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Front cover: Female grass snake (Natrix helvetica) from Vejo, Rome. See article on page 69.

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Discovering the biogeographic history using predefined areas and explicit geographical data in the South American *Liolaemus elongatus* group (Iguania: Liolaemidae)

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The genus *Liolaemus* includes 268 species, classified in two subgenera, *Eulaemus* and *Liolaemus sensu stricto*. The latter is formed by 12 monophyletic groups; one of them being the *Liolaemus elongatus* group, distributed in South America. We studied the biogeographic history of the *L. elongatus* group. We obtained a phylogenetic hypothesis recovering five main clades: the *L. punmahuida*, *L. elongatus sensu stricto*, *L. kriegi*, *L. petrophilus* and *L. capillitas* clades. Based on that hypothesis we obtained a time calibrated tree. The ancestral ranges were estimated applying three methodologies: DEC, DEC+j (using predefined areas) and GEM (using explicit geographical data). Our results show that the ancestral area of the *L. elongatus* group was located in central Argentina, and its divergence began around 11.5 Mya. From here, a combination of events (founder events and/or vicariances) led the species to their current distribution. Despite their differences, DEC+j and GEM show congruent results.

Keywords: biogeography, divergence times, ancestral area, lizards

INTRODUCTION

The *Liolaemus* Wiegman, 1834 genus includes small and medium size lizards and is the second most diverse iguanian group in the world. It comprises ~268 species (Abdala & Quinteros, 2014; Abdala et al., 2016; Verrastro et al., 2017; Gutierrez et al., 2018) and only the genus *Anolis* (Daudin, 1802) is more diverse. Laurent (1983; 1985; 1995) initially, and later Etheridge (1995), split the genus into two main groups, subgenus *Liolaemus (sensu stricto)* or "Chileno group", and *Eulaemus* or "Argentino group", distributed mainly to the west and east of the Andes, respectively. This proposal has been supported by many phylogenetic analyses based on both molecular and/or morphological evidence (Schulte et al., 2000; Espinoza et al., 2004; Esquerré et al., 2019; Pyron et al., 2013; Zheng & Wiens, 2016).

Liolaemus sensu stricto subgenus has been the object of many phylogenetic studies, which proposed hypotheses recognising several subgroups within. Two independent studies recovered twelve monophyletic groups inside the *L. sensu stricto* subgenus: Lobo (2005) and Díaz Gómez & Lobo (2006), (see also Lobo et al., 2010; Abdala & Quinteros, 2014). One of those groups is the *L. elongatus* group, which is distributed in central-south Argentina and Chile. Specifically, this group ranges from Tafi del Valle in northern Argentina, along the Andes

mountain range in Chile and Argentina until the province of Chubut, in southern Argentina.

Species in the *Liolaemus elongatus* group are characterised by large stout bodies and they are usually saxicolous, viviparous, and omnivorous (Ceí, 1986). The *L. elongatus* group is formed by endemic species like the majority of the lizards of austral distribution (Medina et al., 2017). The *L. elongatus* group was primarily defined by Ceí (1974) including three species: *L. elongatus* (Koslowky, 1986), *L. petrophilus* (Donoso-Barros & Ceí, 1971), and *L. austromendocinus* (Ceí, 1974), using morphological characters. Many posterior analyses confirmed the monophyly of the group, including some recent ones which are based on molecular data (Avila et al., 2004; Lobo et al., 2010; Abdala & Quinteros, 2014). In the last 15 years, many new species belonging to this group have been described (Abdala et al., 2010; Avila et al., 2010; Avila et al., 2012; Avila et al., 2015; Troncoso et al., 2016) increasing the number of species to 23.

Several hypotheses have been proposed on the constitution of the *Liolaemus elongatus* group (Morando et al., 2003; Avila et al., 2004; Lobo, 2005; Díaz Gómez & Lobo, 2006; Lobo et al., 2010). The most recent taxonomic revision of the *Liolaemus* genus is that of Abdala & Quinteros (2014). They perform an update of the study of Lobo et al. (2010), proposing the same groups but including all the new species described at

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that time. Avila et al. (2015) recovered four clades inside the *L. elongatus* group (that resemble the complexes of Morando et al. (2003): the *L. elongatus*, *L. kriegi*, *L. petrophilus*, and *L. punmahuida* clades). The same groups (with the exception of *L. punmahuida* clade) are recovered by Medina et al. (2015), who studied in detail the *L. kriegi* complex. Escobar-Huerta et al. (2015), Troncoso et al. (2016) and Esquerré et al. (2019) recovered the same clades as Avila et al. (2015).

Over the years, many authors have made biogeographic general assertions on the *Liolaemus* genus, generally without formal methodology to support these. Many of these hypotheses will be tested in the present study. For example, Cei (1979) characterised Patagonia as an active center of speciation and dispersion, including *Liolaemus* as an example of recent adaptive radiation. There have also been studies with formal methodology, although these have focused mainly on larger groups, with only a few *L. elongatus* representatives. Since the publication of these studies, many new species of the *L. elongatus* group have been described. Young-Downey (1998) performed a Brooks Parsimony Analysis (BPA; Brooks 1990), over a phylogeny of *Liolaemus*. Later, Schulte et al. (2000) performed a molecular based phylogeny of *Liolaemus*, where they arrive at several general conclusions on the genus, for example that subsequent events of dispersals across the Andes mountain range followed by vicariations have shaped the genus over its history. Lobo (2001), in a phylogeny of the *L. chiliensis* group assigned to the species studied the distribution areas defined by Roig-Juñent (1994), found that major groups correspond to these areas, although in that analysis, the *L. elongatus* group is not recovered as monophyletic. Díaz Gómez & Lobo (2006) were the first to perform formal biogeographical analyses in which an ancestral area is assigned to the *L. elongatus* group, although the focus of this work is more general, that is, the *L. sensu stricto* group. They obtain an ancestral area for the *L. elongatus* group that they call “Andes”, using three different methodologies: Fitch optimisation (Fitch, 1970), DIVA (Ronquist, 1997) and Weighted Ancestral Areas (Hausdorf, 1998). This area corresponds to northern Payunia and southern Central Monte, in the Andes mountain range. The most recent biogeographical analysis where the members of the *L. elongatus* group were included is Esquerré et al. (2019), where they use almost all the species members of the group and predefined larger areas at the South American level, since the objective of their study is a much larger group (Liolaemidae). They obtained “central Andes” as the ancestral area of the *L. elongatus* group. The divergence times obtained by the different authors for the clades of the *L. elongatus* group are detailed in Table 1.

In the present study, we inferred a DNA-based phylogeny and estimated divergence times in a time calibrated tree. We used this time calibrated tree and applied different methodologies to reconstruct the ancestral range of distribution in order to identify the main biogeographic events. We study the biogeographic history of the *L. elongatus* group applying three

methods: DEC (Dispersal-Extinction-Cladogenesis model, (Ree et al., 2005), DEC+j (Matzke, 2014), and GEM (Geographically explicit Event Model; Arias, 2017). Applying those methods, we try to elucidate the factors which promoted the diversification of the species members of the *L. elongatus* group. The comprehension of the diversification can provide valuable information not only about the date of origin of the taxonomic groups, but also the impacts of climatic and geological events in it (Weir, 2006), as well as rates of speciation and extinction (Weir & Schulte, 2007; Mercer & Roth, 2003). Moreover, the time and mode of diversification can be studied in the group of *L. elongatus* to test the biogeographical hypothesis in a more quantitative way. The results obtained here will be compared with previous studies, as the processes driving evolution tend to affect many of the taxa inhabiting the same region. This will allow us to test if taxa with different life histories share a common biogeographical history. It should also be noted that the particular environment of these lizards (saxicolous), and the fact that they inhabit rocky outcrops on both sides of the Andes mountain range, make them a good candidate for understanding the role of geological processes (e.g. the uplift of the Andes) in shaping diversity (Esquerré et al., 2019). Additionally, this group of lizards is biogeographically interesting given the wide range of habitats they use, as well as there being many syntopic species -a phenomenon that must be explained either through sympatric speciation or dispersal. It is to be noted that, in the last categorization (Abdala et al., 2012), five species (included in this work) have been declared vulnerable. Therefore, conclusions reached here may prove to be a valuable contribution to the conservation of these endangered species since information about their distributions is incorporated. Unlike previous biogeographical studies, which have focused on divergences deeper in the tree of Liolaemidae, we will focus on the divergences happening at derived nodes within this group. We include almost all known species and populations, which will result in a more robust phylogeny than previously attained, as well as a more complete sample of the group’s distribution. Since biogeographical methods are dependent on phylogenies and distribution records, the hypothesis we arrive at are likely to be more reliable.

