li and Xie, 2004). Although the distribution of the species has been simulated at a global scale (Ficetola et al., 2007) to predict areas susceptible to invasion, little is known about its potential distribution in China and how future climate scenarios will influence its distribution. We therefore modeled the potential distribution of bullfrog based on current climatic models and projected the results onto future climate scenarios (2050 and 2070) under two emissions scenarios, RCP4.5 (a radiative forcing of 4.5 W/m^2 at the end of 2100) and RCP8.5 (a radiative forcing of 8.5 W/m^2 at the end of 2100). Our main aims were to describe the current potential distribution of the bullfrogs in China and to model its distribution under future climate change scenarios.

MATERIALS AND METHODS

We collected individual records of bullfrogs in China from: 1) the relevant literature (n = 83 records); 2) the Global Biodiversity Information Facility database (GBIF, http://data.gbif.org, n = 6 records); and 3) our own field investigations (n = 6 records). We used Arcgis 10.2, combined with Google Earth, to extract the longitude and latitude coordinates and discard duplicate records (Warren and Seifert, 2011). All the distribution points with a spatial resolution of 30 arc-sec are buffered in GIS to ensure that only one point exists within the range of 30 arc-seconds (approximately 1 km × 1 km). Totally, we achieved 95 individual records of bullfrogs in China.

We downloaded climate data with a spatial resolution of 30 arc-sec from the Worldclim database (http//www.worldclim.org/bioclim). We used Arcgis 10.2 to unify all the factors into the same coordinate system and extent (Tang and Yang, 2006). As our base map, we used a 1: 4,000,000 map of China as original map from the national basic geographic information system (http://nfgis.nsdi.gov.cn).

We prepared a total of 22 layers of variables (19 environmental variables and 3 topography variables), that mainly reflect seasonal variation in temperature and precipitation (Hijmans et al., 2005), and topography factors (elevation, aspect and slope). We extracted their values at each distribution point and we calculated the pairwise Pearson product-moment correlation coefficients. In the cases where two variables were inter-correlated to a high degree ($r > 0.75$, Nori et al., 2011a, b), we selected the most important biologically factors (Bourke et al., 2018). We selected 6 final bioclimatic variables and 3 topography variables that did not show high correlation with other variables ($r < 0.75$) (Nori et al., 2011a, b). The final variable set included "Annual Mean Temperature" (bio1), "Mean diurnal range of temperature" (bio2; the mean of monthly maximum temperatures minus the monthly minimum temperatures), "Isothermality" (bio3, Mean Diurnal Range/(Max Temperature of Warmest Month-Min Temperature of Coldest Month)×100), "Mean Temperature of Wettest Quarter" (bio8), "Annual Precipitation" (bio12), and "Precipitation Seasonality" (bio15, Coefficient of Variation), elevation, aspect and slope. To estimate the influence of global climate change on the potential distribution of the species, we modeled the distribution for three different time slices: present, 2050 and 2070. The climate data was available from the Worldclim data (http//www.worldclim.org/bioclim). Due to the large effect of different Atmosphere Global Circulation Models (AGCMs) in species range projections (Diniz-Filho et al., 2009), we selected three different AGCMs (BCC-CSM1-1, ACCESS1-0 and IPSL_CM4) for each time slice with each climate models involving two future emissions scenarios developed by IPCC's Fifth Assessment Report (RCP4.5 and RCP8.5) (http//www.worldclim.org/bioclim). The selected AGCMs have different equilibrium climate sensitivity values ranging from 0.9 °C to 4.8 °C.

Maximum Entropy Modeling (Maxent) is a useful method to simulate the potential habitat redistribution under climate change, due to high predictive accuracy and strong stability (Phillips et al., 2006; Steven et al., 2006; Wisz et al., 2008). We used a maximum entropy approach to model climatically suitable areas of bullfrogs in China using Maxent 3.3.3e (www.cs.princeton.edu/~shapire/maxent), and we validated the model using a cross-fold approach (Hijmans, 2012). We randomly selected 75% of bullfrog records for model training (Bourke et al., 2017) and the remaining 25% for model testing, with a logistic output format ranging from 0 (unsuitable environmental conditions) to 1 (optimal) (values near 0.5 representative of average habitat quality; Phillips and Dudík, 2008). Jackknife tests were run to measure variable importance (Phillips et al., 2006). In addition, a bias file was included in the run to represent sampling effort to reduce the sampling bias and increasing speed (Young et al., 2011).

The accuracy of the model was evaluated by using the area under the receiver operating characteristic curve called AUC (Swets, 1988), commonly recognized as the optimal model prediction since it is unaffected by the threshold value and insensitive to incidence of species (Fielding and Bell, 1997). AUC scores quantify the SDM's ability to differentiate between random prediction (AUC = 0.5) and perfect identification of suitable grid cells (AUC = 1.0) (Hanley and McNeil, 1982; Phillips et al., 2006; Wang et al., 2007). After converting the Maxent output avg.asc into raster format, we reclassified the results of Maxent with thresholds in ArcGIS (Lu et al., 2012) and divided the suit bal environmental conditions into 4 levels based on the fitness index size (Wang et al., 2007; Zhai and Li, 2012) with low potential (< 0.2), moderate potential (0.2-0.4), good potential (0.4-0.6), high potential (> 0.6) (Yang et al., 2013).

To test for possible differences of the predicted distribution under different climate scenarios, each out of the twelve maps was compared to the current distribution map using Map Comparison Kit software (version 3.2.3; MCK, 2017) and an overall similarity index was computed between a map pair. We applied the "fuzzy numerical" algorithm as these maps were numerical (Visser and de Nijs, 2006; Falaschi et al., 2018).

RESULTS

We obtained a good SDM performance with an average test AUC value of 0.867, which indicated that the prediction has high credibility. Analysis of variable contributions revealed that the "Annual Precipitation" had the highest explanative power, explaining 34.7% of the variation, followed by "Mean Diurnal Range" (33.9%), "Elevation" (20.4%) and "Annual Mean Temperature" (3.1%), suggesting that the geographical distribution of bullfrog was most affected by these four factors.

The results from Maxent analysis showed at present there were many areas unsuitable for habitation by bullfrogs: Inner Mongolia, Gansu, Qinghai and Tibet. Overall, mainly the center, east, southeast and the southwest of China were suitable area of bullfrog survival, with a small number of suitable areas in Xinjiang, Ningxia, Jilin, Liaoning and Heilongjiang (Fig. 1).

The AUC values were above 0.8 in all of the models, indicating that the prediction results have high credibility. Generally, climatically suitable areas may become narrower as the invasion begins to retract in the southeast coastal the north of the north China plain, Sichuan basin and the middle and lower reaches of the Yangtze River (Fig. 2; Table 1). Only minor differences were observed in model projection onto climate change scenarios derived from BCC-CSM1-1, ACCESS1-0 and IPSL_CM4 (Fig. 3; Table 1), and these differences and similarities were also confirmed by the fuzzy numerical comparison performed in MCK: similarity maps (Fig. 4) showed only slight differences between current distribution map and these future distribution maps with the similarity index rose from 0.552 to 0.773.

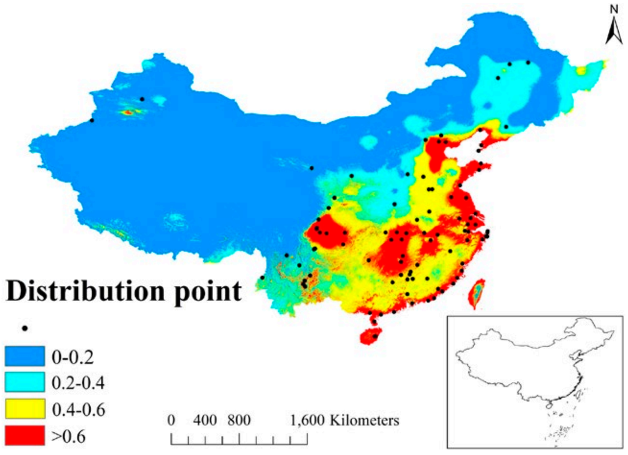

Fig. 1. Map of the suitable distribution of bullfrog in China (Present).

DISCUSSION

We investigated the current potential and future distribution for bullfrogs under different climate change scenarios. The results show that under the current climatic conditions, bullfrogs have a wide range of potential distribution in China, located in the center, east, southeast and southwest China, with only a small number of suitable areas in north China including Xinjiang, Ningxia, Liaoning, Jilin and Helongjiang. Generally, our models also revealed that global climate change is likely to shrink slightly the extent of suitable habitat under future scenarios.

Compared to Ficetola et al. (2007), who found that bullfrogs are mainly distributed in eastern China, our study results extend its distribution area to central China, with a few locations in the west and northeast China, which may represent new invasion areas. This can be explained by the facts that some new invasion sites have been found in China recently (Fei et al., 2012).

The current distribution pattern of bull frogs in China can mainly be explained by precipitation and temperatures. Previous study also showed that bullfrog presence seems to be positively related to precipitation (Ficetola et al., 2007). The availability of water (including the presence of permanent wetlands) for breeding are commonly recorded important environmental features needed for the presence of bullfrogs (Maret et al., 2006) and their tadpoles' growth, development and metamorphosis (Govindarajulu et al., 2006). In addition, Mean Diurnal Range also influences the distribution of bullfrogs. This is also similar to the results from Ficetola et al. (2007) and, indeed, Bullfrog is a 'warm-adapted species' (Bachmann, 1969; Harding, 1997). Besides, previous studies showed that the current distribution of bullfrogs in China is also explained by 1) the proximity to the frogfarms, from where bullfrogs can escape: most of the bullfrog farming in China is surrounded by highly suitable habitats, and the frogs can establish wild population there (Wu et al., 2004; Li and Xie, 2004); 2) the abandonment/release of bullfrogs mainly by religious groups, which also led to the establishment of new wild population, e.g., in Yunnan

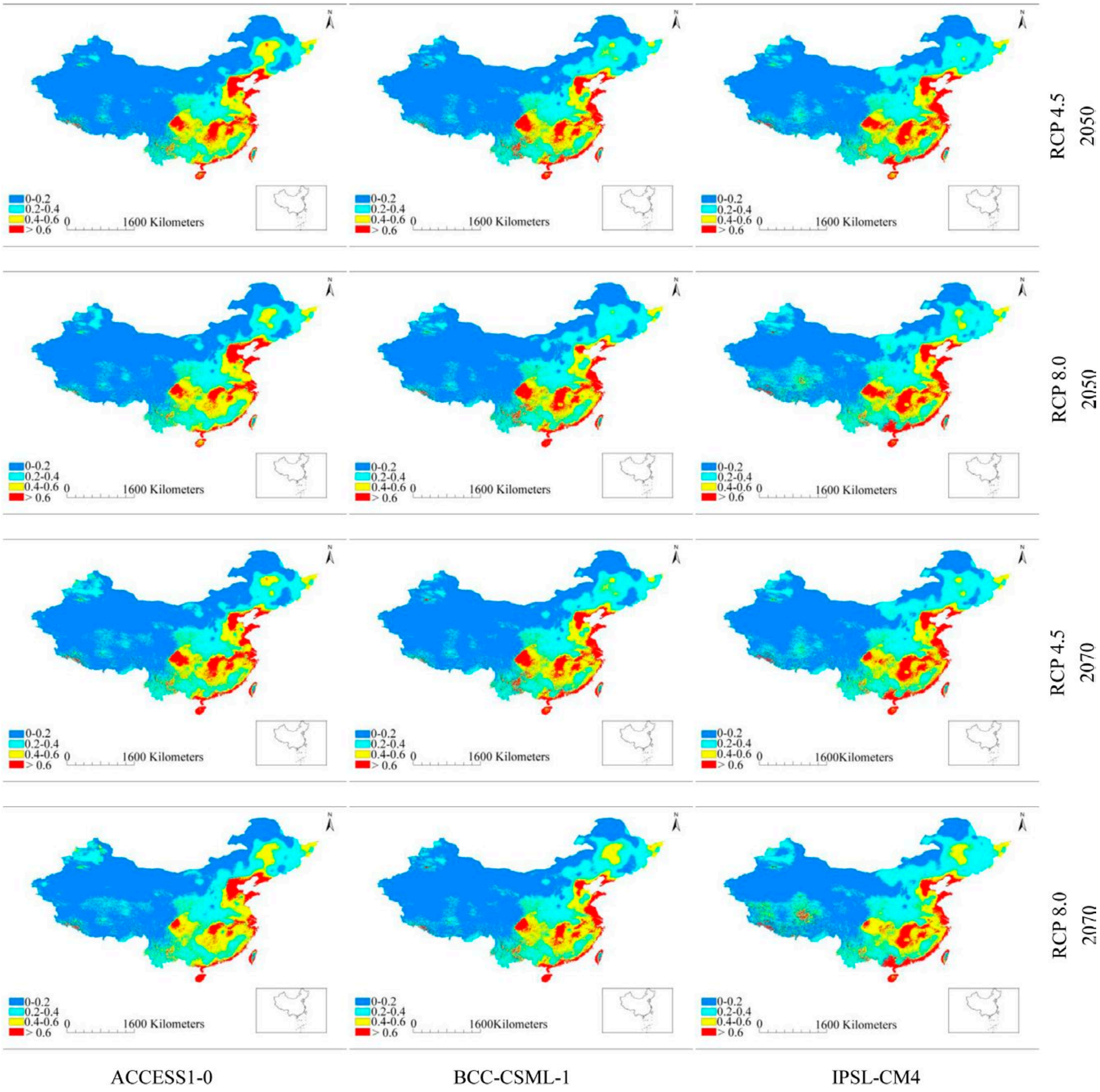

Fig. 2. Maps of the potential suitable distribution of bullfrog in China in 2050 and 2070.

and Sichuan (Wu et al., 2004; Li and Xie, 2004).

As shown by the fuzzy numerical comparison performed in MCK, slight differences between current and future distribution maps have been observed. Also, projecting bullfrogs' climatically suitable areas on future climate change scenarios (RCP4.5 and RCP8.0) indicated that climatically suitable areas will become narrower in China. The potential habitats of bullfrogs in China will retreat to the most suitable area including the north of the north China plain, Sichuan basin and the middle and lower reaches of the Yangtze River (Fig. 2), where bullfrog farming is particularly common (Fei et al., 2012).

Biological invasions are complex and the potential habitat distribution is determined by a variety of factors (Li et al., 2009). In this study, we only considered the effect of the climate and terrain, but we did not consider the effect of the other factors including the vegetation cover, biotic interactions with other species, species

Table 1. Changes in the potential distribution area under climate change in 2050 and 2070.

Climate scenarios	Atmosphere Global Circulation Models (AGCMs)	Area of the most suitable zone (the red part of the map)/ Million square kilometers	Percentage (%)
Present		**82.27201**	**9.19%**
2050RCP4.5	ACCESS1-0	66.06528	6.88%
	BCC-CSML-1	68.44306	7.12%
	IPSL-CM$	80.92153	8.42%
2050RCP8.0	ACCESS1-0	59.2675	6.18%
	BCC-CSML-1	69.33708	7.22%
	IPSL-CM$	89.20736	9.28%
2070RCP4.5	ACCESS1-0	67.75792	7.05%
	BCC-CSML-1	72.75847	7.57%
	IPSL-CM$	78.15097	8.13%
2070RCP8.0	ACCESS1-0	55.53778	5.78%
	BCC-CSML-1	60.97194	6.35%
	IPSL-CM$	78.69361	8.19%

migration capacity, species evolutionary adaptations, and human exploitation of wild populations, on the potential distribution of the bullfrog. If these factors were fully considered, the predicted results could have been more closely related to the current distribution of species (Graham and Hijmans, 2006).

To effectively prevent further invasions of bullfrogs in China, management policies should be more pragmatic, preventing new introductions within suitable habitats and eradicating populations when possible. Based on the predictions on bullfrog potential habitats from SDMs, the authorities should consider the model results to focus the management strategies on these potentially sensitive regions. In addition, authorities should tighten control of bullfrog farming to prevent their escape. In addition, frog factories could be moved to areas which are surrounded by unsuitable habitats of bullfrogs, which would reduce a lot the possibility of survival of escaped captive individuals.

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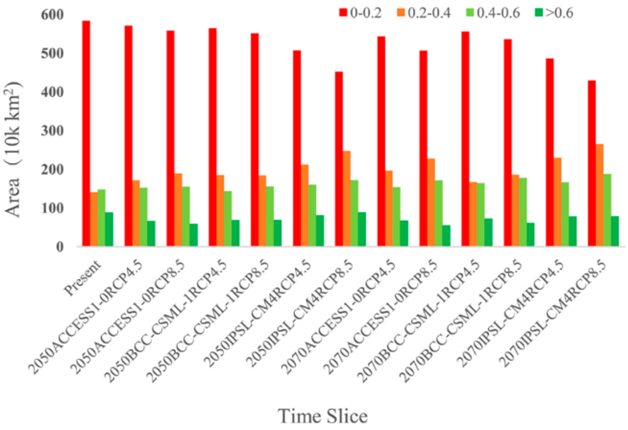

Fig. 3. Comparison of potential suitable distribution of bullfrog at present, in 2050 and in 2070 under future climatic conditions with low potential (< 0.2), moderate potential (0.2-0.4), good potential (0.4 – 0.6), high potential (> 0.6).

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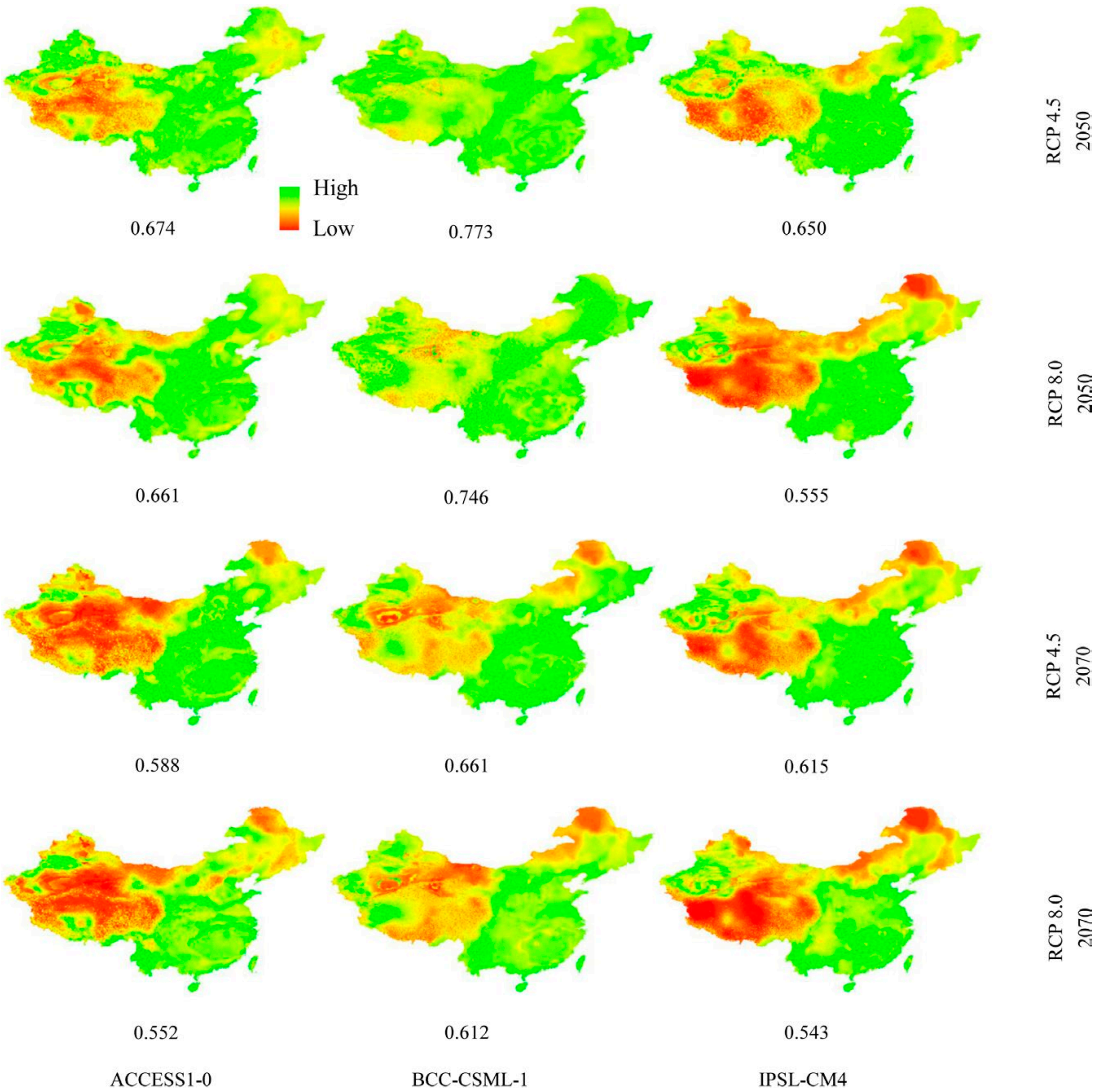

Fig. 4. Similarity maps of the fuzzy numerical comparison between current distribution map and future potential distribution maps under future climatic conditions performed in MCK with similarity index of each map

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A bibliometric-mapping approach to identifying patterns and trends in amphibian decline research

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Abstract. Amphibian decline is one of the most severe and well-documented examples of immediacy of the ongoing biodiversity crisis. In this study we analyze the scientific literature on amphibian declines with a view to identifying and analyzing emerging patterns within this large, and growing, body of knowledge. Focussing on peer-reviewed papers published between 1977 and 2018, we identified a total of 2,619 scientific publications, from the Scopus database. Using a visual-clustering approach, which allows us to investigate patterns and trends of research topics, we developed term-maps to identify how key terms cluster into research areas, and whether geographic patterns exist in the scientific literature on this subject. Overall, the average annual growth rate of the number of studies published on amphibian declines was 109.3%, with a decisive increase since 2001. The term map analysis revealed four main research areas: i) ecology; ii) causes and consequences of pollution; iii) global threats and extinction risk; and iv) pathogens (which includes many current publications, especially those focused on chytridiomycosis). We confirm the existence of a geographical research bias in the study of amphibian decline, with studies mainly located in western countries, which harbour a smaller number of threatened species. In contrast, studies on threatened species are consistently underrepresented. The literature on amphibian declines has increased at a greater rate than general amphibian literature, highlighting the possibility that the observed amphibian decline has been a driver for amphibian research.

Keywords. Global amphibian decline, chytridiomycosis, scientific literature, term map, threatened species.

INTRODUCTION

In the framework of Holocene extinction (see, e.g. Ceballos et al., 2017), the global amphibian decline is likely one of the most severe examples of a clade specific biodiversity crisis (reviewed in Ficetola, 2015). Its severity is due both to the number of species that have already gone, or are likely to become, extinct (e.g. Alroy, 2015) and to widespread ongoing population declines (Houlahan et al., 2000). Amphibian declines are multifactorial, including global changes (climate change, emerging infectious diseases, …), local causes (habitat destruction, invasive species, …), and meso-scale agents (environmental toxins, habitat fragmentation). Furthermore, due to their typical life history, ecology and physiology, amphibians are sensitive to changes of both aquatic and land environments, making them bio-indicators at global level.

In view of their ecological importance and susceptibility to anthropogenic driven change, it is striking that the relative importance of amphibian decline as a topic in scientific literature has largely increased since 1973 (Fig. 1), when the first paper dealing with the subject was pub-

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lished (Beebee, 1973). The purpose of our paper is to provide an analytical overview of the production of scientific research that has dealt with amphibian declines in this time period. To our knowledge, there are only few bibliometric/scientometric studies on amphibians (Gardner et al., 2007; Ohmer and Bishop, 2011; Campos et al., 2014; Ficetola, 2015; Mormul et al., 2017), and those that do exist are all focused on quantitative elements of amphibian declines' studies. Such kind of studies can be useful for making a point and addressing future researches.

Rather than being a purely quantitative analysis of the literature, our analysis takes a different approach, using science mapping (or bibliometric mapping), which is a methodology that produces a spatial representation of the relations among the units of interest extracted from literature (key-words, authors, countries of origin, …) (Costa et al., 2019). Basically, such analytical approach produces a bibliometric network which allows us to spatially structure patterns and trends of research, which is the aim of our study. Research trends are detected by bibliometrics and by quantitative methods that analyze scientific publications as an information process. Once patterns and dynamics in scientific publications are identified, they can be used as a proxy for the development of the investigated disciplines (van Raan, 2004). Thus, bibliographic analysis is a powerful tool to measure scientific production of research and trends, facilitating better direction of science-based strategy to fill knowledge gaps and better complete the scientific information upon which policies are developed. By using such approach we aim to describe how the interest on amphibian declines has changed over time, accounting for topics' trends and geographic pattern which accompanied such changes. In doing so, it is inevitable referring to IUCN Red List Threat categorisation, which is a point of reference in the field of conservation. Our aim is not to discuss the IUCN strategies and monitoring, rather we used IUCN categories for a better comprehension of our findings, in particular for evaluating wheter research efforts are consistent with species' conservation status. While, of course, we can only depict what has been done, we expect that understanding patterns and trends underlying published research will help the planning of future studies and bring new questions.

MATERIALS AND METHODS

Database search

The Scopus database was consulted on July 20th 2018 and used to retrieve bibliographic records related to Amphibian Decline research for the period 1977-2018. To identify relevant amphibian decline publications, the following keywords were used in the combined fields of title, abstract and keywords (*per* publication): "Amphibian" AND "Decline". As the Scopus search was conducted in July 2018, publications from that year were not present in the Scopus database and as a consequence, the frequency of word occurrence in our search may be underestimated. The search was restricted to publications (Articles, Reviews, Book Chapter and Books) written in English. Before starting with the analysis in VOSviewer, a thesaurus file (supplementary text file S4) was created to ensure consistency for different term spelling and synonyms (an example: we used "agriculture" for more terms, such as "agricultural activity", "agricultural landscape", "cattle"). Only terms occurring at least nine times were extracted from the retrieved publications.

Bibliometric mapping and clustering

Bibliometric maps were created on retrieved publications, using the VOSviewer software version 1.6.5.0 (freely available at www.vosviewer.com). The software was specifically developed for creating, visualizing and exploring sciences' bibliometric maps basing on VOS (Visualization Of Similarities) mapping technique, that is closely related to the multidimensional scaling method (van Eck and Waltman, 2010; for further explanation on the method see van Eck and Waltman, 2011; van Raan, 2014; Nardi et al., 2016; Costa et al., 2019). Using VOSviewer we produced term maps. A term map, also called co-word map, is a two-dimensional representation of a research field, in which strongly related terms are located close to each other and the weaker the relationship between terms, the larger the distance is between them. The diameter of the circle representing a given term indicates the number of publications where it appears (in order to avoid overlapping labels, only a subset of all labels is displayed in the maps).

Once terms are in the map, the next step was to identify clusters of related terms. The software uses a weighted and parameterized variant of modularity-based clustering called VOS clustering technique (Waltman et al., 2010; Waltman and van Eck, 2013). The assignment of terms to the same cluster depends on their co-occurrences in the title or abstract of publications. A cluster that is made up of terms of the same colors represents a research theme in which one or more research topics can be identified. The same approach was applied on the bibliographic information on the same publication dataset to observe the countries of the co-authorship map with the aim of observing the collaborative clustering of the countries based on amphibian decline publications.

A term citation map was produced; this map analyzes the scientific impact of specific topic, whereas a term year map performs a timeline analysis of the research topics. More specifically, in the *term citation map,* the color of a term is determined by the normalized average citation impact of the publications where the term occurs, thus reflecting the average citation impact for the term. Therefore, a blue (cold) or red (hot) term indicates that publications in which the term occurs have low and high average citation impacts, respectively (van Eck et al., 2013).

A *term year maps* was also produced; in this map the color of a term indicates the average publication year of all the publications in which the term occurred. As for the *term citation map*, we used colors that range from blue (mean year term presence 2008 or earlier), to green (2010) to red (2012 or later). Therefore, blue terms were those occurring mainly in older publications, while red terms occur mainly in publications that were more recent.

Maps of amphibian decline publications both globally, and restricted to only EU countries and the UK (which for the duration of this study was part of the EU) were constructed using color intensities to illustrate the number of publications. The number of publications was counted considering the host institution of all the co-authors of a paper, hence a single paper may include multiple countries as its country of origin for this purpose. Further, a collaborative clustering map of the countries based on amphibian decline publications was also produced reporting the spatial relationship among countries.

In order to evaluate if and how the conservation status of species biased the scientific production about species or geographic areas, we used the I.U.C.N. Red List Threat category of the species and the I.U.C.N. spatial data for species' ranges; in the following we will refer to this information as IUCN (2019). Specifically, we evaluated the percentages of papers dedicated to species belonging to the different IUCN categories, and evaluated the relation between the numbers of threatened species a given continent harbours and the number of papers actually dedicated to species harboured in that continent.

RESULTS

Amphibian decline publication trends

A total of 2619 scientific publications fitted our search method based on relevance for the terms "amphibian" and "decline": 91.2% of the publications were regular articles, 7.4% review papers, 1.3% book chapters and the remaining 0.1% were books. The studies were published in 157 journals. Most commonly reported journals were Biological Conservation (n = 120; 5.8%), PLoS One (n = 106; 5.1%), Diseases of Aquatic Organisms (n = 103; 5.0%), Conservation Biology (n = 101; 4.9%) and Journal of Herpetology (n = 90; 4.3%). Figure 1 depicts the numbers of publications about amphibian declines from 1977 to 2018 July and their relative contribution to the total amount of literature about amphibians (i.e., number of publications in the Scopus database from a search with only "amphibian" as a keyword). It is possible to observe a decisive increase since 2001, with a light reduction in the frequency of keyword terms in 2014 followed by a recovery. The average annual growth rate (geometric mean) for the whole period has been 109.3%, being 107.8% until 2000 and 111.3% thereafter. Although the literature about amphibians increased by a factor of five in 40 years, the percentage of publications on amphibian declines increased more than four times in the same time period.

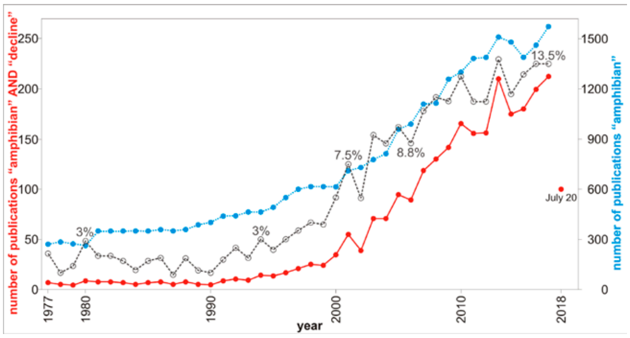

Fig. 1. Trend in amphibian publications from 1977 to 2018. Continuous (red) line is for the number of publications including both "amphibian" and "decline" (left y-axis), dotted (blue) line is for the number of publications including "amphibian" only (right y-axis), dotted (grey, with empty circles) line is for the annual percentage of "amphibian" and "decline" studies out of the total "amphibian" studies (some reference percentages is reported in the graph) (2018 publications were underestimated being not yet all inserted in the Scopus database at the time we consulted it).

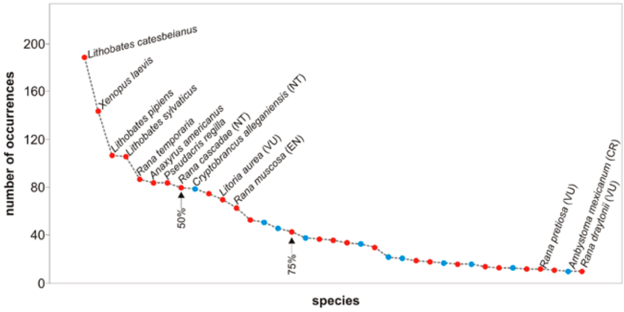

Fig. 2. Occurrences of amphibian species in the publication analyzed (analysis is based only on species with more than 10 occurrences; only the first eight species and non-Least-Concern species are shown; arrows mark the 50% and 75% of total occurrences); red dots are for anurans, blue dots for urodeles, no caecilian species occurs in more than 10 studies.

The species which occurred in at least 10 publications were 37, representing 12 families of amphibians. Eight species (belonging to four families) appear in half of the total number of studies in which single species were cited (Fig. 2), 75% is achieved with eight more species; 81% of the species have been assessed as Least Concern in the IUCN Red List. No caecilian species occurs more than 10, "caecilian" occurs 13.

Amphibian decline research topics and their citation impacts

In the *term map* (Fig. 3) the 579 terms displayed on the map are grouped in four clusters, which partial-

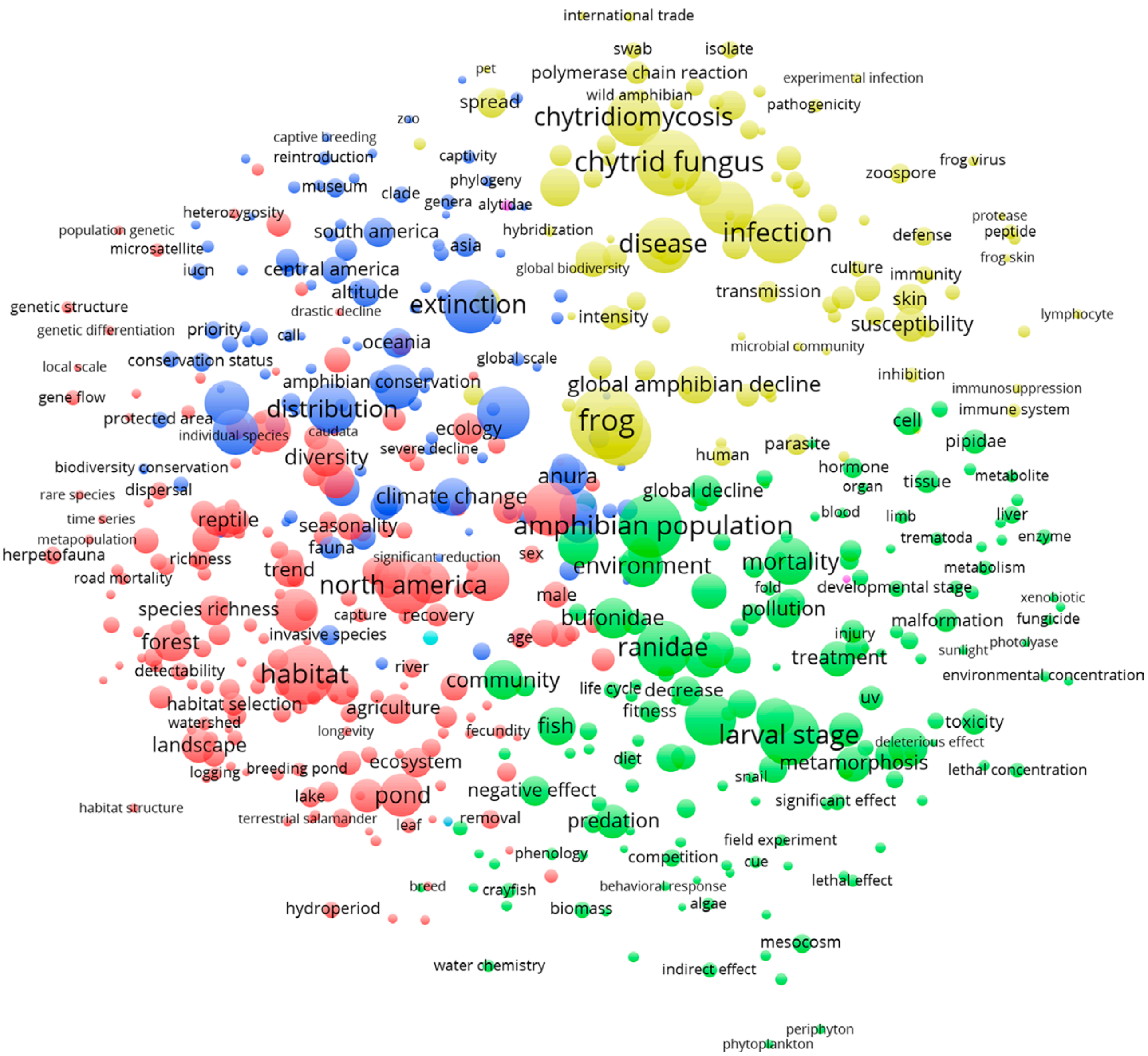

Fig. 3. Term map based on amphibian decline publications. Different colors represent the terms belonging to different clusters.

ly overlap and whose colors were arbitrarily chosen for visual purposes. The red cluster is characterized by terms related to ecology (e.g., "habitat", as well as various kind of habitats, habitat features and alterations, "population dynamics", "climate", "relationship"), but also "diversity" and "cause". Amphibian groups most typical of this cluster are Salamandridae, Ambystomatidae and "salamander" in general, whereas the geographic regions present are Europe and North America.

Green cluster is characterized by terms expressing altered environment ("agrochemical", "pollution", "stressor", …), and its "negative effect" on "amphibian population" ("mortality", "survival", "toxicity") as well as "physiology" and body organs ("muscle", "kidney", "liver", "malformation"), based on studies mostly on "larval stage" and "embryonic stage" and which take into account amphibian populations. Ranidae, Bufonidae and Hylidae characterize this cluster, which is not clearly geographically distinct.

The blue cluster is related to "extinction" and "risk" and "threat" to "amphibian species" and their "distribution", as well for "biodiversity", and what can be done to counteract them ("conservation", "conservation planning"). Important terms in this cluster are also "climate change" and "temperature". Apart from "Anura", no sys-

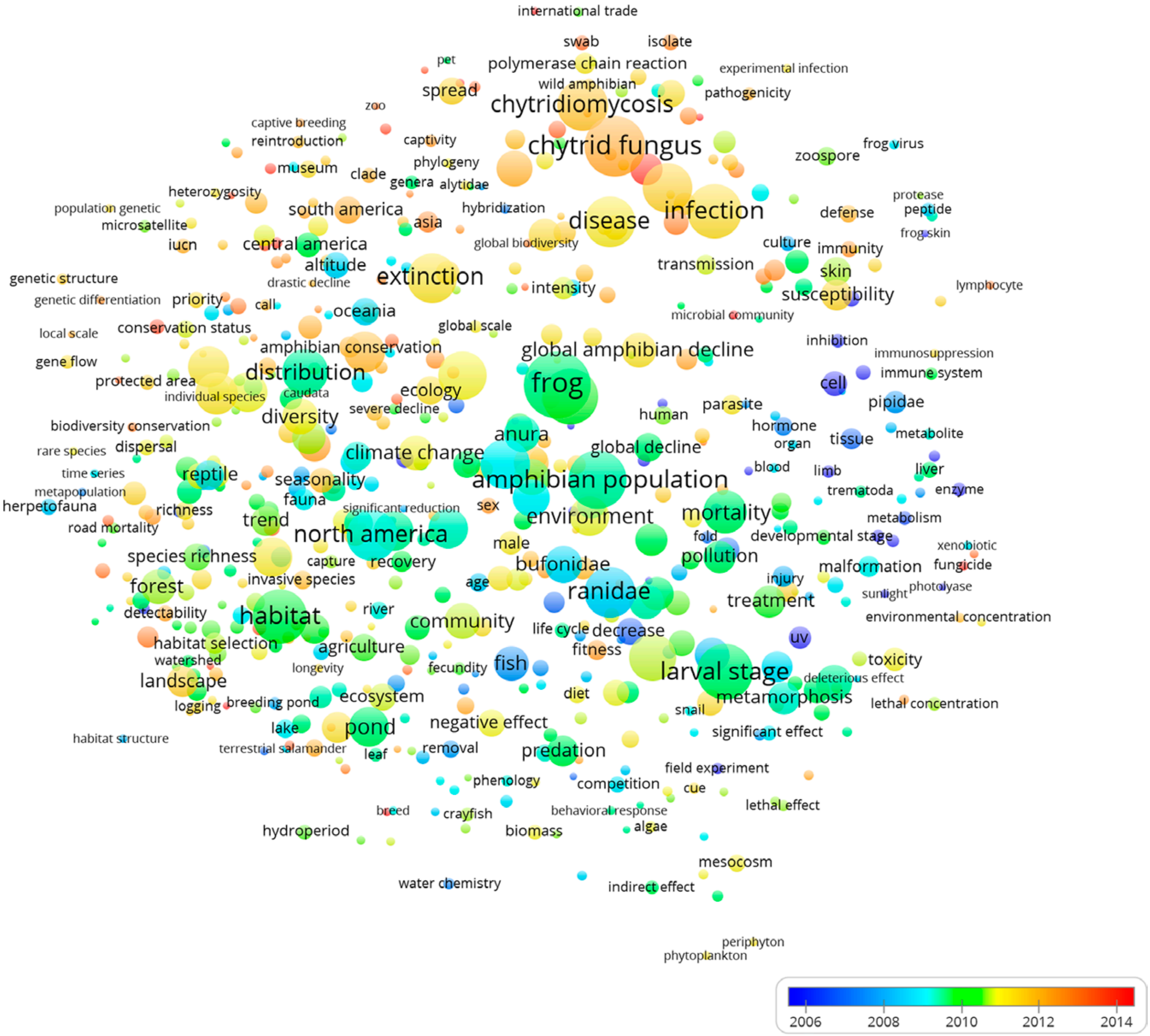

Fig. 4. Term year map based on amphibian decline publications. The scale represents the earlier (blue) or more recent (red) years when the term appeared.

tematic group is typical of this cluster. All continents (but Europe and North America, which are in the red cluster) are represented in this cluster.

The yellow cluster can be considered as the "disease" and "pathogen" cluster. Chytridiomycosis, and related terms (such as "Bd dynamics", "chytrid fungus", "zoospore") especially characterize this cluster. "Iridoviridae" and "virus" are present too. Techniques used for studying diseases are represented ("PCR", "culture", "swab", …), as well as body organs and structures targeted by diseases. "Population decline" and "global amphibian decline" are two important terms in this cluster. No families are represented in this cluster, but "frog" is the term with most weight in the analysis. No geographic area is represented in this cluster.

Figure 4 shows the *term year map* (the terms maintain the same position of the *term map*, Fig. 3). The colors show that terms used in early publications about amphibian declines (blue) are terms related to the proximate anatomical and physiological evidences of threats to amphibian ("cell", "tissue", "metabolism",…), Bufonidae and Ranidae were the most represented amphibians taxa, Oceania has been mostly studied in these early publication, as well as the occurring of "decrease", fishes

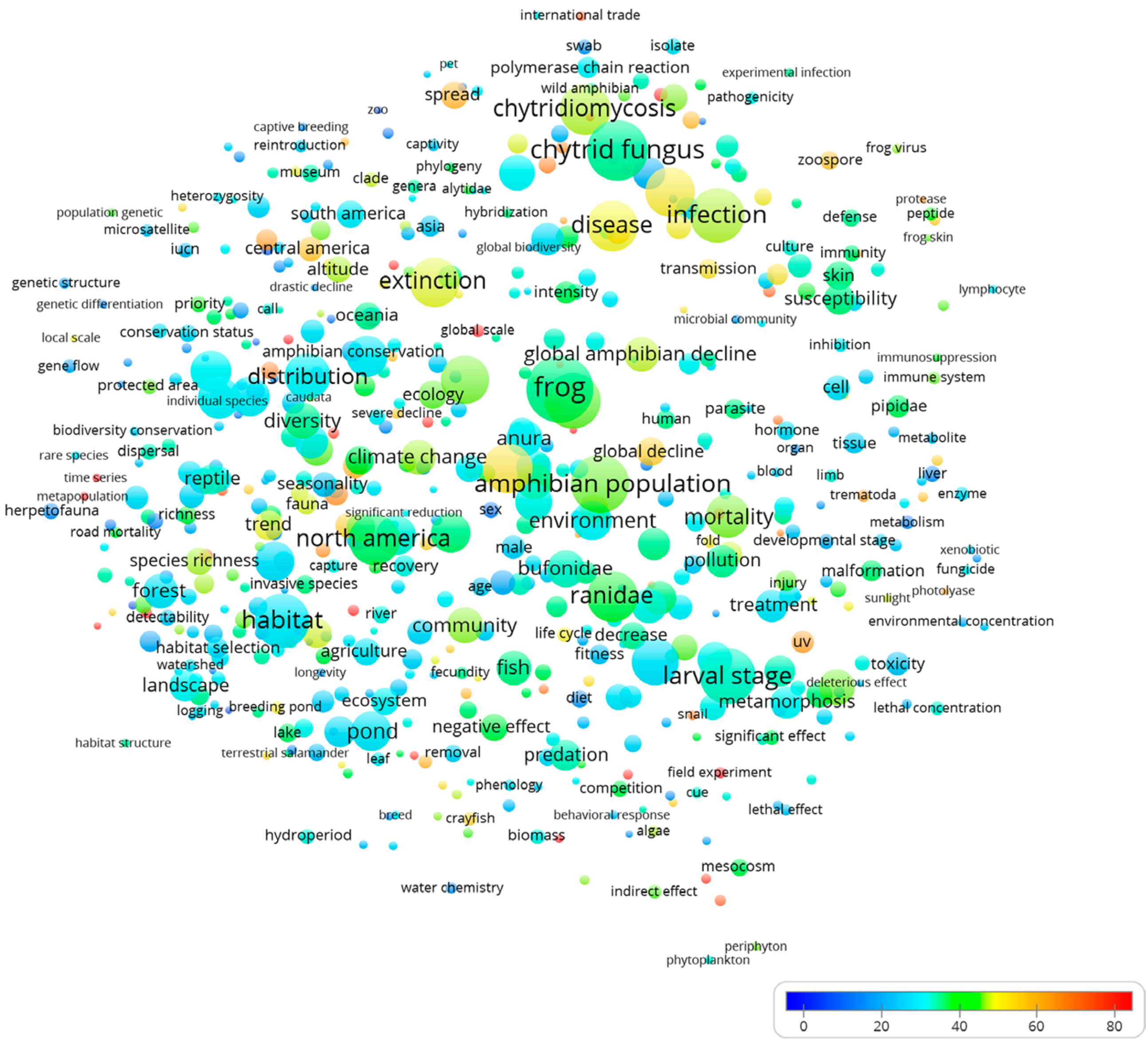

Fig. 5. Term average normalized citation map based on amphibian decline publications. The scale represents the average normalized citation rate, lower (blue) or higher (red).

and UV radiations decreased later. The terms covered in more recent publications (red) were mostly related to chytridiomycosis and diseases in general, but important terms are also "extinction", "amphibian conservation"; Asia and South America have been studied only recently. Terms covered in the middle (green) of the referred period (2010) are related to distribution, habitat, amphibian population, larval stage and frog.