MATERIAL AND METHODS

Phylogenetic Analyses

We included the sequences of nuclear gene KIF24, mitochondrial gene CytB and ribosomal gene of the subunit 12s from Morando et al. (2003), Avila et al. (2004), Fontanella et al. (2012), Medina et al. (2014), Olave et al. (2014), Avila et al. (2015), Medina et al. (2015), Medina et al. (2017) and Medina et al. (2018). Accession numbers of sequences used and specimens studied are listed in Supplementary Material S1. We performed three analyses, one under Parsimony, one under Maximum Likelihood (ML) and the other under Bayesian MCMC. We included all described species members of

Table 1. Previous studies showing the methodology used and divergence times obtained for the clades of the *Liolaemus elongatus* group. (*) Studies based on major scales to genres.

	Schulte (2013)*	Medina et al. (2014)	Zheng & Wiens (2016)*	Medina et al. (2017)	Esquerré et al. (2019)*
Methodology Groups	Relaxed clock	Strict clock	Penalised likelihood	Relaxed clock	Relaxed clock
<i>L. elongatus</i> group	16 Mya	-	12.30 Mya	-	13 Mya
<i>L. punmahuida</i> clade	-	-	-	-	2 Mya
<i>L. elongatus-petrophilus</i> clade	Not recovered	3.75 Mya	10.54 Mya	2.25 Mya	9.50 Mya
<i>L. petrophilus</i> clade	Not recovered	-	Not recovered	1.60 Mya	8.20 Mya
<i>L. capillitas</i> clade	Not recovered	Not recovered	6.01 Mya	-	4.50 Mya
<i>L. elongatus-kriegi</i> clade	Not recovered	1.90 Mya	4.74 Mya	1.60 Mya	5.80 Mya
<i>L. kriegi</i> clade	6 Mya	1.10 Mya	2.93 Mya	1.35 Mya	4 Mya
<i>L. elongatus sensu stricto</i> clade	Not recovered	-	2.93 Mya	1.30 Mya	4 Mya

the group (a total of 23), with the exception of *Liolaemus antonietae*, *L. janequeoae*, and *L. lonquimayensis*, (for which no published sequences were available), plus five populations of uncertain taxonomic status.

Parsimony analysis was performed with TNT 1.5v (Goloboff et al., 2003; Goloboff & Catalano, 2016) using heuristic search using Tree Bisection Reconnection, with 500 addition sequences saving 50 trees in each sequence. Also, we performed an analysis under the New Technology Search (Sectorial search, Ratchet, Tree Drifting, and Tree Fusing) implemented in TNT, with 50 initial add sequences and finding the minimum tree length at least 20 times. The support was measured under Bootstrapping standard, with 500 replicates.

ML analyses were conducted using RAxML v7.0.4 (Stamatakis, 2006) on the platform CIPRES Science Gateway (Miller et al., 2010) based on 1000 rapid bootstrap analyses, and the GTRGAMMA evolution model. We also obtained bootstrap support values with RAxML v7.0.4 (Stamatakis, 2006) based on 1000 quick replicates and a GTRGAMMA model for all genes.

Time of divergence estimates

Tree topology, age of nodes and substitution rates were simultaneously estimated using Bayesian MCMC (Markov chain Monte Carlo) approach as implemented in BEAST v2.5 (Bouckaert et al., 2019). We used the fossil from the *Eulaemus* clade, representing the earliest record of this subgenus (Albino, 2008) to place a mean prior of 20 Mya on the tree height. A lognormal prior is typically most appropriate for the majority of fossil calibrations (Hedges & Kumar, 2004) because it assumes that the divergence event actually occurred sometime before the appearance of the fossil. Under this model, fossils thus represent a hard lower bound and a soft upper bound on a given divergence event. The Yule prior assumes a constant lineage birth rate for each branch in the tree and is considered most suitable for trees describing the relationships between individuals from different species (Ho et al., 2005). An uncorrelated lognormal distributed relaxed clock (UCLD) model was employed, which allow evolutionary rates to vary along branches within lognormal distributions (Drummond et al., 2006). The calibration was conducted for all genes jointly.

Three independent runs of 10 million generations each were performed with sampling every 5000 generations. The three separate runs were then combined (following removal of 10 % burn-in) using Log Combiner v2.0 (Drummond & Rambaut, 2007; Rambaut & Drummond, 2008). Adequate sampling and convergence of the chain to stationary distribution were confirmed by inspection of MCMC samples using Tracer v2.0 (Drummond & Rambaut, 2007). The effective sample size (ESS) values of all parameters were greater than 200, which were considered a sufficient level of sampling. The sampled posterior trees were summarised using Tree Annotator v2.0 (Drummond & Rambaut, 2007; Rambaut & Drummond, 2008) to generate a maximum clade credibility tree (maximum posterior probabilities) and calculate the mean ages, 95 % highest posterior density (HPD) intervals and posterior probabilities for each node. The topology recovered was visualised with Fig Tree v1.2 (Rambaut & Drummond, 2008).

Biogeographical analyses

We applied three different methods to assess the historical distribution processes of the species members of the *Liolaemus elongatus* group. Two use predefined areas (DEC and DEC+j), and the other uses explicit geographical ranges (GEM).

Dispersal-Extinction-Cladogenesis (DEC; Ree et al., 2005) establishes ancestral ranges of the species included in a phylogenetic tree, evaluating different scenarios. DEC is a dispersal-extinction-cladogenesis model for geographic range evolution that specifies instantaneous transition rates between discrete states (ranges) along phylogenetic branches and apply it to estimating likelihoods of ancestral states (range inheritance scenarios) at cladogenesis events (Ree & Smith, 2008). DEC is an attempt to implement DIVA in a maximum likelihood context.

The DEC+j (Matzke, 2014) model is implemented by assigning a parameter, *j*, to specify the weight of each jump dispersal event in the cladogenesis matrix. To calculate the probabilities of a particular range inheritance event at cladogenesis, conditional on a particular ancestral range, the weight of each of the allowed range inheritance events is divided by the sum of the weights.

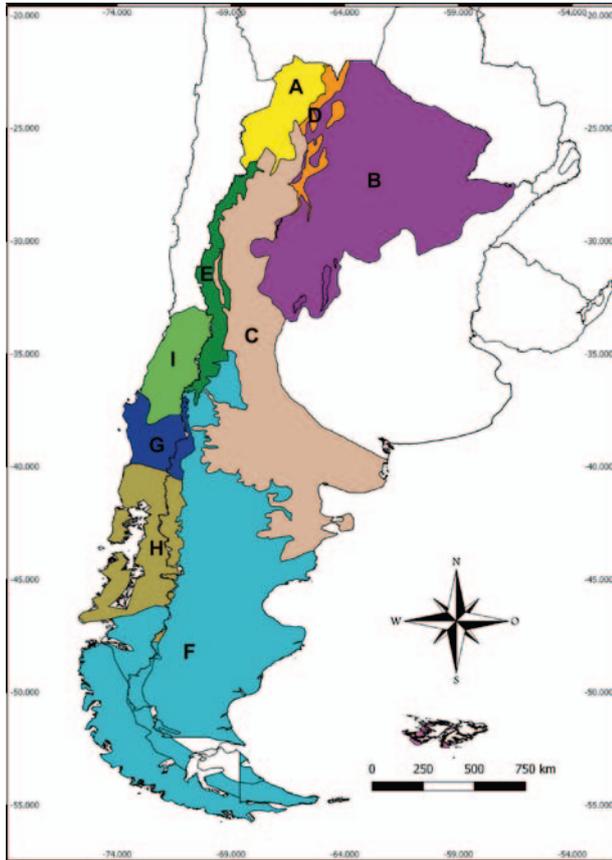


Figure 1. Map of Argentina and Chile showing the biogeographic regions used. Biogeographic regions of Arana et al. (2017) employed in DEC analyses. **A:** Puna Province, **B:** Chaco Province, **C:** Monte Province, **D:** Yungas Province, **E:** Cuyan High Andean Province, **F:** Patagonian Province, **G:** Maule Province, **H:** Valdivian Forest Province, **I:** Principal Cordillera of the Andes.

Conversely, Geographical Explicit Model (GEM, Arias, 2017) uses explicit geographic ranges by drawing actual occurrence data on a grid, instead of using predefined areas. The software then uses these ranges to assign a scenario for every node, choosing between four possibilities: vicariance, founder event, sympatry and point sympatry, and then specifies an ancestral area, accordingly. Each event has a cost which is related to the distribution of the daughter nodes. In order to find the less costly reconstruction, the software performs a heuristic search. GEM can be seen as an attempt to merge the ideas of Hovencamp (1997; 2001) and Ronquist (1997).

The main difference between DEC, DEC+j and GEM lies in the fact that GEM, which is a novel methodology, does not use predefined areas, but distributions drawn on a grid. Because of the, GEM basically only assumes the presence of a species in the corresponding cell in which the actual coordinate of the species lies. Using grid cells of adequate size, this assumption can be thought of as conservative. When using DEC and DEC+j, on the other hand, one has to assume that a given species is present in the whole area where the coordinate lies, whether this represents an accurate description of the actual

range of the species or not. Of course the danger is overestimating the range of the species. This is a known limitation of these methods, which is why we intend to compare these results with those of GEM.

DEC methodology

Since DEC uses pre-defined areas, in order to optimise the ancestral areas of distribution, we used the Geological Provinces proposed by Arana et al. (2017). In this paper, diverse regions of Argentina were distinguished by combining climatic, geologic, and biotic criteria. The Provinces used in the present study were chosen based on the georeferenced current distribution of the species included, being other areas too extensive for the current distribution. The Provinces used were: A: Puna Province, B: Chaco Province, C: Monte Province, D: Yungas Province, E: Cuyan High Andean Province, F: Patagonian Province, G: Maule Province, H: Valdivian Forest Province, I: Santiago Province. The last three (G, H and I) were taken from Morrone (2006) and combined with those from Arana et al. (2017) to include all the individuals with distribution to the east and west of the Andes (Fig. 1).

To infer the processes that moulded the current distribution of the species of the *Liolaemus elongatus* group, we used a parametric method implemented in Lagrange vC++ (Ree & Smith, 2008). This method is based in Dispersal-Extinction-Cladogenesis models which require information about a single ultrametric dated phylogeny and distributional information of extant species. Based on previous studies (Chacón & Renner, 2014; Portelli & Quinteros, 2018) we used a constrained adjacency matrix (Matrix I. Table 2). This matrix assigns user-defined probability values to dispersal events between areas. Values included were: 0.8 for adjacent areas; 0.6 for areas separated by one intermediate area; 0.4 for areas separated by two intermediate areas; 0.2 for areas separated by three intermediate areas; and 0.08 for areas separated by four intermediate areas. We also used a second matrix (Matrix II. Table 3) to apply dispersal through geographic barriers cost. As such, we consider the probabilities of the first matrix plus a value, assigned to a geographic barrier (the Andes mountain range), close to 0 (0.001).

DEC+J methodology

To obtain the ancestral areas with DEC+J, the BioGeoBears 0.2.1 package (Matzke, 2013) implemented in R 3.0.2 (R Core Team) was used. For this test, the tree generated in Beast was used previously and the values of the following parameters were used, dispersion (d) = 0.8, extinction (e) = 0 and a third parameter whose value is predetermined by the method corresponding to speciation by founder effect (J) = 0.001. Furthermore, we use the same matrices (Tables 2 and 3) used for the DEC dispersion.

Finally, DEC and DEC+J were compared by Likelihood Ratio Test and Akaike Information Criterion (AIC wt), which gave relative probability values of both analyses. This was also obtained from the BioGeoBears package.

Table 2. Probabilities of dispersal cost between the areas (A_I) employed in the DEC analysis. Matrix I. See “Materials and Methods” for area names.

	A	B	C	D	E	F	G	H	I
A	1	0.6	0.8	0.8	0.8	0.6	0.4	0.4	0.6
B	0.6	1	0.8	0.8	0.6	0.6	0.4	0.4	0.4
C	0.8	0.8	1	0.8	0.8	0.8	0.6	0.6	0.6
D	0.8	0.8	0.8	1	0.6	0.6	0.4	0.4	0.4
E	0.8	0.6	0.8	0.6	1	0.8	0.6	0.6	0.8
F	0.6	0.6	0.8	0.6	0.8	1	0.8	0.8	0.8
G	0.4	0.4	0.6	0.4	0.6	0.8	1	0.8	0.8
H	0.4	0.4	0.6	0.4	0.6	0.8	0.8	1	0.6
I	0.6	0.4	0.6	0.4	0.8	0.8	0.8	0.6	1

Table 3. Probabilities of dispersal cost between the areas (A_I) employed in the DEC analysis. Matrix II. See “Materials and Methods” for area names.

	A	B	C	D	E	F	G	H	I
A	1	0.6	0.8	0.8	0.8	0.6	0.001	0.001	0.001
B	0.6	1	0.8	0.8	0.6	0.6	0.001	0.001	0.001
C	0.8	0.8	1	0.8	0.8	0.8	0.001	0.001	0.001
D	0.8	0.8	0.8	1	0.6	0.6	0.001	0.001	0.001
E	0.8	0.6	0.8	0.6	1	0.8	0.001	0.001	0.001
F	0.6	0.6	0.8	0.6	0.8	1	0.001	0.8	0.001
G	0.001	0.001	0.001	0.001	0.001	0.001	1	0.8	0.8
H	0.001	0.001	0.001	0.001	0.001	0.8	0.8	1	0.6
I	0.001	0.001	0.001	0.001	0.001	0.001	0.8	0.6	1

GEM methodology

GEM can assign one of four possible states to each node: vicariance, founder event, sympatry or point sympatry. Each event has a cost depending on the cell distribution of the sister nodes which come from it. According to this, an ancestral range is assigned to each node, as well as an event, which indicates what event happened at that node. The final cost of the reconstruction will be the number of cell changes in range, plus the cost of each event. A heuristic search is performed in order to find the least costly reconstruction. The possibilities for these are: vicariance, founder event, sympatry and point sympatry. Since this is a relatively new methodology, we will attempt to summarise it, although a more in-depth explanation can be found in the original paper (Arias et

al, 2017).

- Vicariance: the ancestral area will be equal to the sum of the ancestral ranges of two sister nodes. The number of overlapping cells of the two nodes will be given as extra cost to the event. This is due to the assumption that, in order to get this overlap, one or both of the sister nodes must have crossed a barrier which caused the allopatry in the first place.

- Founder event: considering two sister nodes, one will inherit the ancestral area, while the other (the one born out of the founder event), occupies new territory. Since founder event is supposed to happen at a given spot, after the crossing of a barrier has occurred, the bigger the area of the node originated by founder event, the less likely it is for the event to have happened. Therefore, the cost of the event is given by the amount of cells occupied by the node which does not inherit the ancestral area, minus one.

- Sympatry: in this case, both sister nodes inherit the ancestral range. The event will be given no extra cost only if both sister nodes share the exact same range. Any cells that are not shared by both nodes will be taken as extra cost.

- Point sympatry: when one of the descendant nodes inherits only a point of the ancestral range, instead of the whole range. In this case, the cost assigned is similar to the founder event, that is, the number of cells occupied by the node that does not inherit the whole range, minus one.

We ran the data with GEM as implemented in *evs* program, using a raster grid with pixels of 0.5° x 0.5° degrees, with a filling of 1. Costs were set as follows: vicariance and founder events to a default cost of 1, while sympatry and point sympatry events were set to a cost of 1.5. This is due to the unique set of factors which must happen for sympatric speciation to take place. If an alternative explanation for current distributions is possible, then this must generally be preferred, although sympatry will be considered in light of very strong evidence. In order to avoid having too large ancestral areas, we penalised them using $Z = 10$. The search was made with the flipping algorithm applying 10 independent runs each with 10,000 flip replicates (for a grand total of 100,000 flips replicates).

RESULTS

Table 4. Divergence times obtained for the *Liolaemus elongatus* group and its internal clades with their corresponding highest posterior density intervals (HPD).