The *term citation map* (Fig. 5) revealed as highly average age normalized cited terms (reddish colors) "international trade", "mass mortality" (in the yellow cluster in Fig. 3), "spatial pattern", "global scale", "alien species" (blue cluster), "rapid decline", "local population", "time series", "metapopulation" (red cluster), "ecological community", "field experiment" (green cluster). Although the "hot" term are rather scattered among the clusters, the red cluster in Fig. 3 appears to be the one with more terms having medium-low average normalized citation rate.

Geography of literature

Studies were carried out mainly in North America (40%), then in Europe (17%) and Oceania (12.6%) (Fig.

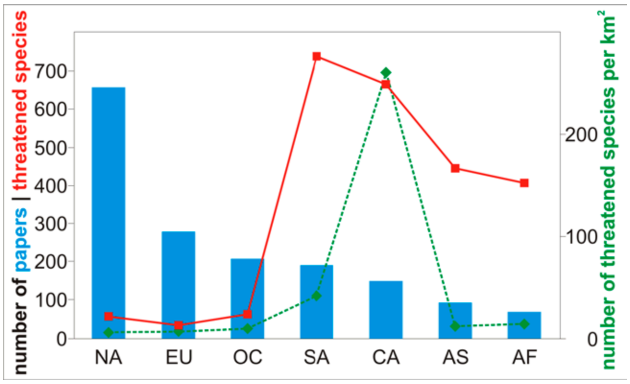

Fig. 6. Number of publication about amphibian decline (histogram) according to the continent were the studies have been carried out (NA: North America, EU: Europe, OC: Oceania, SA: South America, CA: Central America, AS: Asia, AF: Africa; analysis is based only on species with more than 10 occurrences), and the respective number (continuous red line) and richness (species per km^2, dotted green line) of threatened species (according to IUCN, 2019).

6). Figure 6 also shows that the number of studies is not commensurate with the number of IUCN threatened species (i.e., assessed as Critically Endangered, Endangered or Vulnerable) per continent (Spearman R = -0.61, P = 0.15), and even less with their continental richness (Spearman R = -0.71, P = 0.07). A consistent bias is evident when considering the species' ranges of species with more than 10 occurrences in amphibian declines literature (supplementary figure S1).

Amphibian declines research was published by authors working in 106 different countries, many of which based in EU (supplementary figure S2), with three main collaborative clusters (supplementary figure S3).

DISCUSSION

Our analyses outline how scientific papers on the subject of amphibian research have increased greatly in the past four decades. Further, our analyses highlight the important topics underpinning this increase, and the geographic and taxonomic trends within it. The first paper we found in the Scopus database which knowingly dealt with amphibian declines dates back to 1973 (Beebee, 1973; it was not included in the analyses), but papers per year were still few until the first half of '90. Then, the explicit interest in this topic increased: the studies increased to more than 50 per year in 2001 - it is worth highlighting that *B. dendrobatidis* was first discovered in 1998 (Berger et al., 1998) - and then doubled in 2007 and almost quadrupled in 2013, being produced at a consistent rate of approximately 200 per year since then. Notably, although the interest about amphibians steadily increased in the last 40 years, the contribution of amphibian decline studies to amphibian literature show a positive "allometric mean" (Fig. 1). The observed increase in papers on amphibians highlights the raised profile and interest in this global phenomenon, which is probably one of the most serious current biodiversity crises. It is also possible to consider that the observed amphibian declines of the past decades have been a driver for the general interest in amphibian research.

The vast majority of papers deal with Least Concern species (IUCN, 2019). This is even more surprisingly considering that LC species are about 50% of the amount of assessed species. On the other hand, this is not necessarily a negative sign, because the status of any species requires studies to be conducted, and the stages of species can change over time. Furthermore, information from most commonly abundant and widespread species are applicable also for the conservation of other species more difficult to be studied. It is also worth considering that many species that are listed as Least Concern on the Red List may have suffered serious localized declines, and even extinctions (such as the European midwife toad, *Alytes obstetricans*; Bosch et al., 2001; Rosa et al., 2013).

Geography seems to be a very important reason for Threatened species to be less studied then LC ones, as the areas which harbour a greater number of Threatened species were underrepresented in the literature: most publications were about North America, Europe and Australia, even if they harbour only 6.5% of the total amount of Threatened amphibian species (IUCN, 2019). Such bias, which has already been highlighted (see Ficetola, 2015, for a review), is rather consistent with the country of origin of researchers (Fig. S2), suggesting the relevance of economic influence on research. Wealthier countries will generally have most funds available for research, and the cost of conducting research outside of the funding country is more expensive and operationally difficult. The collaborative cluster mostly reflects geographic distance and historic relationships with those funding countries (Fig. S3). Interestingly, Asia and South America are getting more attention in recent years (Fig. 4), and Central America has high citation impact (Fig. 5), likely as a result of close relationships with the USA.

We identified four main research clusters in amphibian decline studies. One is mostly about ecology (red cluster, Fig. 3), with studies mostly based in North America and Europe and especially focused on the decline of newts and salamanders. It is an "evergreen" area of studies, but with some current topics of interest related to advance in ecology and methodology, such as landscape ecology, species distribution, CMR and occupancy studies (Fig. 4). A second area groups studies mostly about causes and consequences of pollution on amphibians, especially frogs and toads (green cluster). It is a rather "cold" area of interest, meaning that its topics have been less studied in recent years. A third area clusters studies on global threats to amphibians and extinction risk, and conservation of biodiversity. The topics this group clusters together are relatively new (e.g., the first systematic categorization of amphibian extinction risk was only conducted as recently as 2004), and are much more commonly investigated in current times than they were historically. However, the most current topics are grouped in the yellow cluster, representing the area of studies about pathogens, especially chytridiomycosis, and their role in amphibian declines.

In his review paper on the subject of amphibian declines, Storfer (2003) suggested four major areas of research in order to understand and facing decline, namely: well-grounded statistically monitoring techniques, metapopulation- and landscape-level researches, using molecular genetics, and using multi-factorial approaches in research. Actually, "detectability", a basic fact to be taken into account when monitoring decline, has few occurrences (47), as do "occupancy" (78) "capture-mark-recapture" (10), and "long-term monitoring" (52). Nevertheless, detection probability is increasingly taken into account, as we have seen just above. This is consistent with the finding of Ficetola (2015), who pinpoints that methods for taking into account detection probability have been developed relatively recently, and not immediately implemented in accessible softwares. However, those detection probability terms strongly characterize the red cluster, being far in the left corner of the graph (due to low occurrence, their labels are not visible in the figures). This means that almost exclusively ecological studies use robust methods for quantifying the scale of amphibian declines at a population level. "Landscape" as a term gets more attention (161), and even if it characterizes ecological studies, as well as less commonly occurring "land use" (69), "connectivity" (40) and "metapopulation" (15), it has connection with other research areas. Terms relative to molecular ecology have in general low occurrences; however, this term is present in ecological studies (red cluster, seven terms for an amount of 219 occurrences) and in studies about pathogens (yellow cluster, seven terms for an amount of 320 occurrences). Assessing whether multi-factorial approaches have been used is not feasible basing on our analyses.

The causes of amphibian declines are still under research, and complex interactions and local-scale factors make it difficult to identify precisely the drivers underpinning a given decline. However, there is a general agreement that climate change, land-use change and chytridiomycosis (as well as other parasites and pathogens) play a role (Hof et al., 2011). It has been debated whether chytridiomycosis (and climate change) has received more attention than other threats to amphibians, in particular habitat change (Gardner et al., 2007; Ohmer and Bishop, 2011), thus producing a research-bias. The term-map analysis shows that actually chytridiomycosis is a very important and ongoing topic of research, earning - *de facto* - its own cluster. Climate change received less research attention, but remains an important term, as well as "temperature", in the global-threat blue cluster; furthermore climatic variables may also be taken into account in ecological studies (red cluster). Terms linked to land use change, such as "habitat alteration" and "habitat loss", appear in ecological studies, although the lower occurring "deforestation" is considered a global threat for amphibians (blue cluster).

Amphibian decline has an ongoing increasingly scientific interest for researchers, so it is likely to be a driver for more general, fundamental amphibian research. Our analyses partly confirm previous findings based on different statistics and on literature surveys focused on specific topics, and partly identify additional information and research trends, identifying gaps in our knowledge too. We characterized four main areas of research: ecology, cause and consequence of pollution, global threat and conservation of biodiversity, and pathogens. The last one, mostly identifiable with studies on chytridiomycosis, appears as the most active area of research, in spite of direct threats to amphibians, such as altered environment and pollution, mostly acting at local scale, which got more attention in the past. Likely, the interest in localised threats has downsized because there is an increasing body of evidence that global changes are happening at unprecedented rates, and that they represent concrete threats for natural systems, including amphibians (IPBES, 2018). Nonetheless, detecting local threats is still important, because amphibian declines have not only multiple causes, but they also interact differently at different geographic scales (Campbel Grant et al., 2016). A second finding of our analyses is that species and population monitoring increasingly takes into account detectability, but that using such robust approaches is mostly limited to ecological studies. Instead, incorporating detectability into studies on, for example, infectious disease will furnish information on the actual occurrence of infection among populations, on real prevalence within populations, and a more reliable representation of both infected population and host-pathogen dynamics.

Our analyses confirm a geographical research bias which appears the main reason for Threatened species being less studied than LC ones. Filling this gap requires specific attention, both because of the vulnerability of the species to be studied and the ecosystems they inhabit, and because they occur mainly in less well-developed countries, with less access to research funds. Increasingly, the disparity in funds available for western countries' researchers could cause a kind of colonialism in science (Hodgetts et al., 2019). Co-production of research (Nature, 2018), that is - in this case - the carrying out of researches jointly with local researchers and communities, from planning the study to publication and follow-up, could be a way to avoid this form of colonialism.

Lastly, despite the role the amphibian decline is playing in herpetological and biodiversity and conservation studies more in general, and despite the relative importance of "mainstream" topics as climate change and land-use change we found in these studies, it is noteworthy the very low frequency and importance in our survey of word as "government", "mitigation", "urbanization", "human health", "risk assessment", "culture", "(international) trade", "pet", and even the absence in our network of word such as "education" and "policy" (or related terms). This shows that the social and political impact of (studies devoted to) amphibian declines is still insufficient.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 8478.

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Food composition of a breeding population of the endemic Anatolia newt, *Neurergus strauchii* (Steindachner, 1887) (Caudata: Salamandridae), from Bingöl, Eastern Turkey

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Abstract. The study presents data on the food composition of a breeding population of the Anatolia newt *Neurergus strauchii* (Steindachner, 1887), from Bingöl, Turkey. A total of 953 prey items were determined in the food contents of 46 individuals (18 males and 28 females) examined in the study. Insecta (63%) and Malacostraca (35%) constitute 97% of the food composition by number. While the most frequently encountered prey groups in the diet are Diptera (87%), Amphipoda (85%), *Neurergus* eggs (74%), and Coleoptera (37%), the comparison of food composition by volume is Amphipoda (40%), *Neurergus* eggs (41%), and Diptera (10%) respectively. No significant difference in food niche was observed between the sexes. The species generally fed on aquatic, poor-flying or slow-moving invertebrates.

Keywords. Ecology, diet, trophic niche, dipterans, amphipods, oophagy.

INTRODUCTION

Understanding the feeding relationships in amphibian communities is of fundamental interest to herpetologists and ecologists due to their key role in aquatic ecosystems (Hirai and Matsui, 1999). Quantitative information about the role of amphibians in ecosystems is extremely important, particularly in their capacity as consumers in both aquatic and terrestrial habitats (Whiles et al., 2006). Furthermore, determining their feeding relationships in this trophic network is an essential step to understand the life cycle of the species and population fluctuations, as well as to develop successful conservation strategies (e.g., Duellman and Trueb, 1986; Cogălniceanu et al., 2001; Solé and Rödder, 2010).

The Middle Eastern newt genus *Neurergus* comprises five stream-dwelling species [*Neurergus strauchii* (Steindachner 1887), *N. barani* Öz 1994, *N. crocatus* Cope 1862, *N. kaiseri* Schmidt 1952, and *N. microspilotus* (Nesterov 1916)] distributed in Anatolia, Iran, and Iraq (Leviton et al., 1992; Papenfuss et al., 2009; Sparreboom, 2014; Rancilhac et al., 2019). The Anatolia Newt, *N. strauchii*, is endemic to eastern Turkey (Schmidtler and Schmidtler, 1970; Öz, 1994; Tok et al., 2016; Yıldız et al., 2018). Due to its narrow area of occupancy, which is less than 2000 km^2, its fragmented distribution, and habitat destruction, the species is listed as vulnerable in the IUCN Red List of Threatened Species (Papenfuss et al., 2009).

While *N. strauchii* inhabits small, cool mountain streams at altitudes between 950 and 1900 m above sea level, it overwinters on land not far from streams under stones and in burrows (Schmidtler and Schmidtler, 1970; Başoğlu et al., 1994; Bogaerts et al., 2012). Being semi-aquatic salamanders adapted to a high-elevation climate,

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adult Anatolia newt emerge from hibernation in early spring and thereafter migrate into streams that form during or after snowmelt where they will mate and breed (Bogaerts et al., 2010; Schneider and Schneider, 2010).

The species is widely studied with respect to distribution (e.g., Schmidtler and Schmidtler, 1970, 1975; Yıldız et al., 2018) and taxonomy (e.g., Steinfartz et al., 2002; Pasmans et al., 2006; Özdemir et al., 2009; Olgun et al., 2016; Rancilhac et al., 2019). Unfortunately, there are still limited data on its ecology (e.g. Schneider and Schneider, 2010; Bogaerts et al., 2006, 2010, 2012). Whilst limited information is available regarding the food habits of *Neurerus* species in captive (Bogaerts et al., 2012) and natural populations (Farasat and Sharifi, 2014), there are no data on the food habits of *N. strauchii* in nature. Therefore, the present study aimed to determine the food habits of a breeding population of the Anatolia newt from Bingöl, Turkey.

MATERIAL AND METHODS

In the study, the ingested food items of 46 aquatic adults (18 males and 28 females) collected between mid-May and early June 2014 from Soğukpınar Village in Genç – a district of Bingöl, Turkey (38°42'N, 40°27'E, 1,075 m a.s.l.) were examined. The newts were caught by hand and using fish nets in a small stream flowing into the Murat River between 07.00 and 13.00. Following capture, the sex of each individual was recorded and secondary sexual characters, snout-vent length (distance from the tip of the snout to the cloaca, SVL) and total length (from the cloaca to the tip of the tail, TL) were measured by using a caliper with a precision of 1 mm and recorded. Within an hour following capture, the stomach contents were extracted in field by flushing the stomach with pressurized water. The obtained food contents were preserved in a 70% ethanol solution for further analysis (Solé and Rödder, 2010; Çiçek, 2011).

The food contents were identified to the least inclusive possible taxonomic level. Vegetal materials, sand and small pebbles were also encountered in the food contents of four individuals (frequency of occurrence, F% = 8.7%). However, we concluded that these materials had most likely been ingested accidentally during foraging and were not regarded as food. The following parameters of dietary items were recorded: frequency of occurrence (the frequency of newt stomachs containing a particular prey type, F%), numeric proportion (the number of a particular prey item type relative to all prey items, N%), and volumetric proportion (the volume of a particular prey item as a percentage of all prey items, V%). The prey volume was calculated using an ellipsoid formula (Dunham, 1983): $V = 4/3\pi\ (L/2)\ (W/2)^2$, where V is the prey volume, L is the length of prey item, and W is the width of prey item. To determine the relative importance of each prey category in the diet of the species, the Index of Relative Importance (IRI, Pinkas et al., 1971) was calculated using the following formula: $IRI = (N\,\% + V\,\%)\ F\,\%$, where N % is the numerical proportion of each prey item in the diet; V % is the volumetric proportion of each prey item in the diet; and F % is the relative frequency of occurrence of prey. The higher value indicates the greater importance of a particular prey item in the overall dietary composition.

Trophic niche overlap was calculated using Pianka's index (O, 1973). This index ranges from 0 (no similarity) to 1 (identical). Food-niche breadth was determined using Shannon's index (H, Shannon, 1948). All niche calculations were made using "EcoSimR vers. 1.0" package (Gotelli et al., 2014) in R vers. 3.6.1 (R Core Team, 2019). The sexes were compared by t-test, and Mann-Whitney U tests were performed using the Deducer statistical package (Fellows, 2012) in the same version of R. Mean values are provided with their standard deviations.

RESULTS

The average *Neurergus strauchii* body length (SVL) was 70.3 ± (SD) 2.84 mm (range = 63-75 mm) for males and 71.2 ± 3.01 mm (65-77 mm) for females. The average total length (TL) was determined as 146.8 ± 7.08 mm (134-159 mm) in males and 148.3 ± 5.77 mm (136-158 mm) in females. No statistically significant difference was observed between the sexes in terms of their sizes (SVL, $t = 0.983$, $P = 0.332$; TL, $t = 0.016$, $P = 0.987$).

In the food content of 46 individuals, a total of 953 prey items, with body lengths ranging from 3 to 55 mm, were determined with a median number of 18 (range = 1-50) dietary items per individual. The median number of prey items was 16 (1-50) for males and 17.5 (1-39) for females. While the number of prey items was slightly greater in females, there was no significant difference between the sexes (Mann-Whitney U test, $Z = -0.452$, $P \leq 0.652$).

Among the prey taxa shown in Table 1, the highest frequency of occurrence (F% > 30%) in the food composition was observed for Diptera (87%), Amphipoda (85%), *Neurergus* eggs (74%), and Coleoptera (37%). Diptera (46%) and Amphipoda (34%) were the most abundant prey groups (N% > 20%). The greatest prey volumes (V% = 20%) were accounted for by Amphipoda (40%) and *Neurergus* eggs (41%). More active prey items such as adult Diptera, Coleoptera, Hemiptera, Lepidoptera, and Orthoptera were less frequently encountered in the food contents. Larval prey accounted for 36% of dietary items in number, 93% in frequency, and 10% in volume in the total food content. Vegetal fragments (e.g., leaves, roots, and seeds) and inorganic material were observed in 4.4% of the newts and were considered to have been ingested accidentally during foraging. In addition, the presence of shed-skin remains was detected in the diet of two individuals (F% = 4.4%). Amphipoda (IRI = 6827), Diptera (5386), *Neurergus* eggs (3739) and Coleoptera (318) were the most important prey categories in Anatolia newts (Table 1).

Table 1. The food composition of 46 (18 males and 28 females) *N. strauchii* individuals from Eastern Anatolia, Turkey. F: Frequency of occurrence, N: Numeric proportion, V: Volumetric proportion, IRI: Index of Relative Importance.