Clades	Times of divergence (Mya)	95 % HPD (Mya)
<i>L. elongatus</i> group	11.5	14.2-5.9
<i>L. punmahuida</i> clade	4	4.47-0.59
<i>L. elongatus-petrophilus</i> clade	10.1	15.6-4.6
<i>L. petrophilus</i> clade	7.7	10.4-5.01
<i>L. capillitas</i> clade	4.25	7.9-0.6
<i>L. elongatus-kriegi</i> clade	6	11.02-1.01
<i>L. kriegi</i> clade	3	3.12-0.95
<i>L. elongatus sensu stricto</i> clade	4.16	7.32-1.01

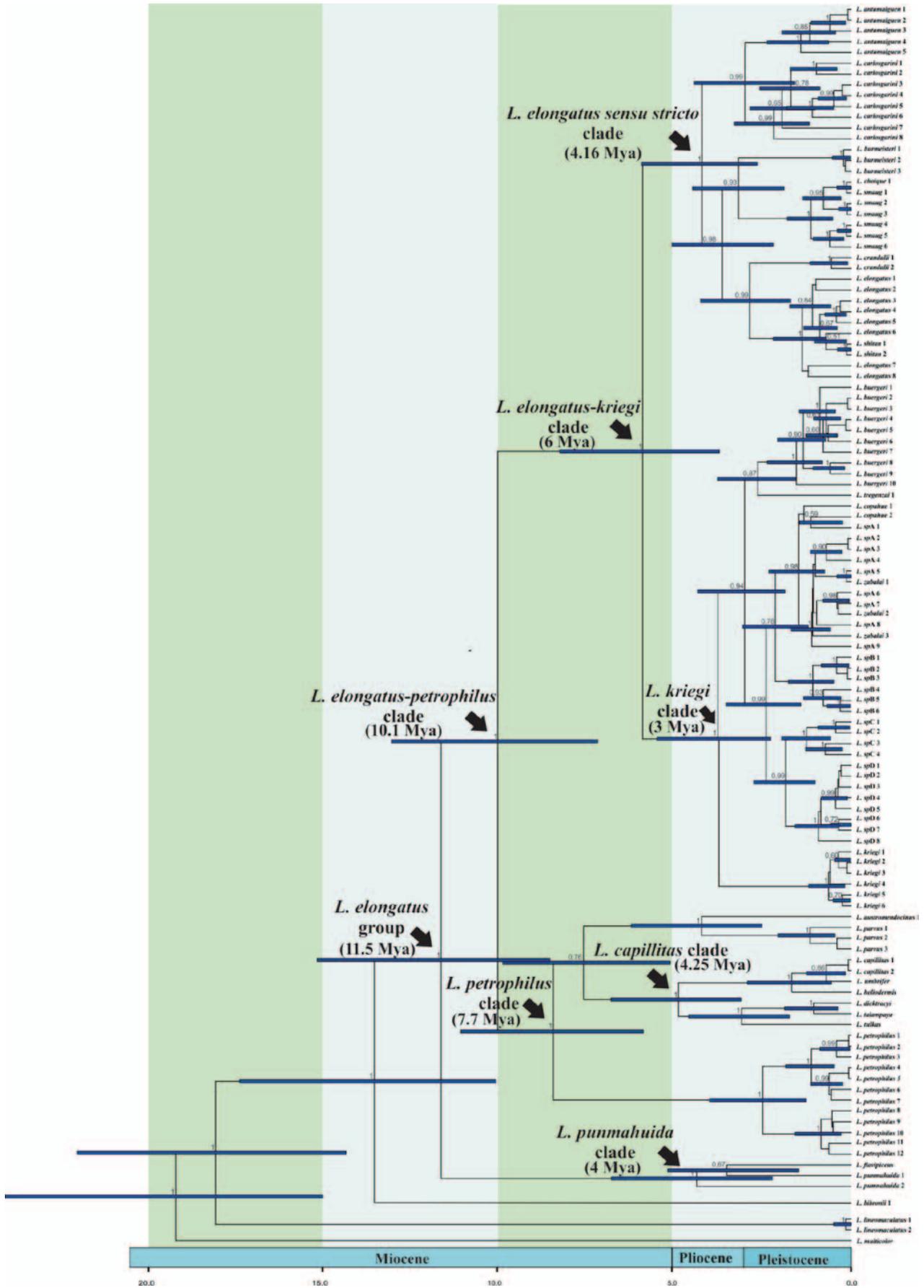


Figure 2. Main clades of the *L. elongatus* group. Times of divergences estimates for the *L. elongatus* group, under BI topology Ultrametric tree scaled in Myr. Numbers and horizontal bars on nodes represent posterior probabilities values and 95 % credibility intervals.

Table 5. Ancestral ranges obtained for DEC, DEC + j and GEM with their corresponding probabilities (P DEC and P DEC + j) of the *L. elongatus* group and their internal clades. Ancestral ranges obtained by DEC and DEC + j: C: Monte Province; F: Patagonian Province; CF: Monte and Patagonian Provinces; FG: Patagonian and Maule Provinces. Ancestral ranges obtained by GEM: CM: Central Monte; NCP: Norther Central Patagonia; Pa: Payunia.

Clades	Ancestral range DEC	P DEC	Ancestral range DEC+j	P DEC+j	Ancestral range GEM
<i>L. elongatus</i> group	CF	0.87	CF	1	NCP
<i>L. punmahuida</i> clade	F	1	F	1	Pa
<i>L. elongatus-petrophilus</i> clade	CF	1	CF	0.89	NCP
<i>L. petrophilus</i> clade	CF	1	CF	1	NCP
<i>L. capillitas</i> clade	C	1	C	0.74	CM
<i>L. elongatus-kriegi</i> clade	F	0.86	F	1	Pa
<i>L. kriegi</i> clade	FG	0.78	FG	0.82	Pa
<i>L. elongatus sensu stricto</i> clade	F	0.86	F	1	Pa

Table 6. Likelihood ratio test and Akaike information criterion (AIC) obtained through an analysis of BioGeoBears in R.

Modelo	lnL	p	d	e	j	AIC
DEC	-10.12		0.034	0.032	0	26.34
DEC+j	-7.23	0.009	0.018	0	0.39	19.76

Phylogeny

We obtained a time calibrated tree with the aim of dating the diversification events of the *Liolaemus elongatus* group. The clade topology recovered with BEAST is the same as those recovered under Parsimony and ML (Fig. 2). The main clades recovered were: 1) the *L. punmahuida* clade is the sister clade of the rest of the *L. elongatus* group; and 2) the *L. elongatus-petrophilus* clade, which includes three clades: the *L. kriegi*, *L. elongatus sensu stricto*, and *L. petrophilus* clades. The *L. kriegi* and *L. elongatus sensu stricto* clades were found to be sister clades (*L. elongatus-kriegi* clade hereafter). Within the *L. petrophilus* clade, we found the previously defined *L. capillitas* clade to be monophyletic group. The list of species belonging to each clade is available in the Supplementary Material S2.

Divergence time estimates

Our results show that the divergence of the *Liolaemus elongatus* (Table 4) group probably began during the Middle Miocene around 11.5 Mya (95 % highest posterior density interval- HPD: 14.2- 5.9). Inside, the clades that belong to the *L. elongatus* group have their origin in the Late Miocene to the Middle-Late Pliocene, with the exception of a few species which have their origin in during the Pleistocene.

The *Liolaemus punmahuida* clade, which includes *L. flavipiceus* and *L. punmahuida* began its diversifications around 4 Mya (95 %HPD: 4.47-0.59). The *L. elongatus-petrophilus* clade, diverged around 10.1 Mya (95 %HPD: 15.6-4.6). The *L. petrophilus* clade diverged around 7.7 Mya (95 % HPD: 10.4- 5.01) in the Lower Miocene, and inside the *L. capillitas* clade around 4.25 Mya (95 % HPD: 7.9- 0.6). The *L. elongatus-kriegi* clade diverged around 6 Mya (95 % HPD: 11.02- 1.01), giving two clades: the *L. elongatus sensu stricto* clade, which diverged at 4.16Mya (95 % HPD 7.32- 1.01) and the *L. kriegi* clade which

diverged around 3 Mya (95 % HPD 3.12- 0.95).

Ancestral distribution reconstruction

When comparing the DEC and DEC+j analyses (Table 5), the latter showed a better fit to the analysis, using values expressed with a likelihood ratio test and the Akaike information criterion (AIC). The likelihood ratio favours DEC+j when compared to DEC ($p = 0.009$), and in that it presents a better fit of AIC (AIC = 19.76) (Table 6).

The preferred biogeographical model was DEC+j; therefore, the results and discussion will be based on this. The results obtained by DEC can be seen in the Supplementary Material section (Fig S1 and S3 File).

The biogeographic reconstructions recovered with DEC+j show that the ancestral range of the *Liolaemus elongatus* group is most likely the combined Monte and Patagonian Provinces (Range CF; P:1). From here, the *L. elongatus* group diversifies into two clades: *L. punmahuida* and the *L. elongatus-petrophilus* clade. The *L. punmahuida* clade originates from the ancestral range Patagonian Province (Area F; P:1). The ancestral range of the *L. elongatus-petrophilus* clade is most likely the combined Monte and Patagonian Provinces (Range CF; P:0.89).

Inside the *Liolaemus elongatus-petrophilus* clade, the ancestral range of the *L. petrophilus* clade is most likely the combined Monte and Patagonian Provinces (Range CF; P:1). Inside the *L. petrophilus* clade, the ancestral range of the *L. capillitas* clade is most likely the Monte Province (Area C; P:0.74).

Conversely, the group sister of the *Liolaemus petrophilus* clade is the *L. elongatus-kriegi* clade, and the ancestral range of it most likely to corresponds the Patagonian Province (Area F; P:1). The ancestral range of the *L. kriegi* clade is most likely the combined Patagonian and Maule Provinces (Range FG; P:0.82). Finally, the ancestral range of the *L. elongatus sensu stricto* clade is most likely the Patagonian Province (Area F; P:1).

GEM results

The search with GEM found 354 different reconstructions with a cost of 67. All the reconstruction yielded 22 founder events and no vicariations. The number of sympatry events varied between 7 and 8, and the number of point sympatry events varied between 2 and 3. In order to process this amount of information, we made a consensus of the main nodes, those being: *Liolaemus elongatus* group, *L. elongatus-petrophilus* clade, the *L. capillitas* clade, *L. elongatus-kriegi* and *L. elongatus sensu stricto* (Fig. 4). All reconstructions assign the same events to the main nodes, although they vary in the direction of one founder event in the *L. capillitas* clade. This indicates that although there are many reconstructions, these are minor variations at very terminal nodes (Fig. 5).

We obtained the ancestral area of *Liolaemus elongatus* group, located in northern Central Patagonia, and from that node, a founder event occurred that originated the *L. punmahuida* clade in the Payunia region. The *L. elongatus-petrophilus* clade inherited the ancestral area of the *L. elongatus* group, and from this node a new founder event towards Payunia happened which originated the *L. elongatus-kriegi* clade. The *L. petrophilus* clade, on the other hand remained in the area of its immediate ancestor. In the *L. elongatus-kriegi* clade, whose ancestral area is in Payunia, a speciation by sympatry occurred, which originated the *L. elongatus sensu stricto* and the *L. kriegi* clades. Finally, within the *L. petrophilus* clade, whose ancestral area is in northern Central Patagonia, there is a founder event which originated a clade formed by *L. austromendocinus* + *L. parvus* and the *L. capillitas* clade in the central Monte region. Within the *L. capillitas* clade, a founder event which split the northern clade in northern Monte region (*L. capillitas*, *L. heliodermis* and *L. umbrifer*) from the southern clade (*L. dicktracyi*, *L. talampaya* and *L. tulkas*), the latter having inherited the area of its immediate ancestor.

DISCUSSION

Phylogeny

Since it was first proposed by Cei (1974), until the actual proposals (Medina et al., 2014, 2015, 2018; Avila et al., 2015; Troncoso et al., 2016; Esquerré et al., 2019, among others), the *Liolaemus elongatus* group has shown many changes in its taxonomic composition and phylogenetic relationships. In fact, since the identification of the *L. elongatus* group, four monophyletic groups have been identified within it (*L. elongatus*, *L. kriegi*, *L. petrophilus*, and *L. punmahuida* clades). The relationship of these three groups varies in the different proposals. Our results are congruent with those of Morando et al. (2003), Avila et al. (2004), Medina et al. (2014), Escobar Huerta et al. (2015), Troncoso et al. (2016), Zheng & Wiens (2016), Medina et al. (2017) and Esquerré et al. (2019) where the *L. elongatus* clade is sister to *L. kriegi* clade, and those are sister to the *L. petrophilus* clade (Fig. 2). Other relationships are found in Schulte (2013), Avila

et al. (2015), Medina et al. (2015), Medina et al. (2018; *L. elongatus* clade, *L. petrophilus* clade, *L. kriegi* clade). In all studies, including the present, the *L. punmahuida* named as such by Avila et al. (2015) clade is recovered as sister to the whole group.

Divergence time estimates

In general, previous biogeography studies that have included representatives of the *Liolaemus elongatus* group were mainly focused on larger groups, such as *L. sensu stricto*, *Liolaemus*, or even Liolaemidae. This work focuses exclusively on the biogeographic history of this unique group of lizards, which is part of an extensive monophyletic group studied both from a phylogenetic perspective (Schulte, 2013; Zheng & Wiens, 2016; Esquerré et al., 2019) and a biogeographical perspective (Díaz Gómez & Lobo, 2006; Esquerré et al., 2019) making it comparable with previous studies. The divergence time was calculated using BEAST and the divergence events were estimated with DEC, DEC+j and GEM.

The correct dating of nodes is important in order to assess possible geological events and paleogeographical changes that might have induced speciation events. Previous works have dated some of the nodes that we have dated here (Table 1), which we must necessarily compare these results in order to evaluate the validity and the confidence of these estimates. Schulte (2013) proposed a phylogeny for Liolaemidae where he dated the main nodes, including *Liolaemus elongatus-petrophilus* clade (although in this work *L. curis* was included in the group, which was recovered as a member of the *L. bellii* group by Abdala & Quinteros, 2014). Schulte (2013) estimated that the age of the *L. elongatus* group is approximately 16 million years, which is just outside our HDP (10.5 million years, 95 % HPD: 15.6-4.6). Schulte (2013) used several mitochondrial genes for this estimate, although only seven taxa of the *L. elongatus* group were used. He did not include *Cytb* genes in his analysis. Nevertheless, it must be noted that Schulte (2013) did not recover the clades that have been recovered consistently by different authors since Avila et al. (2015), as has been mentioned previously, and were also recovered in our own analysis. As he did not recover the same clades as us, we cannot further compare node estimates. Medina et al. (2014) used terminals mainly within the *L. kriegi* clade to date nodes, using *Cytb* sequences. Their results are largely congruent with ours, albeit she obtains slightly younger nodes for the *L. elongatus-petrophilus*, *L. elongatus-kriegi* and *L. kriegi* clades. Our divergence estimates mostly agree with those of Zheng & Wiens (2016) (see supplementary material therein). Although they did not work with the entire group, their representatives are enough to recover the internal clades of the *L. elongatus* group. Our mean estimates and those of Zheng & Wiens (2016) are close (his estimates are within our 95 % HPD), although they obtain slightly older dates. For example, their estimate for the *L. elongatus* group is 12.3 Mya, while we get only 11.5 Mya. They also date the *L. elongatus-petrophilus* clade at 9.9 Mya, while we get 10.1 Mya. Finally, the most recent work where the nodes

of the *L. elongatus* group are estimated is Esquerré et al. (2019) (see supplementary material therein). The estimates of the main group are within the HPD 95 % range of our work. The dates obtained by Esquerré et al. (2019) are the most similar to ours. The estimates of the main group are within the HPD 95 % range of our work. The dates obtained by Esquerré et al. (2019) are the most similar to ours. Their estimate for the *L. elongatus* group is 13 Mya, while we get only 11.5 Mya. They also date the *L. elongatus-petrophilus* clade at 9.5 Mya, while we get 10.1 Mya. For its sister clade, the *L. punmahuida* clade obtained 2 Mya and we date it in 4 Mya. For the *L. elongatus-kriegi* clade they estimate 5.8 Mya and we obtain 6 Mya. The *L. petrophilus* clade is dated at 8.2 Mya while we date 7.7 Mya. For the sister clades *L. elongatus sensu stricto* and *L. kriegi* they obtain 4 Mya for each, while we date 4.16 Mya and 3 Mya, respectively. Continuing with the comparison, in Esquerré et al. (2019), 95 % HPD ranges are slightly higher than ours. For the *L. elongatus-petrophilus* clade they obtain a range of 13.5 Mya to 6.75 Mya, while our range is 13 Mya to 7.5 Mya, and for the clade *L. elongatus-kriegi* they obtain a range of 8 Mya to 4 Mya and our range is 7.5 Mya to 4 Mya, are some examples. Here we can observe that although the of *L. elongatus* group only represents 9 % of the totality of species included by Esquerré et al. (2019), we find more similarities than differences. At the same time, said differences expressed in longer divergence times as HPD ranges may be due to the amount of information analyzed by Esquerré et al. (2019) that increases the variability of the data with which they worked.

Naturally, the differences in node ages found between our own study and any other must come from a difference either in the methodology the datasets (see Table 1). On the other hand, there are in some cases striking similarities despite these differences, which attests to the robustness of these conclusions. We will consider these differences next.

Schulte (2013) uses a relaxed clock to analyse a large number of taxa and genes. Nevertheless, he only included seven species from the *Liolaemus elongatus* group, which could explain the discrepancies with our own study. Medina et al. (2014), on other hand, uses a strict clock to analyse four species within the inner *L. kriegi* clade. Both the lack of representatives from the *L. elongatus sensu stricto* clade, and the low number of overall representatives of the group could explain the differences in our study, although there is some concordance. Later, in their paper, Zheng & Wiens (2016) analyse a large amount of information (studied Squamata and 52 genes) and eleven representatives of the *L. elongatus* group, but unlike Schulte (2013), they chose to use the penalised likelihood method (Sanderson, 2002), which compensates for the large number of taxa and genes. As mentioned in the previous paragraph, their results are much more similar to ours. Medina et al. (2017) analyse eleven species of the *L. elongatus* group through a relaxed clock to analyse their data. Some species fall within the range of our results. Finally, Esquerré et al. (2019) use a relaxed clock to analyse their

data. Given that they included most species of the group, the ages obtained are largely concordant with ours, our within our ranges.