Prey Taxa	F (%)			N (%)			V (%)			IRI
	Females	Males	Overall	Females	Males	Overall	Females	Males	Overall	
MOLLUSCA: GASTROPODA	8 (28.6%)	1 (5.6%)	9 (19.6%)	10 (1.7%)	1 (0.3%)	11 (1.2%)	62 (0.9%)	4 (0.1%)	67 (0.6%)	29.9
Lymnaeidae	6 (21.4%)	-	6 (13.0%)	7 (1.2%)	-	7 (0.7%)	48 (0.7%)	-	48 (0.4%)	14.8
Planorbidae	2 (7.1%)	1 (5.6%)	3 (6.5%)	3 (0.5%)	1 (0.3%)	4 (0.4%)	15 (0.2%)	4 (0.1%)	19 (0.2%)	3.8
ANNELIDA: CLITELLATA	3 (10.7%)	-	3 (6.5%)	3 (0.5%)	-	3 (0.3%)	124 (1.7%)	-	124 (1.1%)	8.9
Lumbricidae	3 (10.7%)	-	3 (6.5%)	3 (0.5%)	-	3 (0.3%)	124 (1.7%)	-	124 (1.1%)	8.9
ARTHROPODA	28 (100%)	18 (100%)	46 (100%)	567 (97.8%)	372 (99.7%)	939 (98.5%)	7069 (97.4%)	4543 (99.9%)	11612 (98.4%)	55801.7
Chilopoda	-	1 (5.6%)	1 (2.2%)	-	1 (0.3%)	1 (0.1%)	-	4 (0.1%)	4 (<0.1%)	0.3
MALACOSTRACA: ISOPODA	2 (7.1%)	2 (11.1%)	4 (8.7%)	2 (0.3%)	5 (1.3%)	7 (0.7%)	45 (0.6%)	48 (1.0%)	92 (0.8%)	13.2
Oniscidae	2 (7.1%)	2 (11.1%)	4 (8.7%)	2 (0.3%)	5 (1.3%)	7 (0.7%)	45 (0.6%)	48 (1.0%)	92 (0.8%)	13.2
MALACOSTRACA: AMPHIPODA	26 (92.9%)	13 (72.2%)	39 (84.8%)	163 (28.1%)	-	328 (34.4%)	2530 (34.9%)	-	4763 (40.4%)	6827.3
Gammaridae	26 (92.9%)	13 (72.2%)	39 (84.8%)	163 (28.1%)	165 (44.2%)	328 (34.4%)	2530 (34.9%)	2233 (49.1%)	4763 (40.4%)	6827.3
INSECTA	26 (92.9%)	18 (100%)	44 (95.7%)	402 (69.3%)	201 (53.9%)	603 (63.3%)	4494 (61.9%)	710.8 (15.6%)	1872.8 (15.9%)	20301.5
ORTHOPTERA	1 (3.6%)	-	1 (2.2%)	1 (0.2%)	-	1 (0.1%)	6 (0.1%)	-	6 (0.1%)	0.3
EPHEMEROPTERA	4 (14.3%)	1 (5.6%)	5 (10.9%)	11 (1.9%)	4 (1.1%)	15 (1.6%)	24 (0.3%)	10 (0.2%)	34 (0.3%)	20.2
HEMIPTERA	3 (10.7%)	4 (22.2%)	7 (15.2%)	7 (1.2%)	6 (1.6%)	13 (1.4%)	21 (0.3%)	26 (0.6%)	47 (0.4%)	26.8
Notonectidae	3 (10.7%)	4 (22.2%)	7 (15.2%)	7 (1.2%)	6 (1.6%)	13 (1.4%)	21 (0.3%)	26 (0.6%)	47 (0.4%)	26.8
HYMENOPTERA	-	1 (5.6%)	1 (2.2%)	-	1 (0.3%)	1 (0.1%)	-	1 (<0.1%)	1 (<0.1%)	0.2
Formicidae	-	1 (5.6%)	1 (2.2%)	-	1 (0.3%)	1 (0.1%)	-	1 (<0.1%)	1 (<0.1%)	0.2
COLEOPTERA	10 (35.7%)	7 (38.9%)	17 (37.0%)	21 (3.6%)	15 (4.0%)	36 (3.8%)	167 (2.3%)	402 (8.8%)	569 (4.8%)	317.9
Coleoptera sucul	4 (14.3%)	1 (5.6%)	5 (10.9%)	14 (2.4%)	4 (1.1%)	18 (1.9%)	88 (1.2%)	20 (0.4%)	108 (0.9%)	30.5
Coleoptera larva	6 (21.4%)	5 (27.8%)	11 (23.9%)	7 (1.2%)	10 (2.7%)	17 (1.8%)	79 (1.1%)	364 (8.0%)	443 (3.8%)	132.4
Carabidae	-	1 (5.6%)	1 (2.2%)	-	1 (0.3%)	1 (0.1%)	-	18 (0.4%)	18 (0.2%)	0.6
LEPIDOPTERA	3 (10.7%)	4 (22.2%)	7 (15.2%)	4 (0.7%)	4 (1.1%)	8 (0.8%)	11 (0.1%)	20 (0.4%)	31 (0.3%)	16.8
Lepidoptera indet.	1 (3.6%)	2 (11.1%)	3 (6.5%)	2 (0.3%)	2 (0.5%)	4 (0.4%)	1 (<0.1%)	5 (0.1%)	6 (<0.1%)	3.1
Lepdoptera larvae (indet.)	2 (7.1%)	2 (11.1%)	4 (8.7%)	2 (0.3%)	2 (0.5%)	4 (0.4%)	10 (0.1%)	15 (0.3%)	25 (0.2%)	5.5
DIPTERA	24 (85.7%)	16 (88.9%)	40 (87.0%)	298 (51.4%)	143 (38.3%)	441 (46.3%)	933 (12.9%)	251 (5.5%)	1184 (10.0%)	5385.9
Diptera adult	2 (7.1%)	1 (5.6%)	3 (6.5%)	5 (0.9%)	2 (0.5%)	7 (0.7%)	37 (0.5%)	1 (<0.1%)	38 (0.3%)	6.9
Chironimid Larvae (indet.)	18 (64.3%)	11 (61.1%)	29 (63.0%)	116 (20.0%)	34 (9.1%)	150 (15.7%)	390 (5.4%)	104 (2.3%)	495 (4.2%)	1256.4
Culicidae	8 (28.6%)	6 (33.3%)	14 (30.4%)	90 (15.5%)	25 (6.7%)	115 (12.1%)	347 (4.8%)	57 (1.3%)	404 (3.4%)	471.5
Culicid larvae	17 (60.7%)	10 (55.6%)	27 (58.7%)	87 (15.0%)	82 (22.0%)	169 (17.7%)	159 (2.2%)	89 (2.0%)	248 (2.1%)	1164.1
AMPHIBIA: CAUDATA	18 (64.3%)	16 (88.9%)	34 (73.9%)	60 (10.3%)	28 (7.5%)	88 (9.2%)	3332 (45.9%)	1548 (34.0%)	4880 (41.3%)	3738.6
Neurergus eggs	18 (64.3%)	16 (88.9%)	34 (73.9%)	60 (10.3%)	28 (7.5%)	88 (9.2%)	3332 (45.9%)	1548 (34.0%)	4880 (41.3%)	3738.6
Total number of prey items	28	18	46	580	373	953	7255	4548	11803	

The food niche breadth (Shannon's index) was low and quite similar between the sexes (1.62 for males and 1.34 for females). The food spectrum of the newts yielded a moderate niche breadth based on the index value. Prey composition was quite similar between the sexes (Pianka's niche overlap index, 0.68), indicating the use of similar microhabitats for foraging. Despite small differences in percentages, no statistically significant differences were found between the sexes with respect to food contents.

DISCUSSION

Adult *Neurergus* newts mainly feed on aquatic prey items in their larval stages (e.g., Kutrup et al., 2005; David et al., 2009; Covaciu-Marcov et al., 2010; Kopecký et al., 2012; Farasat and Mozafar, 2014). The close relative of *N. strauchii*, *N. microspilotus* predominantly feeds on benthic macroinvertebrates, mainly Mycetophilidae, Baetidae, Corbiculidae, Gammaridae, and Lumbricidae (Farasat and Mozafar, 2014). In this study, the most frequent prey items of Anatolia newts were dipterans, amphipods, coleopterans, and their own eggs, whereas the greatest prey volume was attributable to amphipods, *N. strauchii* eggs, and dipterans. The dietary components of Anatolia newts are similar to those of other species of newts (Farasat and Mozafar, 2014). The utilization of amphibian eggs as food is beneficial for several reasons, one of them being the low energy consumption needed for a large volume of food (Denoël and Demars, 2008). The sampling was done during the breeding period of the species and there were many egg clutches in the study area. Eggs were identified in the food contents of 34 individuals (74%). The consumption of amphibian eggs has also been recorded in other newt species (Covaciu-Marcov et al., 2010; Kopecky et al., 2012; Farasat and Mozafar, 2014), though Farasat and Mozafar (2014) reported *N. microspilotus* to consume its own eggs at a lower rate. Our observation in the field showed that the prey spectrum in the environment of the species was limited and that the easily obtained eggs could substitute for potential prey requiring greater foraging effort. It has been shown that in captivity, *N. strauchii* will eat a wide variety of food items offered, even dead unnatural food items, such as slices of liver and octopus (Bogaerts et al., 2012). This means that they are certainly able to identify and eat non-moving foods like an egg in their natural habitats. Under poor environmental conditions, this could be an effective strategy to avoid starvation.

There were no sex-specific differences detected in the food composition of *N. strauchii* in Anatolia. This also coincides with many studies on the same issue reporting that there is no difference between the sexes (e.g., Covaciu-Marcov et al., 2010; Farasat and Mozafar, 2014). This indicates that both sexes use the same habitat for foraging. However, some species (e.g. *Lissotriton vulgaris*, *Lissotriton montandoni*, *Triturus cristatus* group) exhibit sex-specific differences in the feeding strategy and a certain level of trophic selectivity (Covaciu-Marcov et al., 2002, 2010) depending on newt size variation (David et al., 2009; Covaciu-Marcov et al., 2010).

Data suggest that amphibians exhibit two modes for foraging: sit-and-wait foraging and active foraging. While actively foraging, amphibians wander around the environment in search of prey, thus spending considerable energy in the search phase but little energy in the capture phase of foraging (Vitt and Caldwell, 2014). They mostly encounter and consume non-moving or slow-moving prey items (Pianka, 1966) and generally use their visual and olfactory senses while foraging (Vitt and Caldwell, 2014). Their food niche breadth is rather narrower than that of sit-and-wait predators (Perry and Pianka, 1997). Having its diet based mainly on composed slow-moving aquatic invertebrates, the Anatolia newt could be considered an active forager.

As stated in the study, the Anatolia newt's food items comprise slow-moving aquatic invertebrates, particularly aquatic dipterans, amphipods, and coleopterans, as well as their own eggs. There is no significant difference in food composition between the sexes. The species is an active predator usually feeding on poor-flying or non-flying prey items. It has low food niche breadth, mostly limited to the classes Insecta and Malacostraca. The abundance of these prey taxa in habitats might potentially indicate streams habitable by the Anatolia newt within its range. The data on the biology and ecology of the Anatolia newt are still insufficient to identify the ecological pressures on populations. Unfortunately, further studies are necessary to determine its ecology, habitat, and distribution pattern.

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Stomach histology of *Crocodylus siamensis* and *Gavialis gangeticus* reveals analogy of archosaur "gizzards", with implication on crocodylian gastroliths function

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Abstract. Two groups of extant Archosauria, Crocodylia and Neornithes, have two-chambered stomachs and store gastroliths inside their "gizzards". Morphological similarities of the "gizzards" lead some previous studies to assume that the presence of this structure, organ "gizzard" is synapomorphic to Archosauria. However, the homology of archosaur "gizzards" had never been tested. This study provides general histological descriptions of stomachs of two crocodylian taxa, *Crocodylus siamensis* and *Gavialis gangeticus*, to determine the homology of crocodylian and neornithine "gizzards". Our study demonstrates that both *Crocodylus siamensis* and *Gavialis gangeticus* have longer, more complex glands in the fundic stomach (crocodylian "gizzard") than in the pyloric stomach. Additionally, we found that compound glands are present in the fundic stomach of *Crocodylus siamensis.* Therefore, crocodylian stomach histomorphological structures are concordant with those of other non-avian reptiles, despite the unique gross morphology. The pyloric regions of non-avian reptile stomachs are known to be homologous with the pyloric regions of mammalian stomachs as well as neornithine ventriculus (neornithine gizzard). Therefore, crocodylian and neornithine "gizzards" are morphologically analogous but not homologous. The presence of PAS-positive layer in the pyloric stomach of *Gavialis gangeticus*, which resembles the koilin layer of neornithine ventriculus, further supports this interpretation. At the same time, however, the similarity in gastroliths mass/body mass ratio and the correlations between gastroliths occurrence and diet types suggest that crocodylian gastroliths might have contributed to the digestion of ingesta, even though crocodylian and neornithine "gizzards" are not homologous.

Keywords. Histology, gizzard, gastroliths, Crocodylia, stomach.

INTRODUCTION

Crocodylians present the most complex stomach known in existing members of non-avian reptiles (hereafter referred to as reptiles) (Owen, 1866; Richardson et al., 2002). Crocodylian stomachs are composed of two distinct units: fundic and pyloric chambers. Neornithes (a least inclusive clade of living birds), the closest living relatives of crocodylians, also have two-chambered stomachs: a glandular stomach (proventriculus) and a muscular stomach (ventriculus or gizzard) (Ziswiler and Farner, 1972; Denbow, 2015). While the proventriculus excretes the mucus, pepsin, and hydrochloric acid necessary for chemical digestion, the ventriculus performs mechanical digestion of ingesta. Some neornithines, mostly herbivores, consume stones and store them inside gizzards as gastroliths (geo-gastroliths, Wings, 2007) to aid gastric mechanical digestion (Fritz, 1937; Hetland et al., 2003; Jin et al., 2014). Several crocodylians are also known to contain gastroliths inside their fundic stomachs (e.g., Corbet,

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1960; Cott, 1961). Since both crocodylians and neornithines have two-chambered stomachs and store gastroliths inside them, some studies refer crocodylian fundic stomach as a “gizzard” (Reese, 1915; Grigg and Gans, 1993).

Based on the phylogenetic bracket of the two-chambered stomachs, together with the generality of gastroliths among archosaurs including non-avian dinosaurs, (e.g., Kobayashi et al., 1999; Cerda, 2008; Lee et al., 2014), neornithine style muscular “gizzard” had previously been considered as a plesiomorphic feature of Archosauria (Varricchio, 2001; Fritz et al., 2011). However, the homology of avian and crocodylian “gizzards” is considered ambiguous (Schwenk and Rubega, 2005). While some studies considered that crocodylian “gizzards” are homologous with neornithine gizzards (Varricchio, 2001; Fritz et al., 2011), some studies refute the homology (Jones, 1861; Huang et al., 2016). Additionally, the functions of crocodylian gastroliths are still under debates (e.g., food processing, hydrostatic function, accidental intake; Cott, 1961; Davenport et al., 1990; Taylor, 1993; Wings, 2007; Uriona et al., 2019).

Previous studies on crocodylian stomach microstructures were based only on *Alligator mississippiensis* (Eisler, 1889; Reese, 1915; Staley, 1925). This lack of knowledge of crocodylian stomach structures cannot allow determining the plesiomorphic status of archosaur “gizzard”. Our study provides the first histomorphological information of the stomachs of *Crocodylus siamensis* and *Gavialis gangeticus* to test the homology of crocodylian and neornithine “gizzards”. Besides, this study conducts analyses that provide new implications of the digestive function of crocodylian gastroliths. Neornithine gastrolith mass is known to be correlated with a body mass (Wings and Sander, 2007), and the relationship is utilized as a proxy for the digestive use of dinosaur gastroliths (Wings and Sander, 2007; Cerda, 2008; Lee et al., 2014). Furthermore, avian dietary habits are strongly related to the occurrence frequencies of gastroliths (Best and Gionfriddo, 1991; Gionfriddo and Best, 1996; Gionfriddo and Best, 1999). Our study tests if crocodylian gastroliths have the same relationship as observed in neornithines to assess the digestive function of crocodylian gastroliths. The clarifications of archosaur “gizzard” homology and the crocodylian gastroliths functions are expected to contribute to better understandings of crocodylian physiology and the evolutionary history of the archosaur digestive system.

MATERIAL AND METHOD

Corpora of four juvenile individuals of captive *Crocodylus siamensis* which were dead during winter are provided from a local farmer Koike Wani Sohonpo Co. Ltd. in Shizuoka Prefecture of Japan, and four stomachs of captive post-mortem *Gavialis gangeticus* are provided from Atagawa Tropical & Alligator Garden in Shizuoka Prefecture of Japan (Fig. 1). All the specimens were stored frozen before sampling. Small segments were sampled from the greater curvature wall, ventral wall, and pyloric wall of the stomach. The segments are fixed in 10% formalin neutral buffer solution, then dehydrated in ascending grades of ethyl alcohol, cleared with xylene, and embedded in paraffin. Sections were cut at 3μm in thickness and stained with Haematoxylin-Eosin (HE), Periodic Acid Schiff (PAS), and Alcian-Blue (AB) pH 2.5 for general histological observations. To avoid confusion due to different terminologies used in previous studies, this study uses the term “gizzard” for a stomach chamber that may possess gastroliths. Terms fundic stomach and pyloric stomach are used for first and second chambers of the crocodylian stomach, respectively. Terms proventriculus and ventriculus are used for first and second chambers of the avian stomach, respectively.

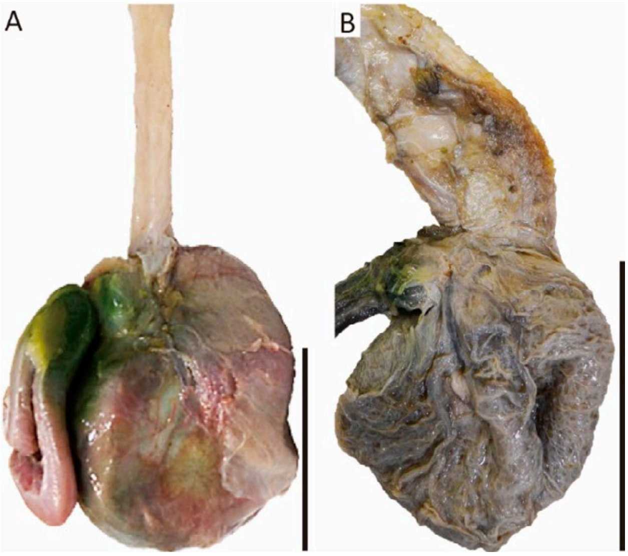

Fig. 1. Stomachs of *Crocodylus siamensis* (A) and *Gavialis gangeticus* (B). Scales: 5cm for A, 10cm for B.

Crocodylian body and gastroliths weights are compiled from previous studies (Corbet, 1960; Cott, 1961; Kennedy and Brockman, 1965; Brazaitis, 1969; Pauwels et al., 2007). Stomach contents of crocodylians are gathered from previous studies (Corbet, 1960; Cott, 1961; Tucker et al., 1996; Platt et al., 2006; Wallace and Leslie, 2008; Platt et al., 2013). Body mass and gastroliths mass are log10 transformed and occurrence frequencies of gastroliths and different food types are arcsine transformed before statistical analyses. Statistical analyses are conducted using the software JMP version 14.3.

RESULTS

The stomach walls of all of the observed specimens are composed of 4 layers: mucosa, submucosa, muscula-

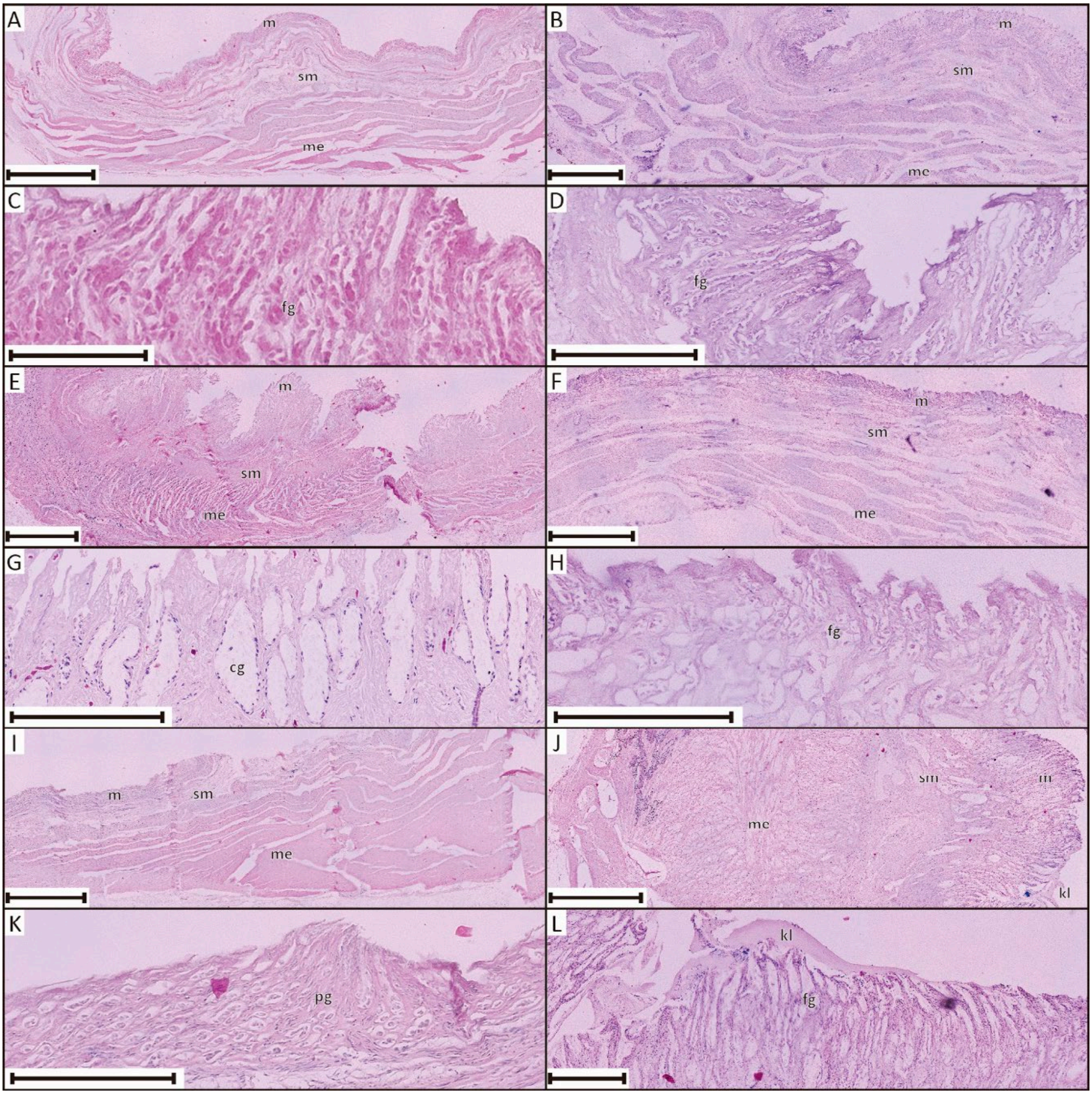

Fig. 2. Histological structures of *Crocodylus siamensis* (A, C, E, G, I, K) and *Gavialis gangeticus* (B, D, F, H, J, L). A-D, greater curvature wall; E-H, ventral wall; I-L, pyloric wall. Abbreviations: cg, compound gland; fg, fundic gland; kl, possible koilin layer; m, mucosa; me, muscularis externa; sm, submucosa; pg, pyloric gland. Scales: 1000µm for A, B, E, F, I, and J; 250µm for C, D, G, H, K, and L.

ris externa, and serosa layers from inner to outer layers (Fig. 2). The greater curvature wall is the thickest among the observed regions (Fig. 1B, 2A, 2B). Submucosa comprises nearly half of the stomach wall in thickness in *Crocodylus siamensis* (~800µm), while the muscularis externa occupies more than half of the wall in thickness in *Gavialis gangeticus* (~2000µm). The gastric folds, supported by thick submucosa, are shorter than wide in both *Crocodylus siamensis* and *Gavialis gangeticus*. The mucosa (~200µm in *Crocodylus siamensis* and ~300µm in *Gavialis gangeticus*) is thinner than submucosa and has long fundic glands in both taxa. The fundic glands are tubular and branched (Fig. 2C, 2D) although postmortem damage obscures the details. The fundic glands are

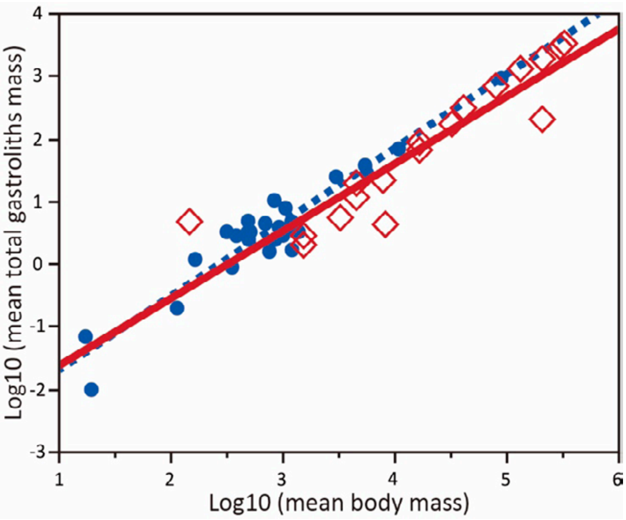

Fig. 3. Relationships of body mass to gastroliths mass in crocodylians (red diamond) and crown birds (blue circle). The red solid line represents the regression line for crocodylians and the blue dashed line represents the regression line for crown birds.

mainly composed of dark oxynticopeptic cells, as previously reported in the stomachs of *Alligator mississippiensis* (Eisler, 1889; Staley, 1925). There are no morphologically distinct mucous neck cells reported in most snakes (Jacobson, 2007).