Ancestral distribution reconstruction

Although currently there is not yet a methodology that is fully accepted to reconstruct biogeographical histories, when there is a complete record of the distribution of all or most of the descendants, and when we have estimates of the divergence of nodes, sometimes patterns can be seen that imply a specific history regardless of the methodology used.

Although GEM analyses have not yet been published, there is a recent study of DEC+j within the *Liolaemus elongatus* group, Esquerré et al. (2019). Although they use more extensive areas, we can find concordance with this work. For example, for Esquerré et al. (2019), the most probable ancestral range for the *L. elongatus* group is what they call the Central Andes or a combination of this area next to Patagonia. While the most probable ancestral range for us is the combination of Monte with Patagonia (Areas C and F respectively; Fig. 3). Here we find a correspondence since the combination of Monte with Patagonia (CF) is part of the distribution that Esquerré et al. (2019) called Patagonia and Central Andes. At the same time, these ancestral ranges (either individual areas or combinations of them) are repeated in the work of Esquerré et al. (2019) and ours for the clades of *L. punmahuida*, *L. elongatus-petrophilus*, *L. petrophilus*, *L. elongatus-kriegi*, *L. kriegi* and *L. elongatus sensu stricto* clades. For the *L. capillitas* clade, Esquerré et al. (2019) locates that the most probable ancestral range is the Altiplanic Andes or the combination of them with the Eastern lowlands, while for us it is the Monte (Area C) or a combination of Monte and Chaco (Areas C and B; Fig. 3). In this case, again the results are concordant since Monte (C) has an area of overlap with the southern part of the Altiplanic Andes and Chaco (B) is part of what is called the Eastern lowlands.

When analysing that the DEC+j studies were quite similar, we can compare this with GEM. DEC+j uses predefined areas as a starting point, while GEM uses species records mapped on a grid. The immediate consequence of this, as can be seen by our results, is that the ancestral areas found by GEM, not being constrained to the user-defined areas, are a lot smaller, and only cover a small portion of the DEC+j areas. Despite this difference, it can be said that in general, GEM does not directly contradict DEC+j, and instead can be seen as a more specific result of what DEC+j shows. The ancestral area for the *Liolaemus elongatus* group obtained by GEM, a portion of northern Patagonia, is part of the ancestral area obtained by DEC+j and by Esquerré et al. (2019). The same can be said of the ancestral areas of *L. elongatus-petrophilus*, *L. punmahuida*, *L. elongatus-kriegi* clades, although as mentioned above, they are actually much smaller areas. On the other hand, as we look at these nodes, we note that the ancestral areas obtained by GEM are much better fitted to elucidate events happening at those nodes. For example, GEM

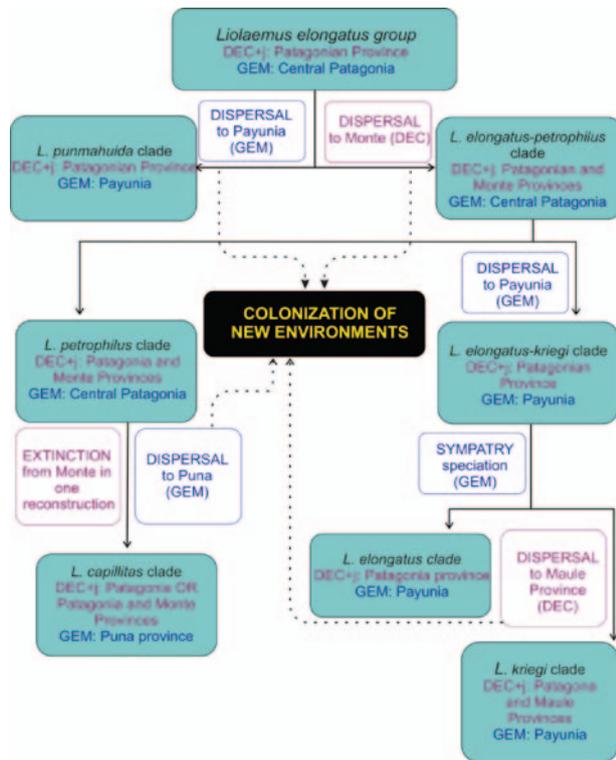


Figure 4. Event flow chart. The main speciation and extinction events, according to our DEC (blue letters) and GEM (red letters) analyses. Solid arrows represent the relationships between clades and dotted arrows indicate the colonisation of new environments.

these lizards to form biological barriers in the absence of geographical ones. There are some studies which show evidence of sympatric speciation (Losos et al., 1998), or sympatric divergence (Huyghe et al., 2010), therefore it would be interesting to see if there are more examples of this kind of speciation in lizards.

Geological and biogeography implications

The historical biogeography of the *Liolaemus elongatus* group provides information about the effect of geological events on its diversification patterns, revealing a complex interaction between dispersion and vicariance. According to our estimates, the *L. elongatus* group began its diversification at a time when the Andes entered its final uplifting phase. During this period (11-3Mya), the Paranaense sea began to retreat, leaving behind plains, which were empty niches ready to be exploited. During this period, the Andes reached its current height and became a barrier to moisture coming from the west, which transformed the areas to the east of the Andes in steppe biome (Donato et al., 2003). Meanwhile, Patagonia is likely to have started experiencing a process of desertification at approximately 16.5 Mya as a result of the Andean uplift (Stern & Blisniuk, 2002), with the severity of this process increasing at approximately 14 Mya (Blisniuk et al., 2005), when a new tectonic pulse increased the efficiency of the orographic effect, limiting the incoming humid winds from the southern Pacific Ocean (Ramos & Ghiglione, 2008). This process generated a marked west–east climatic gradient similar

to the one that characterises present-day Patagonia east of the Andes (Martínez & Kutschker, 2011). This new panorama, in which the cooling and drying of the extra-Andean Patagonia generated the disappearance of the last megathermal taxa and together with an increasing diversity and abundance of xerophytic adapted taxa as pastures (Barreda & Palazzesi, 2007). This set of events facilitated the diversification of the *L. elongatus* group, since when megathermal taxa and potential predators disappeared, small animals such as lizards had greater chances of survival and conquest of new niches. The outcrop of volcanoes east of the Patagonian Andes also facilitated the isolation of populations that at the same time confronted altitudinal changes. All this can explain the unusual number of lizard species in the region (Avila et al., 2013; Corbalán et al., 2011). At least half of the lizard species in the Patagonia area are endemic (Corbalán et al., 2011), and has been proposed as the centre of origin for several lizard genera (Lamborot & Díaz, 1987; Cei & Videla, 2003; Scolaro et al., 2003; Díaz Gómez, 2009). In support of this hypothesis, according to a recent paper by Esquerré et al. (2018) of the Liolaemidae family, the speciation rates have been much higher in the Andes or in adjacent areas. They also found a strong correlation between diversification rates in liolaemids and rising altitudes. They provide a simple explanation for this: the elevation of the Andes provides what they call “sky islands”, that is mountain tops become a novel niche to be inhabited, and at the same time, these become isolated among themselves, as they are surrounded by different environments. The *L. elongatus* group suffered two major speciation peaks during its history (Unpublished data), one corresponds to the Late Miocene and another during the Pleistocene. This corresponds with events described for the Miocene and for a series of events that determined the limits of the distribution of this group of saxicolous lizards. The northern end of the *L. elongatus* group distribution is given by *L. heliodermis* with a possible origin at 1 Mya and whose type locality is found in Tafí del Valle (Tucumán, Argentina). During the Middle and Upper Pleistocene, the Tafí del Valle Formation was formed (Collantes et al., 1993, Sayago et al., 1998). At the same time, in the Quebrada de las Conchas, to the south center of Salta province (located just north of Tafí del Valle), pleistocene formations composed of sand and gravel were defined and no paleontological record is known (Frenguelli, 1936). At an estimated age for the Upper Pleistocene-Lower Holocene, the sedimentary environment of this unit is interpreted as a lake of shallow depth and low salinity, with stages of greater evaporation (Salfity & Monaldi, 2006). The presence of a lake and the rapid formation of Tafí del Valle could explain why *L. heliodermis* has not expanded its distribution range and today is only found in its type locality. On the other hand, there are species which are widely distributed, occupying a range that includes high and low sections of the Andes, such as *L. kriegi* (Patagonia and Maule Province), although they might prove to be in fact a complex of species, including misidentified populations which in fact correspond to