The ventral wall is slightly thinner than the greater curvature wall (Fig. 2E, 2F). The submucosa of the ventral wall is thin, and the muscularis externa comprises the largest proportion of the ventral wall. The gastric folds are well-developed in *Crocodylus siamensis*, but it is absent in *Gavialis gangeticus*. The mucosa is proportionally thinner than it is in the greater curvature wall, resulting in shorter fundic glands than in the greater curvature wall in both taxa. The fundic gland structures are generally the same as those in the greater curvature wall. However, gastric glands in the ventral wall are markedly larger than in the other stomach walls and form a lobule-like compound gland in *Crocodylus siamensis* (Fig. 2G). These gastric glands are separated from each other with thick connective tissue. The lobule-like compound glands could not be observed in *Gavialis gangeticus*, partly because available stomachs are not well-preserved compared to *Crocodylus siamensis*.

The pyloric walls of the two crocodylian taxa are largely different from the greater curvature and the ventral walls in their extremely thick muscularis externa, which represents up to 80% of the stomach wall thickness (Fig. 2I, 2J). On the other hand, the submucosa is reduced, unlike what was observed in the fundic stomach. Muscularis mucosa is also much thicker than in the other two regions. Pyloric glands are simple tubular glands and are significantly short compared to the fundic glands (Fig. 2K). Unfortunately, details of the pyloric glands are not available due to the impact of postmortem damage, especially in the stomachs of *Gavialis gangeticus*. The internal surface of the pyloric wall is locally covered by a PAS-positive layer in *Gavialis gangeticus* (Fig. 2L).

Body mass and gastroliths mass of Crocodylia (Table 1) demonstrates that the average proportion of gastroliths mass relative to body mass is 0.66%. The value is slightly higher than that in neornithines (0.55%), but the difference is not statistically significant (Student's t-test, $P = 0.50$). Regression analysis demonstrates the correlation of crocodylian body mass and gastroliths mass (Fig. 3; $r^2 = 0.84$, $P < 0.001$) as in neornithines (Wings and Sander, 2007). Neither the slope nor the intercept of the regression line differs from those of neornithines ($P = 0.43$ and 0.73, respectively), indicating that the relationship between gastroliths mass and body mass of crocodylians are statistically indistinctive from that of neornithines. Regression analyses on occurrence frequencies of gastroliths and different food types (Table 2) demonstrate that occurrence frequency of gastroliths are positively correlated with those of vertebrates and negatively correlated with those of most invertebrates (Table 3). The correlations are statistically significant ($P < 0.05$) in Insecta, Pisces, Amphibia, and Mammalia.

DISCUSSION

Histological evaluations of *Crocodylus siamensis* and *Gavialis gangeticus* stomachs demonstrate that general stomach morphology is similar to each other. Both taxa have long, tubular branched fundic glands and short, simple pyloric glands (Fig. 2). The result is concordant with the stomach microstructure of *Alligator mississippiensis* as reported in Staley (1925), indicating that members of Crocodylia share generally the same fundic and pyloric gland structures. The long, complex fundic glands and short, simple pyloric glands are also in agreement with general features of reptilian stomachs (Luppa, 1977; Jacobson, 2007). Furthermore, the lobule-like compound fundic glands that are present in the ventral wall of *Crocodylus siamensis* (Fig. 2G) are also reported in fundic stomachs of other reptiles including *Caretta caretta* (Oppel, 1896), *Chamaeleon afticanus* (Hamdi et al., 2014), *Laudakia stellio* (Koca and Gurcu, 2011), *Ophisops elegans* (Çakici and Akat, 2013), and *Varanus niloticus* (Ahmed et al., 2009). Therefore, the present observations demonstrate that general histomorphological structures of crocodylian stomach glands are concordant with those

Table 1. Mean total gastroliths mass and body mass of crocodylians compiled.

Species	Mean total gastrolith mass[g]	Mean body mass[g]	Sample size	Relative weight of gastroliths [%]	Reference (gastroliths)	Reference (body mass)
Crocodile acutus	174.00	32206	2	0.54%	Brazaitis (1969)	Brazaitis (1969)
Alligator mississippiensis	22.00	7800	1	0.28%	Kennedy and Brockman (1965)	Kennedy and Brockman (1965)
Osteolaemus t. tetraspis (Rabi oil fields)	5.54	3241	14	0.17%	Pauwels et al. (2007)	Pauwels et al. (2007)
Osteolaemus t. tetraspis (Loango National Park)	4.33	8193	8	0.05%	Pauwels et al. (2007)	Pauwels et al. (2007)
Crocodilus niloticus 0.5-1.0m	2.04	1524	101	0.13%	Cott (1961)	Cott (1961)
Crocodilus niloticus 1.0-1.5m	11.70	4518	102	0.26%	Cott (1961)	Cott (1961)
Crocodilus niloticus 1.5-2.0m	88.87	16540	76	0.54%	Cott (1961)	Cott (1961)
Crocodilus niloticus 2.0-2.5m	312.50	40900	73	0.76%	Cott (1961)	Cott (1961)
Crocodilus niloticus 2.5-3.0m	700.30	79390	69	0.88%	Cott (1961)	Cott (1961)
Crocodilus niloticus 3.0-3.5m	1321.20	131900	52	1.00%	Cott (1961)	Cott (1961)
Crocodilus niloticus 3.5-4.0m	1906.20	206500	16	0.92%	Cott (1961)	Cott (1961)
Crocodilus niloticus 4.0-4.5m	2940.40	298700	5	0.98%	Cott (1961)	Cott (1961)
Crocodilus niloticus 4.5-5.0m	3356.00	325500	3	1.03%	Cott (1961)	Cott (1961)
Crocodilus niloticus 0.3-0.5m	4.80	146	2	3.29%	Corbet (1960)	Corbet (1960)
Crocodilus niloticus 0.5-1.0m	2.87	1524	23	0.19%	Corbet (1960)	Corbet (1960)
Crocodilus niloticus 1.0-1.5m	19.79	4518	18	0.44%	Corbet (1960)	Corbet (1960)
Crocodilus niloticus 1.5-2.0m	66.80	16540	2	0.40%	Corbet (1960)	Corbet (1960)
Crocodilus niloticus 3.5-4.0m	206.50	206500	1	0.10%	Corbet (1960)	Corbet (1960)

of other reptiles, despite the unique gross morphology of crocodylian stomach among Reptilia.

The general histomorphological features of reptilian fundic and pyloric glands resemble neornithine gastric glands of proventriculus and ventriculus, respectively. Neornithine proventriculus contains highly branched compound glands that compose lobules, and the ventriculus contains simple tubular glands covered by the PAS-positive koilin layer (Ziswiler and Farner, 1972). Through stomach muscle structure comparisons, Pernkopf (1929) suggested that the reptilian pyloric stomach is homologous to the pyloric region of the mammalian stomach, which is homologous to neornithine ventriculus (Smith et al., 2000). The present results suggest that the crocodylian pyloric stomach is homologous with neornithine ventriculus (neornithine gizzard), whereas the crocodylian fundic stomach (crocodylian "gizzard") is homologous with neornithine proventriculus. Since crocodylian and neornithine "gizzards" are not homologous, the previous assumption that "gizzard" is synapomorphic to Archosauria (Varricchio, 2001; Fritz et al., 2011) is dismissed.

Although crocodylian and neornithine "gizzards" are not homologous, the absence of statistical difference in the body mass-gastroliths mass relationship between the two groups suggests the digestive function of crocodylian gastroliths based on previous interpretations (Wings and Sander, 2007). The relationships between gastroliths

Table 2. Stomach contents of crocodylians compiled.

Species	Body Length (cm)	SVL (cm)	Sample #	Occurrence Frequencies (%)										Reference
				Gastroliths	Insecta	Araneida	Crustacea	Mollusca	Pisces	Amphibia	Reptilia	Aves	Mammalia	
Crocodylus niloticus	<100	-	30	76.67	96.55	13.79	10.34	13.79	17.24	24.14	0.00	0.00	6.90	Corbet (1960)
Crocodylus niloticus	100-199	-	25	92.00	77.27	4.55	18.18	9.09	45.45	9.09	0.00	18.18	0.00	Corbet (1960)
Crocodylus niloticus	30-50	-	12	0.00	91.67	16.67	0.00	0.00	0.00	25.00	0.00	0.00	0.00	Cott (1961)
Crocodylus niloticus	50-100	-	142	50.00	82.39	13.38	23.94	7.04	9.86	11.97	2.82	3.52	4.93	Cott (1961)
Crocodylus niloticus	100-150	-	141	67.24	58.16	2.13	28.37	17.73	16.31	12.77	2.84	2.13	10.64	Cott (1961)
Crocodylus niloticus	150-200	-	111	82.29	26.13	0.90	10.81	22.52	37.84	0.90	7.21	9.91	10.81	Cott (1961)
Crocodylus niloticus	200-250	-	117	89.69	10.26	0.00	7.69	26.50	45.30	0.85	5.98	5.98	9.40	Cott (1961)
Crocodylus niloticus	250-300	-	129	100.00	2.33	0.00	3.10	17.83	43.41	0.00	9.30	10.85	12.40	Cott (1961)
Crocodylus niloticus	300-350	-	113	100.00	0.88	0.00	5.31	20.35	42.48	0.00	14.16	9.73	21.24	Cott (1961)
Crocodylus niloticus	350-400	-	49	100.00	0.00	0.00	2.04	12.24	44.90	0.00	20.41	12.24	26.53	Cott (1961)
Crocodylus niloticus	400-450	-	23	100.00	0.00	0.00	4.35	8.70	13.04	0.00	34.78	13.04	47.83	Cott (1961)
Crocodylus niloticus	450-500	-	12	100.00	0.00	0.00	0.00	0.00	33.33	0.00	41.67	0.00	58.33	Cott (1961)
Crocodylus niloticus	-	17.0-38.9	151	3.60	57.10	57.10	0.00	0.00	10.70	7.10	0.00	0.00	3.60	Wallace and Leslie (2008)
Crocodylus niloticus	-	39-66.3	82	20.80	45.80	41.70	8.30	0.00	12.50	4.20	4.20	0.00	4.20	Wallace and Leslie (2008)
Crocodylus niloticus	-	66.4-115.8	53	50.00	20.00	10.00	0.00	0.00	80.00	0.00	0.00	0.00	10.00	Wallace and Leslie (2008)
Crocodylus moreletii	<30	-	71	11.27	84.51	29.58	0.00	2.82	16.90	0.00	0.00	0.00	0.00	Platt et al. (2006)
Crocodylus moreletii	30.1-50	-	117	5.98	91.45	26.50	7.69	5.13	5.98	0.85	0.85	0.00	0.00	Platt et al. (2006)
Crocodylus moreletii	50.1-100	-	121	18.18	68.60	6.61	18.18	20.66	25.62	5.79	6.61	1.65	9.92	Platt et al. (2006)
Crocodylus moreletii	100.1-150	-	63	17.46	34.92	1.59	14.29	41.27	31.75	3.17	3.17	4.76	3.17	Platt et al. (2006)
Crocodylus moreletii	>150	-	48	14.58	12.50	0.00	20.83	70.83	31.25	0.00	2.08	10.42	2.08	Platt et al. (2006)
Crocodylus acutus	-	<15	19	0.00	63.16	0.00	31.58	0.00	5.26	0.00	0.00	0.00	0.00	Platt et al. (2013)
Crocodylus acutus	-	15.1-40	16	0.00	75.00	0.00	68.75	6.25	12.50	0.00	6.25	0.00	0.00	Platt et al. (2013)
Crocodylus acutus	-	40.1-65	28	28.57	14.29	0.00	89.29	3.57	25.00	3.57	0.00	3.57	3.57	Platt et al. (2013)
Crocodylus acutus	-	65/1-90	16	31.25	18.75	0.00	87.50	0.00	0.00	0.00	12.50	12.50	0.00	Platt et al. (2013)
Crocodylus acutus	-	>90	18	16.67	0.00	0.00	94.44	5.56	11.11	0.00	0.00	11.11	0.00	Platt et al. (2013)
Crocodylus johnsoni	-	10-19.9	29	59.00	66.00	48.00	0.00	0.00	10.00	3.00	0.00	0.00	0.00	Tucker et al. (1996)
Crocodylus johnsoni	-	20-29.9	62	89.00	66.00	45.00	5.00	0.00	31.00	7.00	0.00	0.00	2.00	Tucker et al. (1996)
Crocodylus johnsoni	-	30-39.9	49	82.00	59.00	31.00	10.00	0.00	34.00	16.00	0.00	0.00	2.00	Tucker et al. (1996)
Crocodylus johnsoni	-	40-49.9	43	91.00	42.00	40.00	14.00	0.00	21.00	19.00	0.00	0.00	0.00	Tucker et al. (1996)
Crocodylus johnsoni	-	50-59.9	39	87.00	33.00	41.00	5.00	0.00	13.00	15.00	3.00	0.00	3.00	Tucker et al. (1996)
Crocodylus johnsoni	-	60-69.9	24	96.00	29.00	8.00	8.00	0.00	8.00	75.00	4.00	0.00	0.00	Tucker et al. (1996)
Crocodylus johnsoni	-	70-79.9	36	94.00	11.00	11.00	6.00	0.00	3.00	56.00	8.00	0.00	6.00	Tucker et al. (1996)
Crocodylus johnsoni	-	>80	42	91.00	12.00	12.00	10.00	0.00	0.00	52.00	2.00	2.00	10.00	Tucker et al. (1996)

Table 3. Results of regression analyses between the occurrences of different food types and gastroliths.

Group	Coefficient	p-value	R^2	Correlation
Insecta	-0.659	0.002	0.280	Negative
Araneida	-0.222	0.496	0.015	Negative
Crustacea	-0.478	0.065	0.106	Negative
Mollusca	0.000	0.999	0.000	-
Pisces	0.762	0.041	0.106	Positive
Amphibia	0.407	0.228	0.047	Positive
Reptilia	1.342	0.004	0.237	Positive
Aves	0.937	0.110	0.080	Positive
Mammalia	1.398	0.000	0.352	Positive

occurrence frequency with dietary types (Table 3) further support their digestive function. The positive correlations with vertebrate diets, although supported statistically only in mammals, may suggest that gastroliths are possibly beneficial for digesting bones. Although the gastroliths might have not served as "teeth" to strongly grind ingesta as they do in herbivorous birds (Moore, 1998; Moore, 1999), they might have benefited digestion through ingesta mixing and facilitating stomach juice excretion (Wings, 2007). These functions do not contradict with other possible gastroliths functions such as buoyancy control (Taylor, 1993). Therefore, the results of this study suggest a possibility that although crocodylian "gizzard" is not homologous with that of neornithines, their "gizzard" efficiently utilized gastroliths for digestion.

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Does chronic exposure to ammonium during the pre-metamorphic stages promote hindlimb abnormality in anuran metamorphs? A comparison between natural-habitat and agrosystem frogs

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Abstract. Despite their detrimental effects on locomotion, prevalence of hindlimb abnormalities is increasing in anuran populations worldwide. Among others, agrochemical pollution during the larval stage is a potential cause. However, populations exposed to such a strong selective pressure could evolve resistance. In this work, we examine the potential effects of chronic exposure to ammonium during the pre-metamorphic stages of *Pelophylax perezi* frogs on metamorph hindlimb abnormality prevalence, as compared with unpolluted-water-reared conspecifics. We conducted the experiment on tadpoles either from natural-habitat or from agrosystem parents. We detected no effect of chronic exposure to ammonium on hindlimb abnormality prevalence in frogs from either habitat, which suggests that the lack of effect detected is not related to resistance evolved in agrosystem frogs.

Keywords. Amphibian, anuran, anomaly, malformation, *Pelophylax perezi*.

Functioning appendages are fundamental for whole-organism performance of most anurans (Johansson et al., 2010; Zamora-Camacho et al., 2019). Hind limb morphology is directly responsible for locomotion of metamorph (Zamora-Camacho and Aragón, 2019a) and adult anurans (Zamora-Camacho, 2018), either from terrestrial (Gomes et al., 2009) or aquatic environments (Herrel et al., 2012), regardless of their locomotion mode (Enriquez-Urzelai et al., 2015). Therefore, hindlimb abnormality in this group is likely eradicated by natural selection due to its severe negative effects on locomotion (Zamora-Camacho and Aragón, 2019b). Consistently, prevalence of anuran appendage abnormality appears generally below 5% (Ouellet, 2000; Mester et al., 2015).

Nonetheless, limb abnormality rates are increasing in anurans worldwide (Johnson and Bowerman, 2010; Laurentino et al., 2016). These include diverse malformations, such as lacking and extra limbs and digits, as well as fused or misshaped limbs (Johnson and Bowerman, 2010; Reeves et al., 2013). Limb abnormalities are particularly common in metamorphs (Kiesecker, 2002; Piha et al., 2006), seemingly because reduced locomotor performance (Zamora-Camacho and Aragón, 2019b) might cause their death shortly after metamorphosis.

Besides a genetic origin (Droin and Fischberg, 1980), hindlimb abnormalities in anurans have been related to biotic interactions such as predatory pressure (Johnson and Bowerman, 2010) or parasite infections (Roberts and Dickinson, 2012), as well as abiotic factors such as ultra-

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violet-B radiation (Pahkala et al., 2001). However, human perturbance frequently provokes these malformations (Blaustein and Johnson, 2003), which are more common next to roads (Reeves et al., 2008) or in agrosystems (Ouellet et al., 1997; Spolyarich et al., 2011). Agrochemicals such as fungicides (Bernabo et al., 2016), pesticides (Jayawardena et al., 2010), and fertilizers (Xu and Oldham, 1997) increase limb abnormality prevalence in anurans. The aetiology of these malformations is often multiple (Meteyer et al., 2000): trematode infections (Haas et al., 2018) and predator attacks (Reeves et al., 2010) boost the effects of agrochemicals. Albeit, greater selective pressures could also drive the appearance of resistance to the environmental stressors (Miaud and Merilä, 2001), which could eventually reduce the prevalence of abnormalities in agrosystem populations.

Metamorph morphology is often related to tadpole growth history (Tejedo et al., 2000). In this work, we compare the prevalence of hindlimb abnormality in metamorphs of *Pelophylax perezi* (López Seoane, 1885) frogs resulting from tadpoles chronically exposed to ammonium contamination with unpolluted-water-reared conspecifics. Ammonium is among the most common compounds derived from agricultural fertilizers, with several negative effects on amphibian populations (Ortiz et al., 2004). We checked any possible resistance to contamination evolved in agrosystems by applying this treatment to tadpoles from natural habitats and from agrosystems. We expected higher prevalence of hindlimb abnormalities in metamorphs from the ammonium treatment. However, if agrosystem populations have evolved resistance, this effect would be greater in natural-habitat tadpoles.

Pelophylax perezi is a Ranid that occurs naturally throughout the Iberian Peninsula and southern France (Egea-Serrano, 2014), in a wide variety of habitats, but always in or not far from waterbodies, either pristine or polluted (Egea-Serrano, 2014). Indeed, it often inhabits human-altered habitats, such as urban or agricultural environments (Egea-Serrano, 2014).

Fieldwork was conducted in pristine Pinares de Cartaya *Pinus pinea* grove and surrounding agrosystems (SW Spain, 37º20' N, 7º09' W). Agrosystems are about 6 km away from pine grove, and consist of a traditional extensive vegetable crop area that has lately transitioned into intensive plantations regularly added fertilizers and at owners' discretion.

In April 2018, 10 adult males and 10 adult females were randomly caught from each habitat. Capture was manual, and males were recognized for their greyish forelimb nuptial pads and their vocal sacs in the mouth commissures (Egea-Serrano, 2014). Frogs were pooled separately according to their provenance in two adjacent outdoor semi-natural enclosures with ponds (Fig. S1 in Supplementary Material). Ponds were daily checked for the presence of egg masses, which we transferred to the laboratory within 12 hours after they had been laid.