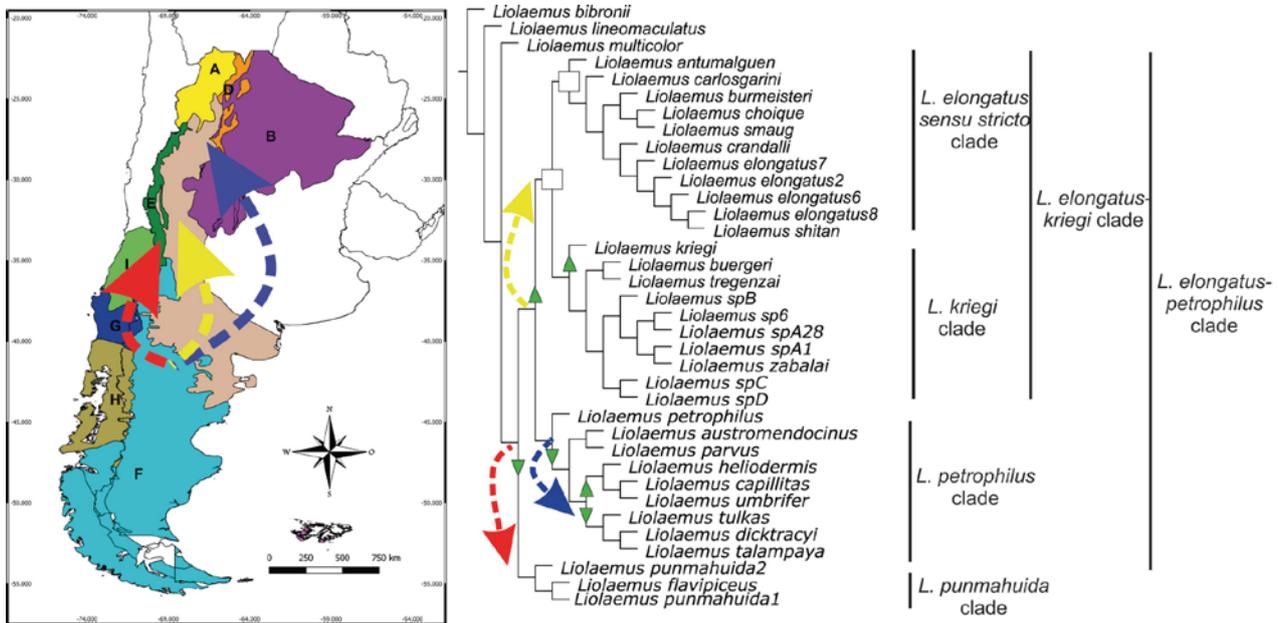


Figure 5. GEM reconstruction. The speciation events in the main clades recovered from GEM are shown here. The green arrow indicates the occurrence of a founder event in the direction of the arrow (i.e. the clade not pointed at inherits the ancestral range). The white square at the node indicates a sympatry event (i.e. both clades inherit the ancestral range). In addition, the events are superimposed on the map of predefined areas used for DEC + j.

novel species or it could be that *L. kriegi* had a recent diversification according to our results and we could be talking about a widely distributed polymorphic species. GEM shows support for this hypothesis in the *L. petrophilus* and the *L. punmahuida* clades, the ancestors of which speciated by founder event from central Andes to Payunia, occupying higher lands.

We find that our results are congruent with many of the previous analyses made on the group. Cei (1979) mentioned in his book the possibility that Patagonia served as a dispersal centre for *Liolaemus* lizards. Therefore, we find it interesting that previous works have found Patagonia as the ancestral area for Liolaemidae, and for groups within Liolaemidae (Díaz Gómez & Lobo, 2006; Portelli & Quinteros, 2018). Similarly, in this study we find that Patagonia, is the ancestral area for the *L. elongatus* group, and for clades within (*L. punmahuida*, *L. elongatus-petrophilus*, *L. petrophilus* and *L. elongatus-kriegi* clades; Fig. 3), which would give further supports to Cei's claims. This can also be seen in Díaz Gómez & Lobo (2006), who obtains similar ancestral areas to ours (northern Patagonia, an area which they call "Andes"). This pattern is also appreciated in a related group of lizards, which share a similar lifestyle: the saxicolous genus *Phymaturus*. In fact, many species of the *L. elongatus* group and *Phymaturus* are found syntopically (for example: *Phymaturus tromen* with *Liolaemus punmahuida*; *Phymaturus verdugo* with *Liolaemus thermarum*; *Phymaturus sitesi* with *Liolaemus crandalli*; *Phymaturus lobo* with *Liolaemus carlosgarini*; *Phymaturus denotatus* and *Phymaturus tromen* with *Liolaemus umbrifer*, among others). According to Esquerré et al. (2019), these two groups must have shared much of their history, as *Phymaturus*

was calculated to be 16 million years old, while the *L. elongatus* group was 12.3 million years old (11.2 in this work). Although, the divergence time is not exactly the same, a correspondence can be observed between the diversification of the palluma group and patagonicus group (members of the genus *Phymaturus*) with the clades of *L. punmahuida*, *L. capillitas*, *L. kriegi* and *L. elongatus sensu stricto* because their diversification would have begun during the Pliocene. We can even find a correspondence between the possible ancestral ranges of some clades. For example, in Esquerré et al. (2019) found that the most probable ancestral range for the palluma group corresponds to the Central Andes or a combination of these with Patagonia that corresponds to our results that show that the most probable ancestral range for the *L. capillitas* clade would be in Monte (C) or a combination of it with Chaco (CB). In support of this hypothesis, both groups have representatives west of the Andes, distributed mostly in southern areas, between 34°-38° latitude, and they share most of their distribution range in the east of the Andes (between 26°-43° latitude).

In spite of the coincidences, we must take into account that by using smaller predefined areas (DEC) than previous studies, as well as by using explicit occurrence data (GEM), our level of detail for each of the ancestral ranges is greater. Therefore, it is easier to assess possible geological events that might have caused the speciation in said areas. Having greater detail of these ancestral ranges, we were able to integrate the analyses into a geological context, which generated a hypothesis for the diversification of the group. This is the first time that such hypothesis, with these level of detail, were produced for the group.

Finally, we can conclude that, although implementing

two different methodologies, we were able to see a similar sequence of events that explains the current distribution of the *Liolaemus elongatus* group. Furthermore, our results are largely consistent with previous studies (at the level of inner clades), but our results expressed with greater detail for the *L. elongatus* group. We believe our study can be used as a basis for future studies within each clade and/or sister species.

For both methodologies, the ancestral area of the *Liolaemus elongatus* group is located in the center of Argentina, more specifically, in the Patagonia region. The diversification of the *L. elongatus* group would have begun along with the final phase of the Andes uplift, with the founding events prevailing over others such as vicariance. All this set of events determined the current distribution of the group, which is why we currently find representatives of the group in the east and west of the Andes.

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Snakes and the Eternal City: variation in community metrics, body size and population density of snakes in contrasted landscapes of suburban Rome (Italy)

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Reports of global declines in animal populations are now numerous and also include snakes, a group of animals now widely regarded as bio-indicators. A prerequisite for any conservation management plan to protect or restore snake populations requires a data base that provides insight into population composition and changes. However, snakes are well known to be particularly difficult to quantitatively sample due to their secretive and elusive nature, and hence accumulating an adequate database for analysis requires long-term field studies that involve intensive searches. Populations of four snake species, *Zamensis longissimus*, *Natrix helvetica*, *Vipera aspis* and *Hierophis viridiflavus* living in two suburban areas of Rome with different extents of habitat alteration (deforestation), Vejo, a less altered site, and Tor Bella Monaca, a high altered site, have been monitored, but with interruptions since 1995. The results indicated that *H. viridiflavus* was the commonest species at both sites. Male bias was found in all four species but especially in *Z. longissimus* and *V. aspis* with detection of juveniles greatest in *H. viridiflavus* and *N. helvetica*. Snout to vent lengths (SVL) of *H. viridiflavus* and *Z. longissimus*, which were present at both sites, were greater at the less degraded habitat of the two study localities. Community metrics indicated that the degraded habitat had lower species richness, evenness, Shannon and Simpson diversity indices, but a higher dominance index. Recapture frequencies of snakes recaptured either once or multiple times were in general greater at Vejo. The highest population densities were found in *H. viridiflavus*, followed by *V. aspis* and *N. helvetica*, which were similar. However, long term trends in densities show declines in *V. aspis* and *N. helvetica* between 1995 and 2019. Population densities were in good agreement with density estimates found in previous studies of snakes in more natural habitats.