In the laboratory, we immediately separated eggs randomly in groups of 15. Each group was placed in an aquarium (Length×Width×Height: 38×27×19 cm) with 6 L of untreated spring water. In half of the aquariums, randomly chosen for each egg mass, we added 178.87 mg of 99.7% pure NH_4Cl, so we obtained a concentration of 10 mg NH_4^+/L. In a previous study on this species, a concentration of 13.5 mg NH_4^+/L caused circa 70% mortality rate in a mid-term experiment on larvae of this species from natural habitats (Egea-Serrano et al., 2009). We chose a concentration slightly lower in order to avoid such mortality rates, while triggering sublethal effects. The other aquaria contained untreated spring water, as a control. Thus, we had 15 aquaria with eggs from frogs from each habitat and treatment, totalling 60 aquaria, in a 2 × 2 factorial experimental design.

Aquaria were kept in shelves in the laboratory until larvae finished metamorphosis. Water was completely replaced twice a week, and each time we maintained the treatment and randomly changed the position of each aquaria within the shelves. A window let natural daylight in, permitting adjustment of circadian rhythms. Because tadpole diet can affect limb abnormality rates in this species (Martínez et al., 1992), all specimens were standardly fed boiled spinach *ad libitum*. In Gosner stage 42, preceding tail resorption (Gosner, 1960), tadpoles were transferred to tilted aquaria to allow them to exit the water as metamorphosis ended.

Some metamorphs presented an abnormality in one of their hindlimbs (Fig. 1). Abnormal limbs were aberrantly inserted in the pelvis with an approximate angle of 270º with respect to the body axis (Fig. 1). Moreover, the knee-joints were unable to fold normally in resting position (Fig. 1). In all cases, only one hindlimb was abnormal in each individual affected, either the right or the left appendage. We calculated the proportion (number of abnormal metamorphs divided by number of total surviving metamorphs) of abnormal-limbed metamorphs from each aquarium.

Data met the criteria of homoscedasticity and residual normality (Quinn and Keough, 2002), so we conducted a two-way ANOVA to test the effects of habitat, treatment, and their interaction on the proportion of abnormal-limbed metamorphs, using the software Statistica 8.0.

The total numbers and proportions of abnormal-limbed metamorphs from each habitat and treatment are in Table S1 in Supplementary Material. The effects of habitat ($F_{1,\,56} = 0.026$; $P = 0.874$; Fig. 2), treatment ($F_{1,\,56}$

Fig. 1. *Pelophylax perezi* metamorph affected by the hindlimb abnormality described, with a measuring tape in cm.

= 0.007; *P* = 0.932: Fig. 2), and their interaction ($F_{1,\,56}$ = 0.914; *P* = 0.343; Fig. 2) on the proportion of abnormal-limbed metamorphs obtained in each aquarium were non-significant.

At the concentration used, chronic exposure to ammonium during the larval stage does not increase the prevalence of hindlimb abnormality in these frogs. However, a subchronic exposure to even lower concentrations of this compound reduces survivorship (Egea-Serrano et al., 2009) and affects behaviour of *P. perezi* larvae (Egea-Serrano et al., 2011). Prevalence of limb abnormality in *Bufo bufo* toad metamorphs were higher following an acute exposure to 100 mg/L ammonium nitrate during the larval stage than following a subchronic exposure to 50 and 100 mg/L ammonium nitrate (Xu and Oldham, 1997). Those results could be a consequence of greater mortality of larvae in the subchronic exposure treatment (Xu and Oldham, 1997), which could mask potential limb abnormalities if future bearers die. Chronic exposure to other pollutants, such as mercury in *Rana sphenocephala* frogs (Unrine et al., 2004), and nickel, cobalt, or cadmium chlorides in *Xenopus laevis* frogs (Plowman et al., 1994), causes malformations in metamorphs. Also, subchronic exposure to carbamate and organophosphate pesticides causes malformations in *P. perezi* (Alvarez et al., 1995).

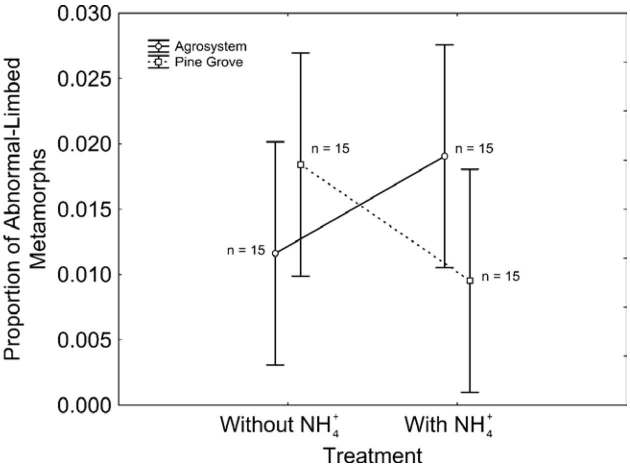

Fig. 2. Effects (mean ± 1SE) of treatment and habitat on the proportion of abnormal-limbed metamorphs obtained from each aquarium, calculated as the number of abnormal metamorphs divided by the total number of surviving metamorphs. Sample sizes indicated represent the number of aquaria in each treatment.

Juvenile *P. perezi* from agrosystems are smaller, and show increased limb fluctuant asymmetry, than conspecifics from natural habitats (Burghelea et al., 2013). However, we detected no effect of habitat on hindlimb abnormality prevalence on either treatment. Aligned with our results, prevalence of limb abnormality was not greater in *Rana temporaria* frogs from agrosystems than from natural habitats (Piha et al., 2006). Nevertheless, these findings contrast with others that detected increased prevalence of limb abnormality close to agrosystems in several anurans (Kiesecker, 2002; Guerra and Aráoz, 2016). Our results do not support the hypothesis of resistance in agrosystem frogs. We obtained an overall prevalence of hindlimb abnormality notably below the 5% detected in other wild amphibian populations (Ouellet, 2000; Mester et al., 2015). Low prevalence in both habitats could be a consequence of the capability of this species to thrive in polluted waters (Egea-Serrano et al., 2008).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < http://www.unipv.it/webshi/appendix > Manuscript number 10016.

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Confirming Lessona's brown frogs distribution sketch: *Rana temporaria* is present on Turin Hills (Piedmont, NW Italy)

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Abstract. The presence of *Rana temporaria* on Turin Hills (Piedmont NW Italy) has been confirmed through morphological and molecular analyses. Breeding individuals of this species were found at two sites and assessed by either morpho-chromatic and genetics. This new finding represents an interesting confirmation of ancient record reported in 1877 by the renowned naturalist Michele Lessona, and highlights that the species is likely quite cryptical and secretive and has a distribution wider than formerly presumed.

Keywords. Common frog, *Rana temporaria*, new findings, Turin Hills, NW Italy.

The common frog *Rana temporaria* shows a conspicuous wide geographical and elevational distribution, being present in most of Europe and in the northern and central regions of western Asia, from northern Spain, France, United Kingdom and the Scandinavian Peninsula and Russia (Urals and adjacent western Siberia) to northern Kazakhstan (Lanza, 1983; Kuzmin, 1999; Lanza et al., 2009; Sillero et al., 2014), from sea level to around 2846 m a.s.l. (Vences et al., 2003, 2013; Maurino and Doglio, 2010; Tiberti and von Hardenberg, 2012; Di Nicola et al., 2019). In southern Europe this species is usually associated to montane habitats, being absent from southern and central Iberia, most of southern Italy and Caucasus, and shows a patchy distribution in the Balkans and in the Mediterranean islands. In Italy it is found on the Alpine and northern Apennine reliefs, with a fragmented and irregular distribution in Arezzo, Florence and Forlí-Cesena provinces, and a relict population on the Monti della Laga (Rieti Province, NE Latium) (Capula and Bagnoli, 1983; Razzetti et al., 2007).

In Piedmont (NW Italy) *R. temporaria* is quite common on the Alps, on northern Apennines and on southern hilly reliefs, known as "Langhe", with scattered, findings at low altitudes (Andreone et al, 1988; Andreone and Sindaco, 1989, 1999). In a pioneer contribution on anuran distribution, the renowned naturalist, writer, lecturer, minister and Darwin translator Michele Lessona published a colour map (realised by his son-in-law L. Camerano) with the inferred distribution of *R. temporaria* in Piedmont and Aosta Valley known at that time. In this map the species was also reported on the hill system bordering the town of Turin, known as "Collina di Torino" (Lessona, 1877). No precise localities or toponyms were provided together with this map, although Lessona provided considerations on abundances, human uses and life history traits on this species in Piedmont. Although Lessona was Turin Zoological Museum's director (Camerano,

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Fig. 1. *Rana temporaria*, male from Turin Hills, Piedmont (NW Italy) (A: dorsolateral view; B: ventral view) (photographs by F. Andreone).

1894), no preserved specimen of this species from Turin Hills is currently present in the historical herpetological collection of Turin University (now hosted by the Museo Regionale di Scienze Naturali: Gavetti and Andreone, 1993), and we did not find any record of *R. temporaria* from Turin Hills on the historical catalogues or quoted by previous authors, such as Camerano (1884) and Tortonese (1953). Until now *R. temporaria* was not reported for Turin Hills, neither during the realization of the herpetological distribution atlas of Piedmont and Aosta Valley (Andreone and Sindaco, 1999), nor in the Italian atlas (Sindaco et al., 2006), and this record was considered anecdotal. This assumption was contradicted by recent observations, reported in this paper. During a survey carried out on the 8th March 2017 to confirm the presence of the Alpine newt (*Ichthyosaura alpestris apuana*) on Turin Hills (Marino, 2018), one of us (DM) reported a single brown frog at Valsalice (altitude: 315 m a.s.l.; coordinates: 45°03’04.5”N, 7°42’46.6”E). This individual corresponded in morphology and colouration to a typical *R. temporaria*. This record was promptly reported on iNaturalist, and further confirmed by the herpetological online community. This individual (a male) was quite large (around 60 mm snout-vent length), had a relatively short snout and hindlimbs, that, adpressed along body barely reached the eye (Fig. 1). The colouration of this individual was brownish, with sparse dark spots on the back, intense purple shading under the throat and missed the yellowish belly and groin shadings typical of the agile frog *R. dalmatina*¸ the other brown frog confirmed in his area. A few days later another female and some males were found around an artificial pond, where typical eggs-clumps were also found. On the 29th March this species was also found at at Reaglie (altitude: 380 m a.s.l.; coordinates: 45°02’44.9”N, 7°44’55.1”E), about 10 Km away from Valsalice (Fig. 2).

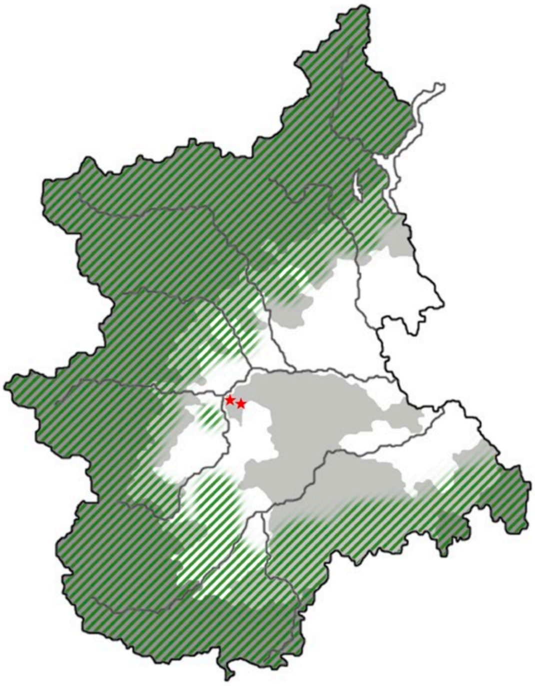

Fig. 2. The distribution of *Rana temporaria* in Piedmont and Aosta Valley (NW Italy) based upon current data. Dark and light gray show Alps, Prealps and inner hills. The transverse (green) bars represent the known distribution. The (red) stars mark the confirmed sites recently found on the hill system near Turin.

To further confirm the species identity we analysed the tissue samples of two adults, one tadpole and one egg. We also took the tissue samples of two adults *R. dalmatina* of Reaglie for comparison. Total genomic DNA was extracted from these samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt extraction protocol (Bruford et al., 1992). We sequenced a fragment of ca. 550 bp of the 3’ terminus of the mitochondrial rrnL gene. Polymerase chain reactions (PCR) were performed in a final volume of 25 µl using 0.75 µl each of 10 pmol primer, 0.4 µl of total dNTP 10 mM (Promega), 0.1 µl of 5 U/ml GoTaq (Promega), 5 µl 5X Green GoTaq Reaction Buffer (Promega) and 4 µl of MgCl2 25mM (Promega). For primers and cycling protocols, see Crottini et al. (2011). Successfully amplified PCR products were treated to inactivate remaining primers and dNTPs. Purified PCR templates were sequenced using dyelabelled dideoxy terminator cycle sequencing on an ABI3730xl at Macrogen Inc. Sequences were checked by eye, edited (when necessary), aligned using the BioEdit sequence alignment editor (version 7.0.5.3; Hall 1999) and compared to the GenBank dataset. All newly determined sequences have been deposited in GenBank (MT459788-MT459793).

All these observations support the presence of *R. temporaria* on the hill system around Turin and confirm old Lessona's ancient maps, providing a significant novelty in terms of species distribution in Piedmont. It remains somehow surprising that no records of *R. temporaria* were reported since Lessona's contribution (including the lack of museum specimens and photographs): we believe this absence might be due to the species overall similarity with the agile frog *R. dalmatina*, and to a deficit of field research during suitable periods and in suitable sites. In fact, *R. temporaria* apparently prefers stream systems (in particular in late winter), which were not so frequently surveyed on the Turin Hills.

Finally, we cannot exclude that in the 19th Century the species was more widespred and common than today, and since then this species has reduced its distribution, as it is already known for *Pelobates fuscus* and *Zootoca carniolica,* two species which survived with patchy populations at a few sites along the Po River (Andreone and Sindaco, 1999). The presence of *R. temporaria* in the hill system of Turin may represent a vestige of an ancient and more widespread distribution range for the species, as already supposed for the Alpine newt (Andreone and Sindaco, 1987; Marino 2018). Further research efforts should be devoted to better characterize the distribution of this species around the hill system of the city of Turin, and between the city and "Langhe", to valorise all remnant populations of this species.

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Phylogenetic relationships of the Italian populations of Horseshoe Whip Snake *Hemorrhois hippocrepis* (Serpentes, Colubridae)

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Abstract. *Hemorrhois hippocrepis* is a colubrid snake with a West Mediterranean distribution. It is widespread in the Iberian Peninsula and Northwest Africa. The only Italian populations are found on the islands of Sardinia and Pantelleria. The phylogenetic relationships of these insular populations have been analysed for the first time on the basis of the mitochondrial DNA cytochrome *b* gene. The sequences were compared with those available from the geographic range of this species. The analyses showed that the Italian samples are part of a lineage that groups Tunisian and East Algerian samples, with which they share the same haplotype. These results strongly support the hypothesis of a recent origin of the Italian populations of *Hemorrhois hippocrepis*, probably determined by human-mediated dispersal from North Africa.

Keywords. Horseshoe Whip Snake, phylogeny, colubridae, cytochrome *b*.

The Horseshoe Whip Snake, *Hemorrhois hippocrepis* Linnaeus, 1758, is a medium to large size colubrid with a Western Mediterranean distribution. It is widespread across the eastern, central and southern Iberian Peninsula, and in the northern mesic parts of Morocco, Algeria, and Tunisia. In Italy, its presence is limited to the islands of Sardinia and Pantelleria (Sindaco et al., 2013).

The first reports of *H. hippocrepis* in Sardinia are found in Gené (1834, 1839) and refer to the southwestern part of the island. Later, Mertens and Wermuth (1960) considered the species extinct, but subsequently, Bruno and Hotz (1976) rediscovered it in a territory between Cagliari and Oristano, and its presence there was also confirmed by Puddu et al. (1988) and Bruno and Maugeri, (1990). *Hemorrhois hippocrepis* in the San Pietro satellite islet was reported by Stefani (1971) and Poggesi et al. (1995) and is still mentioned in recent references (Corti et al., 2006; Di Nicola and Mezzadri, 2018), although its presence is doubtful given the absence of corroborating observations (Corti et al., 2000). More recently, *H. hippocrepis* appears to be present with certainty only a few localities of the Cagliari Province (Corti et al., 2000; Zuffi, 2006; Di Nicola and Mezzadri, 2018; Di Nicola et al., 2019).

The Horseshoe Whip Snake has been known to inhabit the island of Pantelleria (Sicily) since the late

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1800s (Doderlein, 1881; Camerano, 1891; Minà Palumbo, 1893) and it is widespread throughout the island (Cattaneo, 1985; authors' unpublished data).

The population of Pantelleria has been attributed on a morphological basis to the endemic subspecies *H. h. nigrescens* (Cattaneo, 1985), due to the melanotic color morph of adults, the large dimensions of both hatchlings and adults, and to certain distinctive pholidotic features. Later, Corti et al. (2000), in comparing various populations of *H. hippocrepis*, found no substantial morphological differences between the populations of Pantelleria, Sardinia and Tunisia. According to some authors, the Italian populations of Horseshoe Whip Snake may have been introduced by man in historical times (Bruno and Hotz, 1976; Zuffi, 2006; Cattaneo, 2015). Therefore, the ssp. *nigrescens* is currently considered doubtful, and *H. hippocrepis* is widely reported as a monotypic species (Venchi and Sindaco, 2006; Luiselli et al., 2011; Di Nicola, 2019; Di Nicola et al., 2019), as indicated by the genetic studies of Carranza et al. (2006), which however did not include samples from Italian populations.

In this work, we have analyzed for the first time the Italian populations of *H. hippocrepis* from a genetic point of view, with the aim of clarifying their phylogenetic relationships and testing if their origin can be related to human mediated dispersal as we expect.

In order to infer the phylogenetic relationships between the Italian samples and those of the other populations, we analysed the mitochondrial DNA cytochrome *b* (cyt *b*) sequence, a fragment often used for snakes in this type of analysis (Carranza et al., 2004, 2006; Faraone et al., 2020). Tissue samples ranging from 2 to 20 mg were collected from six specimens originating from Pantelleria and one from Sardinia, and stored in ethanol until processing. All snakes were found as roadkills between 2013 and 2019. DNA was extracted as described in Tagliavia et al. (2016). 3 ml of crude lysate were used as template for PCR amplification. Reactions were carried out in 30 ml in presence of 200 mM dNTPs and 0.15 mM primers CB1F and CB2R (respectively: 5'-CCATCCAACATCTCAGCATGATGAAA-3' and 5'-CCCTCAGAATGATATTTGTCCTCA-3') (Korcher et al., 1989). The PCR conditions were 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and by 5 min of final extension at 72 °C. PCR products were analysed by electrophoresis onto 1% agarose gel containing 0.5 µg/mlethidium bromide and sequenced with primer CB1F (BMR Genomics).

Nucleotide sequences, each of about 320 nucleotides, were analysed and manually proofread with the software Chromas v. 2.6.6 (Technelysium Pty. Ltd. 1998, Queensland, Australia). Coding gene fragments of cyt *b* were translated into amino acids to assess the lack of stop codons. Cyt *b* sequences of *H. hippocrepis* were searched and downloaded from GenBank (Nagy et al., 2004; Carranza et al., 2006; Beddek et al., 2018) from the native geographic range of the species (Figs 1 and 2). Additionally, cyt *b* sequences of *Lytorhynchus diadema*, *Hierophis viridiflavus*, *Hemorrhois algirus*, *Coronella girondica*, *Psammophis shokari*, *Rhagerhis moilensis* and *Malpolon monspessulanus* were downloaded and used as outgroup. Nucleotide sequences were aligned using ClustalW with default parameters.

Phylogenetic analysis was performed with the Maximum Likelihood (ML) method and the Akaike Information Criterion, using "Smart Model Selection" (SMS) (Lefort et al., 2017), implemented in PhyML v. 3 (Guindon et al., 2010). Support for nodes was estimated using bootstrapping (Felsenstein, 1985) with 1000 replicates. The most appropriate evolutionary model was the GTR+G+I model (-Log likelihood value -1397.47), with a 0.30 estimate of invariable sites and a 0.819 discrete approximation of the gamma distribution.

Including the outgroups, 67 sequences of 294bp total length were analysed. All the Italian samples showed the same haplotype (GenBank accession numbers MT498647-MT498653), which is also shared with all the Tunisian and the easternmost Algerian samples.

The results obtained confirm the same overall *H. hippocrepis* phylogenetic structure detected by previous studies on mitochondrial sequences (Carranza et al., 2006; Beddek et al., 2018), i.e., the existence of a western clade (subclade A) that includes the Iberian Peninsula, Morocco, western Algeria and non-native populations of the Balearic islands (see Silva-Rocha et al., 2015), and an eastern clade (subclade B) that includes eastern Algeria and Tunisia. The western clade has greater haplotype diversity while the eastern clade is characterized by a single haplotype, shared by all the Italian samples. The phylogenetic structure obtained also reflects the morphological variation within this species. In fact, the eastern populations (Sardinia, Pantelleria and Tunisia) differ from the western ones (Spain, Portugal and Morocco) in that they have a greater number of ventral scales (Cattaneo, 1985; Corti et al., 2000).

The sharing of the same cyt *b* haplotype by the Tunisian and Italian populations of *H. hippocrepis* strongly supports a recent origin of the latter. This was probably determined by human-mediated fauna translocation, as previously speculated (Bruno and Hotz, 1976; Zuffi, 2006; Cattaneo, 2015) and suggested by the biogeographical and historical features of the two islands. Although Sardinian fossil ophidiofauna is well represented and several findings of 'colubrinae' are known, there are no

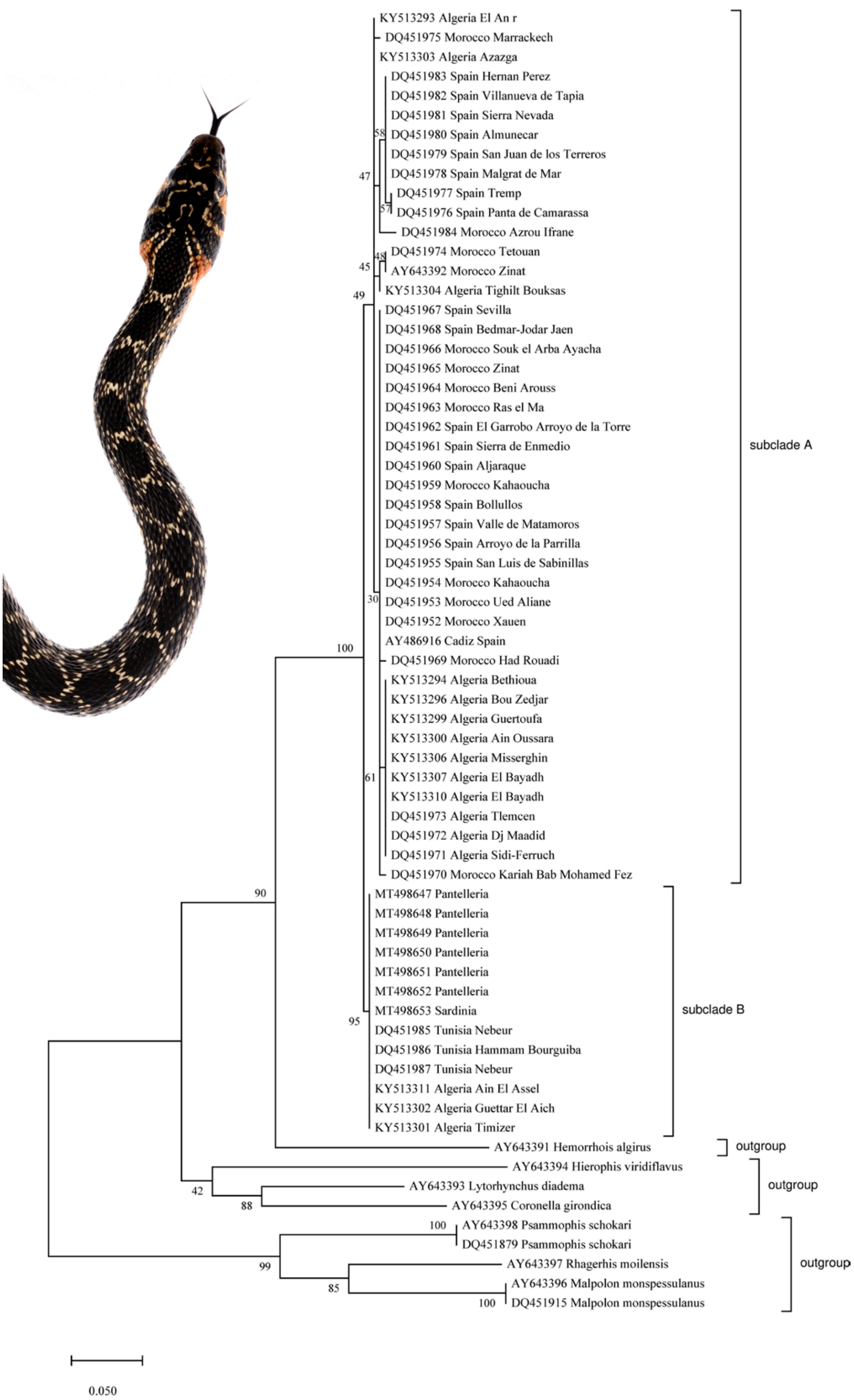

Fig. 1. Maximum Likelihood (ML) tree of *Hemorrhois hippocrepis* inferred from the mitochondrial cytochrome *b* gene. The numbers at nodes are ML bootstrap values. Except for the samples from Pantelleria and Sardinia, the remaining cyt *b* GenBank accession numbers are from Nagy et al. (2004), Carranza et al. (2004, 2006) and Beddek et al. (2018).

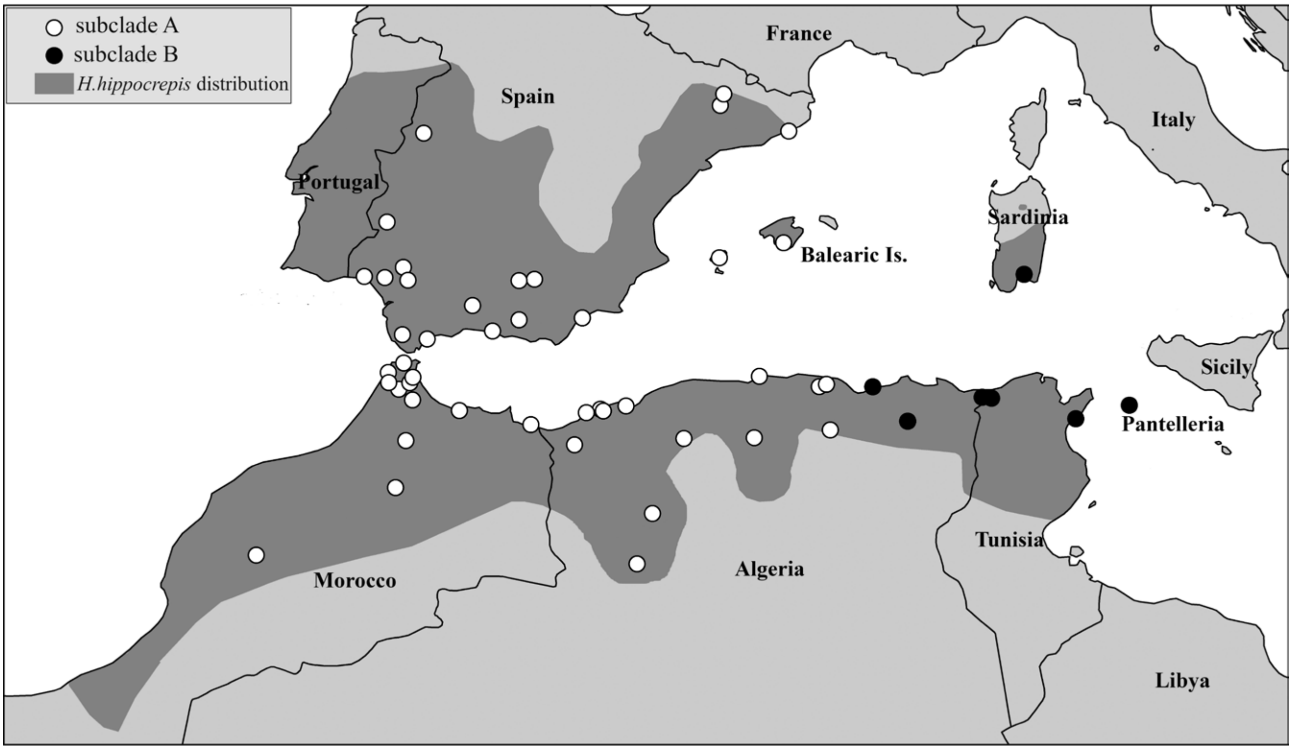

Fig. 2. Geographic range of *Hemorrhois hippocrepis* and distribution of all the samples included in the analysis, distinguished on the basis of the western and eastern lineages, respectively white and black dots.

remains clearly attributable to *H. hippocrepis* (Kotsakis, 1980; Abbazzi et al., 2004; Georgalis et al., 2019). While the only remains of terrestrial vertebrates found in Pantelleria, belong to undetermined birds and Artiodactyla mammals dating back to the Bronze Age and linked to human farming and hunting activities (Wilkens, 1987). Furthermore, *H. hippocrepis* is not listed in the overall fossil register of the Italian reptiles (Delfino, 2006, 2011). Several current reptile species found in Sardinia share close genetic affinities with Tunisian populations, and this suggests they are of recent origin. The origin of the Sardinian populations of *Natrix maura* are not completely clear, due to its slight variation compared to the Tunisian one (Guicking et al., 2008). For other species like *Testudo graeca*, *Chalcides ocellatus,* and *Chalcides chalcides*, human-mediated dispersal appears to be the most probable process involved (Carranza et al., 2008; Fritz et al., 2009; Kornilios et al., 2010). As for Pantelleria, although some studies support ancient Pleistocene contacts with the Tunisian mainland (Pasa, 1953; Bordoni, 1973), and an active colonization of some species has been hypothesized (Lanza, 1973; Stöck et al., 2016), the onset of catastrophic eruptive events dating back to about 45,000 years ago probably caused the extinction of most of the native fauna (Agnesi and Federico, 1995; Massa, 1995; Muscarella and Baragona, 2017). This suggests a subsequent origin of the reptile community through recent passive dispersion mechanisms (Cattaneo, 2015).

Wide cultural and commercial connections between Tunisia, Pantelleria and Sardinia throughout history, especially around the 6th-3rd century B.C.E. under the influence of the Carthaginians (Bechtold, 2013), may have played a role in the human-mediated exchanges of reptile fauna across the Mediterranean basin (Bruno, 1985; Masseti and Zuffi, 2011). It may be in this context that the Italian populations of *H. hippocrepis* originated.

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First karyological analysis of the endemic Malagasy phantom gecko *Matoatoa brevipes* (Squamata: Gekkonidae)

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Abstract. The genus *Matoatoa* includes two Malagasy endemic species, *M. brevipes* and *M. spannringi*. Due to their cryptic behaviour, the two species are known only from a handful of specimens and have been included in few molecular studies. Here we carried out a molecular barcoding analysis using a fragment of the mitochondrial NADH dehydrogenase subunit 2 (ND2) and the first chromosomal analysis of *M. brevipes*. The molecular analysis confirmed the identity of the studied samples as *M. brevipes*. However, the level of genetic divergence (4% uncorrected *p*-distance) between our samples and other sequences of *M. brevipes*, suggests previously unrecognised diversity within the species. The karyotype of *M. brevipes* is composed of 2n = 34 chromosomes: the first pair is metacentric, while all the other pairs are telocentric and gradually decreasing in length (Arm Number, AN = 36). C-banding revealed little evidence of centromeric heterochromatin, while NOR-associated heterochromatin was found on the telomeres of a medium sized telocentric pair. No heteromorphic chromosome pairs were found in the karyotype of the species, suggesting that putative sex chromosomes are at an early stage of differentiation. Karyological comparisons with closely related species were performed with *Christinus marmoratus*, and representatives of the genera *Phelsuma*, *Ebenavia*, *Paroedura* and *Uroplatus*. Comparisons across genera suggest that chromosome diversification in this group of geckos probably occurred by means of chromosome fusions and inversions, leading to a reduction of the chromosome number and the formation of biarmed elements in different species.

Keywords. Chromosomes, leaf-toed geckos, *Matoatoa*, Madagascar, evolution**,** reptiles.

Madagascar is one of the hottest reptilian biodiversity hotspots, including more than 430 reptile species (Glaw and Vences, 2006; Uetz et al., 2019). The mostly endemic reptile fauna of Madagascar is still relatively poorly understood, despite significant progress in the last few decades (Glaw and Vences, 2006), with several new species described every year (Uetz et al., 2019). A significant number of these new reptile species are known only from a few specimens, and despite researchers applying molecular barcoding techniques (Nagy et al., 2012) with large numbers of comparative molecular sequences, the reptile diversity remains significantly underestimated.

Compared to other groups, only a small fraction of reptile species have been studied with cytogenetic methods, despite increasing evidence that species-level diversity is reflected at the karyotype level (e.g. Mezzasalma et al., 2016, 2018; Rovatsos et al., 2017). Malagasy vertebrates display dynamic patterns of chromosome evolu-

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tion including augmentation and reduction of the chromosome number and the independent diversification of sex chromosome systems (e.g. Mezzasalma et al., 2017a; Rovatsos et al., 2017), making the Malagasy reptile fauna an exciting study system for evolutionary cytogenetics.

The genus *Matoatoa* includes two endemic Malagasy gecko species, *M. brevipes* (Mocquard, 1900) and *M. spannringi* Nussbaum, Raxworthy and Pronk 1998. Species of *Matoatoa* are part of a clade including the southern African genera *Afrogecko*, *Cryptactites*, *Kolekanos* and *Ramigekko*, the Australian genus *Christinus* and the more distantly related genera *Afroedura*, *Goggia*, *Phelsuma*, *Paroedura*, *Ebenavia* and *Uroplatus* (Heinicke et al., 2014). Karyological data are currently available for *Christinus marmoratus* and different species of *Phelsuma*, *Paroedura*, *Ebenavia* and *Uroplatus* (King and Rofe, 1976; King and King, 1977; Aprea et al., 1996, 2013). However, there are no available chromosome data from the genus *Matoatoa*. Here we present the results of the first karyological study on the endemic Malagasy phantom gecko *M. brevipes* and a preliminary mitochondrial analysis to provide a taxonomic identification of the sample studied. Finally, we compared our newly generated karyotype data to those from closely related gecko genera and hypothesise how karyotype evolution occurred in the group.

We used a sample from a female specimen of *M. brevipes* collected 20 km south of Tulear (the cell suspension and tissue sample are deposited in the Natural History Museum, London, UK, and in the Department of Biology, University of Naples Federico II, with voucher GA436). The sample was preliminary treated with colchicine (1mg/ml; 0.1 ml/10 gr body weight) and, after two hours, tissue samples with high mitotic indices (intestine and gonads) were placed for 30 min in hypotonic solution (sodium citrate 0.50% + KCl 0.56%) and then transferred in Carnoy's fixative (Methanol + glacial acetic acid, 3:1).

We performed a phylogenetic analysis using a fragment of 561 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2). Genomic DNA was extracted using the standard phenol-chloroform method (Sambrook et al., 1989). For the ND2 analysis we used the primers and PCR parameters reported in Heinicke et al. (2014). After sequencing, the obtained chromatogram (accession number MT579309) was manually corrected and aligned with homologous ND2 segments of *M. brevipes* deposited in GenBank using Chromas Lite 2.2.1 and BioEdit 7.2.6.1 (Hall, 1999). Phylogenetic analysis was performed with Bayesian inference (BI) using MrBayes 3.2.5 (Ronquist et al., 2012), and Maximum likelihood (ML) using MEGA 10.1.7 (Tamura et al., 2013), with 2,000,000 generations and 1000 bootstrap replicates, respectively. In both analyses we included homologous ND2 sequences of representatives of phylogenetically closely related gecko genera: *Christinus alexanderi* (KF666813), *Christinus guentheri* (KF666801), *Cryptactites peryngueyi* (KF666814), *Ramigekko swartgergensis* (KF666793) and *Afrogecko porphyreus* (KF666772). We used as outgroup two homologous sequences of the phylogenetically closely related *Kolekanos plumicaudus* (KF666791, JX041304).

In the cytogenetic analysis, chromosomes were prepared by the scraping + air drying method (see Mezzasalma et al., 2016), and a combination of traditional staining (Giemsa staining at pH7) and banding techniques (sequential C-banding + Giemsa + CMA_3 + DAPI; see Mezzasalma et al., 2013, 2015, 2017b).

In our phylogenetic analysis, the studied female (GA436) is the sister taxon of the other three available ND2 sequences of *M. brevipes* (KF666815, KF666816, and EF490777) (Fig. 1). The uncorrected *p*-distance of ~4%, between our newly generated ND2 sequence and those available from GenBank, highlights the occurrence of intraspecific diversity. In turn, the other three available ND2 sequences of the species do not show any appreciable nucleotide variability (Fig. 1). Potential explanations for the intraspecific divergence in *M. brevipes* may be related to geographical patterns of genetic diversity, however, all three ND2 sequences of the species available from GenBank (KF666815, KF666816, and EF490777) are not associated with specific sampling localities (see Nussbaum et al., 1998; Heinike et al., 2014) and more

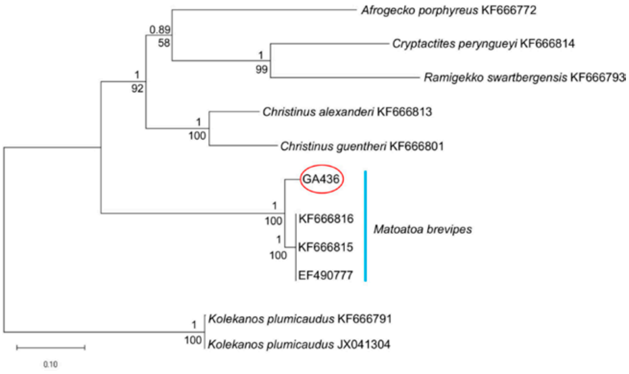

Fig. 1. Phylogenetic tree with ML (1000 bootstrap replicates) and BI (2,000,000 generations), with our newly generated ND2 sequence (GA436) and available homologous sequences of *Matoatoa brevipes* (KF666815, KF666816, EF490777). We included homologous sequences of *Christinus alexanderi* (KF666813), *Christinus guentheri* (KF666801), *Cryptactites peryngueyi* (KF666814), *Ramigekko swartgergensis* (KF666793), *Afrogecko porphyreus* (KF666772) and *Kolekanos plumicaudus* (KF666791, JX041304). Bootstrap and Bayesian posterior values are reported below and above tree branches, respectively.

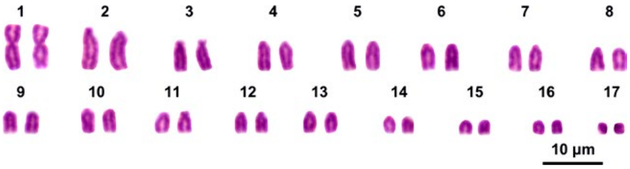

Fig. 2. Giemsa stained karyotype of *Matoatoa brevipes* with 2n = 34 chromosomes.

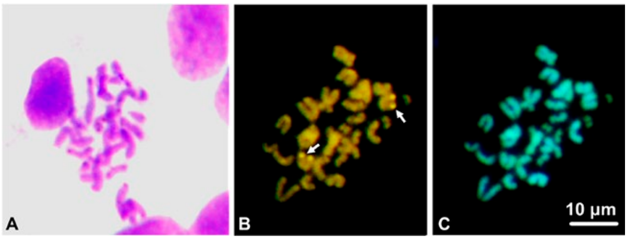

Fig. 3. C-banding + Giemsa (A), CMA_3 (B) and DAPI (C) on metaphase plates of the female specimen of *M. brevipes* GA436, showing low heterochromatin content and absence of completely or largely heterochromatic chromosomes. White arrows in (B) point at NOR-associated heterochromatic blocks. Scale bar = 10 µm.

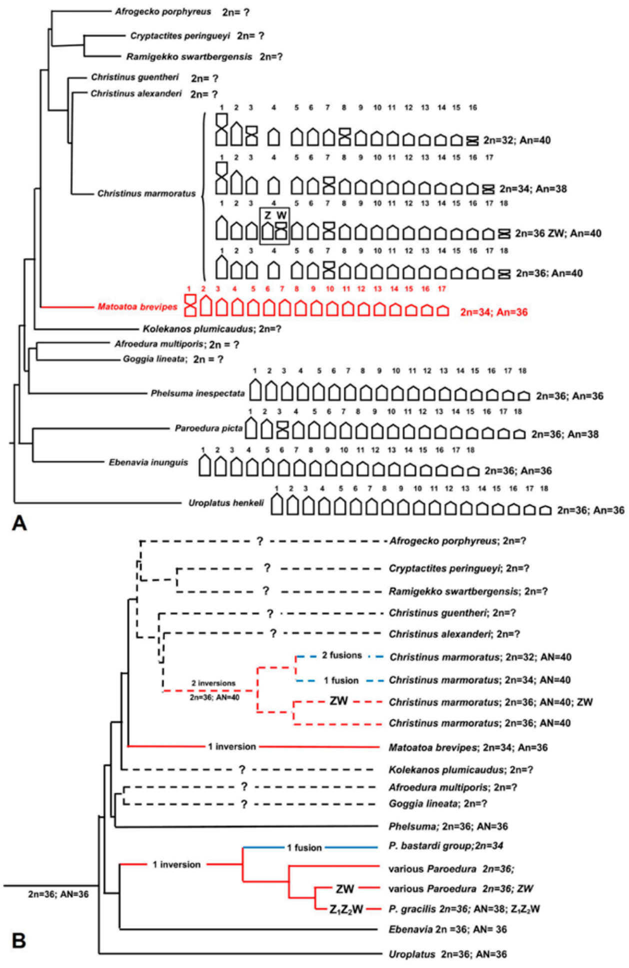

Fig. 4. (**A**) Phylogenetic relationships of circum-Indian Ocean leaf toed geckos (redrawn from Heinicke et al., 2014) superimposed with available haploid karyograms (King and Rofe, 1976; Aprea et al., 1996, 2013; this study). (**B**) Karyotype evolutionary hypothesis and relative chromosome rearrangements.

data are needed to advance hypotheses on the intraspecific molecular diversity of *M. brevipes*.

We found that *M. brevipes* has a karyotype composed of 2n = 34 chromosomes, with one metacentric pair (1^{st}) and 16 telocentric pairs, gradually decreasing in length and without any clear distinction between macro- and micro-chromosomes (Fig. 2). Sequential C-banding revealed a scarce heterochromatin content in the genome of *M. brevipes*, without any completely or largely heterochromatic chromosome (Fig. 3). Centromeric and telomeric heterochromatin was more easily viewed with C-banding + CMA_3. Furthermore, the telomeric heterochromatin of two medium sized telocentric chromosomes was relatively brighter with CMA_3, suggesting the co-localization of the NOR associated heterochromatin (Fig. 3). In fact, C-banding + CMA3 can be a useful technique to localize NORs. Unambiguous co-localization of CMA_3 signals after C-banding and Ag-NOR staining and NOR-FISH signals have been consistently documented in different papers and taxa (see e.g. Pardo et al., 2001; Suman and Kaur, 2013). This is especially true for karyotypes showing low heterochromatin content like squamates and lacking other paired CMA_3+ heterochromatic blocks. Karyological comparisons with closely related genera were possible with *Christinus marmoratus* and different species of *Phelsuma*, *Paroedura*, *Ebenavia* and *Uroplatus*. *Christinus marmoratus* has accentuated chromosome polymorphism, with different populations possessing different chromosome number (2n = 32, 34 and 36) and morphology, with or without differentiated ZW sex chromosomes, and an invariable Arm Number (AN) of 40 (King and Rofe, 1976; King and King, 1977). *Christinus marmoratus* probably includes cryptic species (Kay, 2008), and the variable karyotypes found in different clades are similar to those found in other geckos of the same group in chromosome number (2n = 32-36) and the prevalence of telocentric elements, with one (as in *M. brevipes* and *P. picta*) or more bi-armed chromosomes occurring on different chromosome pairs.

We superimposed the updated chromosomal data on the phylogeny of Heinicke et al. (2014) to explore possible scenarios of chromosome diversification in the group (Fig. 4). Based on the distribution of karyotypes, we hypothesise a putative ancestral karyotype with 2n =

36 and all telocentric elements (AN = 36). In fact, higher numbers of chromosomes and telocentric elements are considered primitive features of lizard karyotypes (see Deakin and Ezaz, 2019). Furthermore, the hypothesised ancestral karyotype of 2n = 36 is the most common karyotype in the focal gecko species and is conserved in all *Uroplatus*, *Ebenavia*, and *Phelsuma* species studied so far (Aprea et al., 1996, 2013 and our unpublished data). Starting from an ancestral 2n = 36 karyotype, multiple fusion and inversion events likely led to the formation of biarmed elements and the reduction of the chromosome number in different clades (Fig. 4). In particular, one fusion between two medium sized telocentric pairs probably originated the karyotype of *M. brevipes* (with 2n = 34 and one metacentric pair). One inversion involving two primitive telocentric pairs may explain the karyotypic variation across species of *Paroedura* (Aprea et al., 2013; Koubová et al., 2014) and a subsequent centric fusion probably shaped the karyotype of *P. bastardi*, reducing the chromosome number to 2n = 34 (see also Aprea et al., 2013; Koubová et al., 2014). In *Christinus marmoratus*, two inversions involving pairs 7 and 18, likely formed the karyotype of 2n = 36 populations, and one and two centric fusions of telocentric elements formed the karyotype of 2n = 34 and 2n = 32 populations, respectively. Similar chromosome rearrangements have been hypothesized in different phylogenetic lineages within the family Gekkonidae, with distinct karyotype formulas progressively diverging from the hypothesized ancestral condition by means of independent chromosome fusions, fissions and inversions (see also Trifonov et al., 2011; Srikulnath et al., 2014).

Concerning sex determination systems, differentiated ZW chromosomes are common in squamates, and have been observed in various *Paroedura* species (Koubová et al., 2014), including a complex $Z_1Z_1Z_2Z_2/Z_1Z_2W$ sex chromosome system in *P. gracilis* (Aprea et al., 2013). However, the female specimen of *M. brevipes* studied here did not possess heteromorphic or largely heterochromatic chromosomes. This suggests that the ZW sex chromosomes are at an early differentiation stage, as has been observed in a 2n = 36 population of *C. marmoratus* (King and King, 1977). Variability in sex chromosome morphology is not unexpected as squamate reptiles can have either XY or ZW sex chromosomes and these conditions can evolve and differentiate rapidly, sometimes occurring within the same taxon (e.g. Gamble, 2010; Koubová et al., 2014; Mezzasalma et al., 2019).

Our results indicate that the NOR loci of *M. brevipes* are probably located on the telomeres of a medium sized telocentric chromosome pair. This contrasts with NOR location in related taxa indicating a remarkable variability in their chromosomal localization in geckos. For example, NORs are on the first chromosome pair in *E. inunguis*, on the last pair in *Paroedura* and on different chromosome pairs in *Phelsuma* and *Uroplatus* (Aprea et al., 1996, 2013 and our unpublished data), highlighting a high genomic mobility of these elements.

In conclusion, the karyotype of *M. brevipes* is composed of 2n = 34 chromosomes with one metacentric and 16 telocentric pairs. Comparison with the available karyotypes of closely related gecko genera allowed us to hypothesise an evolutionary scenario where a karyotype of 2n = 36 with all telocentric chromosomes was the ancestral state of the group. This ancestral state would necessitate chromosome diversification occurring by means of fusions and inversions, leading to a reduction of the chromosome number and the formation of biarmed elements.

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Notes on sexual dimorphism, diet and reproduction of the false coral snake *Oxyrhopus rhombifer* Duméril, Bibron & Duméril, 1854 (Dipsadidae: Pseudoboini) from coastal plains of Subtropical Brazil

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Abstract. Herein, we provide information on diet, sexual dimorphism and reproductive biology of the false coral snake *Oxyrhopus rhombifer* from the southernmost Brazilian coast, Pampa Biome, a region under influence of subtropical climatic domains. The analysis of 142 specimens revealed a marked sexual dimorphism, with significant differences for all the characters analyzed. The diet of the species is composed by small rodents and squamate reptiles, the latter consisting mainly of lizard tails, probably autotomized. Males reached sexual maturity at smaller sizes (SVL) than females (354 mm vs 451 mm, respectively). Secondary follicles were found mainly in spring months and one single female presented 3 oviductal eggs in January (summer). Our results add to the knowledge on biology of snakes in the Pampa morphoclimatic domain.

Keywords. Feeding habits, reproductive biology, Pampa Biome, Squamata, Xenodontinae.

Investigating the natural history of species is essential for understanding ecological processes at different levels and provides crucial information for species conservation (Greene, 1993; Gaiarsa et al., 2013). Regarding Neotropical snakes, the last decades have experienced an increment in the number of studies concerning diverse traits of natural history and sexual dimorphism, but these are still fragmented and incipient, considering the group's high diversity (Barbo et al., 2011; Gaiarsa et al., 2013).

The *Oxyrhopus* genus comprises 14 species of medium-sized pseudoboines distributed from southeastern Mexico to central Argentina (Lynch, 2009; Uetz et al., 2017). Most *Oxyrhopus* species exhibit a coral snake mimetic pattern (Savage and Slowinski, 1992), responsible for the popular name 'false coral snakes'. *Oxyrhopus rhombifer* (Fig. 1) is the southernmost distributed *Oxyrhopus*, ranging from southeastern Brazil to central Argentina (province of Buenos Aires) (Giraudo, 2001). There are few scattered data available *O. rhombifer* diet and reproductive biology (Vidal, 2002; Maschio et al., 2003, 2004; Sawaya et al., 2003, 2008; Gaiarsa et al., 2013) and no data on its sexual dimorphism. Herein, we examined size at sexual maturity, female reproductive cycle, feeding habits and sexual dimorphism of *O. rhombifer* from southernmost Brazilian coastal environments.

Specimens were collected along a stretch of ca. 470 km of the coastal plain of the Rio Grande do Sul state (30°15'44"S, 50°28'03"W; 33°40'27"S, 53°30'52"W), southern Brazil, between August 2008 and October 2017. Sampling method consisted in active search performed by two researchers in 77 field trips with an average duration from two to 11 hours (average of four hours).

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Fig. 1. *Oxyrhopus rhombifer* (unvouchered) from Rio Grande, Rio Grande do Sul state, southern Brazil.

Searches were conducted on foot and by car at low speed (20-40 km/h) during day and night periods. This region is inserted in the Pampa biome, under "subtemperate humid" and "temperate humid" climatic domains according to the regional classification by Maluf (2000). Seasons are well-defined, with monthly average air temperature ranging from 13 °C in the winter (July) to 22 °C in the summer (January). Average rainfall is 1,271 mm and rain is distributed mainly from June to October (Climate-Data, 2019). Predominant vegetation types are shrub grassland, and other phytophysiognomies with less coverage include the coastal peat and sandy forests (*restinga* forests) and psamophyte formations of coastal dunes.

Specimens were euthanized through intraperitoneal injection of pentobarbital sodium 50 mg/ml, in accordance with guidelines from international protocols. This procedure had been authorized in our laboratory since 2006 and it is in accordance with the institutional committee for the use of animals in research (CEUA-FURG). Collection was permitted by the Brazilian environmental agency (ICMBio process nº 43658-1). The snakes had to be euthanized for the analyses of the reproductive condition and digestive tract content and for the availability of specimens and tissues for the projects "Taxonomic revision and systematics of *Oxyrhopus rhombifer* (Serpentes: Dipsadidae)" and "Male reproductive cycle of *Oxyrhopus rhombifer* (Serpentes: Dipsadidae) in Southern Brazil". *Oxyrhopus rhombifer* is classified as Least Concern by IUCN (Arzamendia et al., 2019). All specimens are deposited in the herpetological collection of Universidade Federal do Rio Grande (CHFURG) (Appendix).

Sex was determined through verification of the presence or absence of hemipenes via subcaudal incision. The following measurements were taken from each specimen, with a digital caliper to the nearest 0.01 mm: snout-vent length (SVL), tail length (TL), head length (HL), head width (HW), inter-ocular length (IOL), inter-nostril length (INL). We examined the existence of sexual dimorphism in the following characters: 1) SVL; 2) tail proportion in relation to body length (ratio TL/SVL); 3) number of ventral scales; 4) number of subcaudal scales; 5) head length in proportion to body length (ratio HL/SVL); 6) head width in proportion to head length (ratio HW/HL); 7) inter-ocular length in relation to head length (ratio IOL/HL); 8) inter-nostril length in relation to head length (ratio INL/HL). All database was checked for normality through Shapiro-Wilk tests, which detected normal distributions only for SVL, TL/SVL, and number of subcaudal scales. For these characters, the existence of significance differences between males and females was examined with a Student's *t* test. For the other characters (HL/SVL, HW/HL, IOL/HL, INL/HL and number of ventral scales) we applied a Mann-Whitney test aiming to verify significant differences between the sexes. Only mature specimens were examined for sexual dimorphism in SVL, TL/SVL, HL/SVL, HW/HL, IOL/HL and INL/HL. Mature and immature specimens were pooled for the analysis on sexual dimorphism in the number of ventral and subcaudal scales. Statistical tests were performed in software PAST v.3.25 (significance $P < 0.05$). (Hammer et al., 2001).

A ventral incision was made from the esophageal region to around 5 mm above the cloaca and the digestive and reproductive tracts were externalized for analysis. All stomach and gut contents were removed and identified to the lowest possible taxonomic category. Whenever possible, the direction of prey intake (head first or tail first) was annotated. Numeric abundance (N%) of all consumed taxa was determined as the ratio between the absolute number of prey from one taxon and the sum of prey of all identified taxa (Corrêa et al., 2016).

The following reproductive data were recorded from females: total number of ovarian follicles; number of follicles in secondary vitellogenesis (secondary follicles are defined as enlarged follicles with yolk accumulation; diameter ≥ 9 mm, based on the scatterplot of the largest follicles of all females and on coloration [Almeida-Santos et al., 2014]); diameter of the largest secondary follicle; total number of eggs; occurrence of celomatic fat storage; and largest diameter of the largest egg. Females were considered mature when showing at least one of the following characteristics: 1) secondary follicles; 2) oviductal eggs; and 3) folded oviducts, indicating recent oviposition. Males were considered mature when presented coiled and opaque *ductus deferens*, indicating the presence of sperm. The female reproductive cycle was evaluated by: 1) analysis of the distribution of females

Table 1. Summary statics of the variables analyzed for sexual dimorphism in *Oxyrhopus rhombifer* from southern Brazilian coast (Rio Grande do Sul state) and results of Student'*t* and Mann-Whitney tests performed on the variables. Analyses on SVL, TL/SVL, HL/SVL, HW/HL, IOD/HL and IND/HL were performed in mature specimens. Analyses on number of ventral and subcaudal scales were performed on both mature and immature specimens. See main text for abbreviations; n = number of specimens.

Variable	Males	Females	test value	P value
SVL	445 ± 59 (354-597) n = 52	616 ± 92 (451-758) n = 22	9.58	1.701^{E-14}
TL/SVL	0.276 ± 0.023 (0.226-0.358) n = 52	0.189 ± 0.012 (0.161-0.215) n = 22	16.35	5.902^{E-26}
HL/SVL	0.034 ± 0.003 (0.026-0.041) n = 49	0.032 ± 0.003 (0.027-0.039) n = 22	2.94	0.0032
HW/HL	0.557 ± 0.065 (0.478-0.779) n = 49	0.520 ± 0.061 (0.429-0.675) n = 22	2.38	0.017
IOD/HL	0.0346 ± 0.036 (0.296-0.455) n = 49	0.0317 ± 0.006 (0.246-0.368) n = 22	2.56	0.010
IND/HL	0.202 ± 0.028 (0.153-0.289) n=49	0.184 ± 0.022 (0.138-0.226) n = 22	2.40	0.016
Nº ventral scales	180 ± 6 (162-197) n = 78	192 ± 9 (160-212) n = 51	7.74	9.76^{E-15}
Nº subcaudal scales	65 ± 3 (58-71) n = 81	57 ± 3 (50-63) n = 50	16.06	2.19^{E-22}

Table 2. Absolute number (n) and numeric abundance (N%) of prey items found in digestive tracts of *Oxyrhopus rhombifer* from the southern Brazilian coast (Rio Grande do Sul state), frequency and percentage (between parentheses) of prey intake directions of identified taxa, namely: head first (hf), tail first (tf) and not determined (ND).

Prey	n	N%	hf	tf	ND
Sauropsida: Squamata					
Ophiodes sp. (Anguidae)	2	8.3			2 (100)
Aspronema dorsivittatum (Scincidae)	1	4.2	1 (100)		
Cercosaura schreibersii (Gymnophthalmidae)	7	29.2	3 (42.9)	1 (14.2)	3 (42.9)
Lizard not identified	1	4.2			1 (100)
Snake not identified	1	4.2			1 (100)
Mammalia: Rodentia					
Deltamys kempi (Cricetidae: Akodontini)	1	4.2	1 (100)		
Oligoryzomys flavescens (Cricetidae: Oryzomyini)	3	12.5	3 (100)		
small rodent not identified	8	33.3			8 (100)

carrying secondary follicles and oviductal eggs along the months of the year; 2) analysis of the annual profile resultant from the plot of the largest follicles and eggs of each female (Mesquita et al., 2013; Almeida-Santos et al., 2014). Fecundity was determined by the number of eggs in the oviduct (real fecundity) and the number of secondary follicles (potential fecundity) in the ovarium (Mesquita et al., 2013). The correlation between female SVL and potential fecundity was examined with a simple linear regression analysis (Mesquita et al., 2013).

A total of 141 *O. rhombifer* specimens (89 males, 52 mature and 37 immature; 53 females, 22 mature, 31 immature) were analyzed. Females presented higher mean values of SVL and number of ventral scales. Males presented higher mean values for TL/SVL, HL/SVL, HW/HL, IOL/HL, INL/HL, and number of subcaudal scales (Table 1). Summary statistics for all the variables analyzed for sexual dimorphism are presented in Table 1. Significant differences between sexes were found for all the variables tested (Table 1).

Twenty-four specimens contained 24 prey item each. Prey was comprised of rodents (N% = 50.0) and Squamata reptiles (N% = 50.0) (Table 1). Eight out of nine prey in which the direction of intake could be determined were head-first consumed (Table 2). Eleven out of 12 rodents identified were consumed by mature individuals. Lizards were consumed by both mature and immature individuals. The single snake identified was consumed by an immature individual. Half of the squamate prey consisted of tail fragments of Scincomorpha and Anguidae lizards.

The smallest female exhibiting secondary follicles presented 451 mm of SVL, while the smallest male exhibiting coiled/opaque *ducti deferentes* presented 354 mm of SVL. Secondary follicles were found mainly from

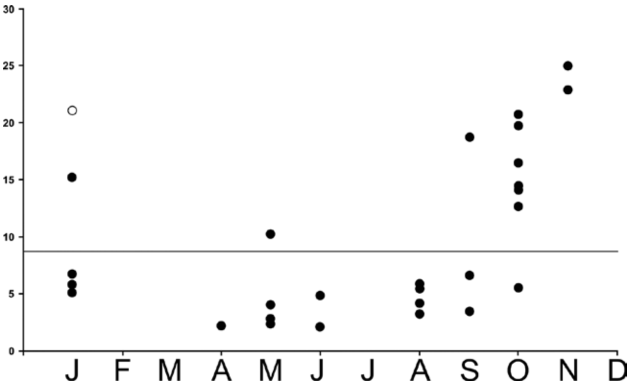

Fig. 2. Monthly variation in diameter (mm) of the largest follicles (black circles) and egg (white circles) of *Oxyrhopus rhombifer* from the southern Brazilian coast (Rio Grande do Sul state). The horizontal line indicates size from which follicles were considered as being in secondary vitellogenesis (secondary follicles).

September to November (spring). A single female presented secondary follicles in January (early summer) and another one showed secondary follicles in May (middle autumn) (Fig. 2). Potential fecundity varied from two to 13 (x = 7.9 ± 3.4 SD; n = 11). One single female, euthanized in January, presented eggs in the oviduct (three eggs). This same female also presented secondary and primary follicles in the ovaries, indicating the possible occurrence of more than one clutch during the cycle. This female did not present expanded oviducts, which indicates that there was no previous egg laying, and the three oviductal eggs may correspond to a single oviposition event. A greater fat storage was observed in mature females during initial and intermediate secondary vitellogenesis while low celomatic fat deposition was found in females in advanced secondary vitellogenesis. We did not find any significant, albeit positive, correlation (r = 0.07; P = 0.84) between female SVL and potential fecundity. An aggregation of three similar-sized males and one larger female, intertwined, but not involved in copulation, was observed in October (early spring).

Our results indicated that sexual dimorphism is very well marked in *O. rhombifer* from southern Brazilian coastal plain, considering that all the variables herein tested were significantly different between sexes. The dimorphism in body size (SVL) is noteworthy, with females significantly larger and showing higher number of ventral scales. Males, however, presented tail proportionally longer, higher number of subcaudal scales, head proportionally longer and wider and greater interocular and inter-nostril measurements. The larger body in females and the tail proportionally longer in males is a well-known aspect in dipsadid snakes (Aguiar and Di-Bernardo, 2005; Balestrin and Di-Bernardo, 2005; Mesquita et al., 2013; Quintela et al., 2017; Quintela and Loebmann, 2019a, b), including the congener *Oxyrhopus trigeminus* (Alencar et al., 2012). Mature females of *Oxyrhopus guibei* also showed SVL significantly larger than mature males (Pizzatto and Marques, 2002). The longer body size in females in species without male-male combat is associated with fecundity so that a larger body can accommodate a larger offspring. Thus, females experience the fertility selection, in which larger bodies take advantage (Shine, 1994). In relation to head dimensions, studies have demonstrated that sexual dimorphism in head traits of snakes are related to differences in the maximum size of preys consumed by different sexes (Shine, 1989; Pearson et al., 2002; Shetty and Shine, 2002; Vincent et al., 2004). A broader analysis of the feeding habits of the species, based on a larger sample of preyed items, could reveal if in fact males feed on larger prey. Another hypothesis to be considered is the use of the head by males for the immobilization of females during mating.

Oxyrhopus rhombifer feeds on small mammals and squamate reptiles in the studied area, which is in agreement with data from other regions where the species occurs (Vidal, 2002; Maschio et al., 2003, 2004; Sawaya et al., 2008; Gaiarsa et al., 2013) and from other *Oxyrhopus* species (Alencar et al., 2012; Gaiarsa et al., 2013). However, the high occurrence of solely lizard tail fragments in the analyzed digestive tracts (25% of all prey items, 50% of Squamata items; Tab. 1), possibly autotomized to avoid predation, is remarkable. Caudal autotomy is a very common defensive mechanism in lizards (Clause and Capaldi, 2006), considering that solely tail fragments have been found in tracts of other xenodontine species such as *Philodryas aestiva* and *P. patagoniensis* (Quintela, pers. comm.).

Females attained sexual maturity at much larger sizes than males, in agreement with *O. guibei* (Pizzatto and Marques, 2002), *O. trigeminus* (Alencar et al., 2012) and other xenodontine genera (Pizzato et al., 2008; Mesquita et al., 2013; Rebelato et al., 2016; Quintela et al., 2017, Quintela and Loebmann, 2019a, b). The small number of mature females in our sample, however, did not allow further analysis or conclusions about the reproductive cycle and fecundity. The occurrence of females showing secondary follicles in spring may indicate a seasonal reproductive cycle, a pattern already recorded for other xenodontines in the studied region (Oliveira et al., 2011; Rebelato et al., 2016; Quintela et al., 2017; Quintela and Loebmann, 2019a, b). A marked greater accumulation of celomatic fat in females during initial and intermediate secondary vitellogenesis when compared to females in advanced secondary vitellogenesis also indicates a capital-breeding strategy (Bonnet et al., 1998), where the consumption of lipidic energy sources occurs along the reproductive cycle (Almeida-Santos et al., 2014). The only data on real fecundity (three oviductal eggs) showed a clutch size smaller than those observed in *O. rhombifer* from other regions (four to 17; Pontes and Di-Bernardo, 1988; Yanosky et al., 1996; Gallardo and Scrocchi, 2006; Gaiarsa et al., 2013), but within the clutch size variation found for *O. guibei* (three to 20; Pizzato and Marques, 2002) and *O. trigeminus* (one to 11; Alencar et al., 2012). Sampling and examination on a greater number of females and histological analyses of male gonads, already in progress, will soon allow a more profound understanding of *O. rhombifer* reproductive biology in the southern Brazilian coast.

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APPENDIX

Specimens examined and deposited in the herpetological collection of Universidade Federal do Rio Grande (CHFURG).

Oxyrhopus rhombifer: Brasil: Rio Grande do Sul: Palmares do Sul (CHFURG 1267, 2548); Tavares (CHFURG 4320, 4321); Rio Grande, Quinta (CHFURG 2015), Parque Marinha (CHFURG 833, 4580), Vila Carreiros (CHFURG 2540, 2963), Distrito Industrial (CHFURG 1313, 1314, 1704, 1846, 1851, 1886, 1888, 1899, 1901, 1904, 1906, 1907, 1908, 1909, 1915, 1959, 1961, 1962, 2385, 3156, 3328, 4561, 5070, 5078, 5243), Senandes (CHFURG 832), Área de Proteção Ambiental da Lagoa Verde (CHFURG 1926, 1963, 1964, 1965, 2975, 3436), Cassino (CHFURG 1120, 1900, 1902, 1905, 3239, 4570, 4582, 4583, 5169, 5170, 5171, 5172, 5174, 5175, 5176, 5177, 5178, 5276, 5404, 5957, 5958, 5959, 5960, 5961, 5962, 5963, 5964), Barra (CHFURG 1910, 3076, 3078, 3079, 3090, 3208, 3274, 3328, 3329, 4419, 4444, 4567, 4578, 4579, 4581, 4584, 4671, 4680, 4687, 4688, 4789, 4790, 4820, 4891, 4902, 4918, 4920, 4921, 5071, 5072, 5073, 5074, 5075, 5076, 5077, 5078, 5079, 5080, 5081, 5082, 5083, 5173, 5201, 5238, 5239, 5240, 5241, 5242, 5243, 5244, 5245, 5246, 5247, 5249, 5250, 5285, 5633, 5966), Estação Ecológica do Taim (CHFURG 1500); Santa Vitória do Palmar (CHFURG 5376).

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