Keywords: snakes, long-term population changes, suburban areas, Rome, Italy

INTRODUCTION

Snakes are an important component of natural ecosystems regulating prey numbers such as rodents, or as prey species themselves (e.g. Greene, 2000). Recent reports of their and other reptiles' global decline are of major concern (e.g., Gibbons et al., 2000; Reading et al., 2010; Goiran & Shine, 2013), particularly as snakes have been defined as indicator species (Landres et al., 1988) for assessing the health of ecosystems. Indeed, research has suggested that predictions of snake extinction risk are underestimated (Böhm et al., 2013), but to understand the level of risk requires data on snake presence,

demography and densities in a variety of different habitats over long time periods (e.g., see Cayuela et al., 2019). Mark, release and recapture (MRR) is a well-known and a frequently employed method of estimating animal numbers and often applied to snakes (e.g., Flatt et al., 1997; Bonnet et al., 2002; McDiarmid et al., 2011) but usually in natural and well-preserved environments (e.g., Webb et al., 2002; Stevenson et al., 2009; Cayuela et al., 2019). However, MRR presents logistical challenges since snakes are usually cryptic, secretive reptiles that are difficult to detect and identify (Kery, 2002; Willson et al., 2011), thus presenting considerable problems when attempting to accumulate an adequate data base for analysis (MacKenzie et al., 2002). Of interest and of

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particular value are snake densities in patches of suitable habitat within and around large cities (Burger et al., 2004; Pattishall & Cundall, 2008), where snakes may form small and isolated subpopulations with little genetic exchange between the various demes, and where inappropriate management of the “green areas” may compromise their survival (e.g. Vignoli et al., 2009; Bonnet et al., 2016). These patches of suitable habitat within large cities are arguably the most rapidly changing environments and the fragmented landscapes they produce have been cited as a major cause of loss of biodiversity (e.g. Fischer, 2000) by changing levels of predation risk (e.g. Evans, 2004), altering the thermal environment (Zhou et al., 2011) and reducing species abundance (Bateman et al., 2014). Therefore, it is reasonable to expect populations in these areas to experience the most rapid changes. However, few studies of reptiles have been carried out in these areas, especially in temperate regions (Capula et al., 1993; Rugiero & Luiselli, 2006; Luiselli & Rugiero, 2007; Simbula et al., 2019), including those of snakes (Tóth et al., 2002; Vignoli et al., 2009). Increasing our knowledge of snake population ecology in suburban areas is a key factor for effective management of their populations to enhance their survival probabilities.

The city of Rome, founded in the 8th century BC, has at present a population of around 4 million people (about 6 million if peripheral areas are included). The first major increase in population and expansion into surrounding areas that we know of began around 200BC and by the time of the first Emperor Augustus in 30BC Rome was the world’s most populous city with an estimated one million or more inhabitants (e.g. Lo Cascio, 1994). The metropolitan expansion, after the end of the World War II was rapid with a resultant massive habitat loss and deforestation in the peripheral zones. This includes localities that once held dense snake populations, which were well known historically as indicated by the toponomastic (place name), for instance the “Serpentara” area because of the reported high snake densities. Rome, therefore, represents a prime region for examining snake presence and numbers in suburban areas with very few previous published studies (e.g. Vignoli et al., 2009). Additionally, recent research has shown that, in an area situated about 50 km north of Rome, changes in local climate (increases in annual temperatures) may be a factor in the decline of aspic viper (*Vipera aspis*) at hibernation dens (Luiselli et al., 2018), and it is therefore conceivable that suburban snake populations in the locality may have experienced similar declines. In this paper we present the first long term population study of snakes in urban environments in two areas of the city: La Storta (hereby VEJO) and Tor Bella Monaca (TBM). This information will not only increase our present understanding of suburban snake populations that were known during antiquity, but will serve as a data base to understand potential impacts of habitat alteration and climatic changes.

The main objectives of the study were:

(i) to estimate species’ presence, population densities, and general demographic aspects (sex ratio, body size, etc) of snakes in Rome’s suburban areas with contrasted habitat characteristics (a well-preserved forest-

cultivation mosaic area versus a totally deforested and heavily degraded area).

(ii) to determine long-term population trends, i.e. identify any decreases in population densities as reported for other snake populations throughout the world (Reading et al., 2010).

Specifically, we investigate the following key questions:

(1) Are community metrics (diversity and dominance) different between the two study areas? We predicted that community evenness should be higher in the well-preserved habitat at VEJO but that species dominance should be higher in the degraded TBM habitat (Magurran, 1988).

(2) Are the body sizes of snakes greater in the well-preserved VEJO habitat in comparison to the degraded habitat at TBM? We predicted that the average body size of snakes should be greater in the well-preserved environment due to greater abundance, diversity and density of prey species, particularly small mammals and saurians.

(3) Is the inter-annual variation in mean body size lower in the well-preserved habitat in comparison to the degraded habitat? We predicted that snake body size should be less prone to inter-annual oscillations in the higher quality habitat due to greater diversity of potential prey species. This should minimise the impact of any simultaneous collapse of prey populations in certain years.

(4) Are population densities declining with time greater in the degraded site than in the well-preserved site? We predicted that, because of heavy habitat loss, the snake populations in the degraded site may be more prone to declines than in the well-preserved site.

METHODS

Taxonomic note

The European herpetological fauna has undergone remarkable taxonomic changes during the last decade. Therefore, in this paper we followed the nomenclatural options as in Di Nicola et al. (2019).

Study areas

Surveys were carried out at two localities situated in the peripheral areas of Rome (Latium, central Italy): Tor Bella Monaca (TBM; 45 ha surveyed) on the north-eastern side of Rome and La Storta (VEJO; 55 ha surveyed), situated just north of the main ring road (Grande Raccordo Anulare). TBM is the altered/degraded site in the present study. It is a highly urbanised suburb in the eastern region of the city and the study site is primarily composed of two narrow strips of hedgerow (less than 10 m width and several hundreds meters length), bisecting wide agricultural land bordered mostly by light suburban areas (Fig. 1A). The primary habitat at VEJO is well preserved and composed of sections of oak woodland interspersed with agricultural land bordered by urban areas (Fig. 1B). Roads that interrupt habitat continuity bisect both VEJO and TBM. The list of potential prey species for snakes (small mammals, saurians and amphibians) at the two

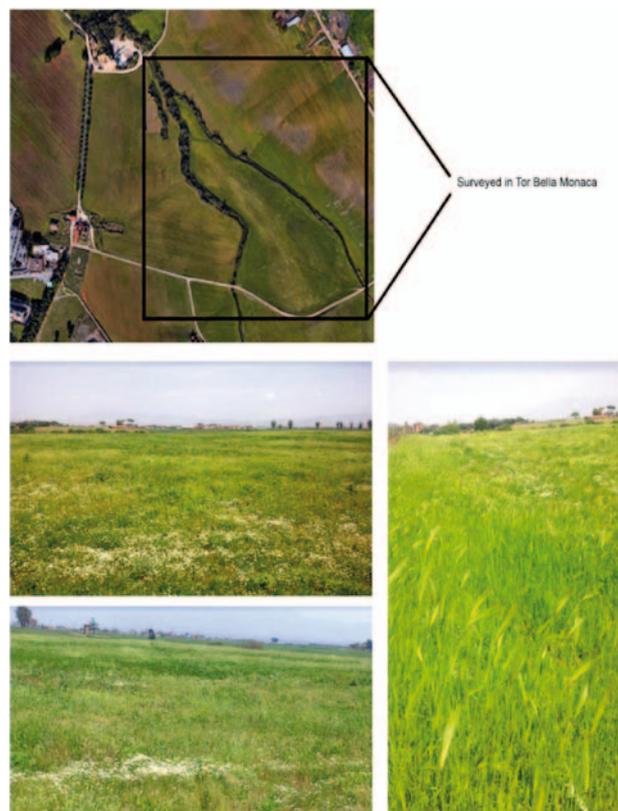


Figure 1A. Google Earth images of the study site at Tor Bella Monaca with photographs illustrating the basic habitat structures.



Figure 1B. Google Earth images of the study site at Vejo with photographs illustrating the basic habitat structures.

study areas is given in Table 1 (bibliographic and personal data being pooled). The available data showed that VEJO has a considerably higher diversity and richness of prey species than TBM (19 versus 12 prey species).

Protocol in the field

In Mediterranean climates, it is frequently the case that snakes cannot be found during summer months (June–September) due to a combination of high temperature ($>30^{\circ}\text{C}$) and prolonged periods of very low rainfall (Brito, 2003; Capula et al., 2014; Luiselli et al., personal observations) and so field-work was confined mostly to spring/early summer and autumn. Surveying varied between (CET) 0855 and 1745 hours. In total, 30601 minutes (510 hrs) were spent in the field searching for snakes at TBM and 34483 minutes (574.7 hrs) at VEJO, with a total of 3–4 field workers per season. Detection was through visual encounter by walking along both sides of the various hedgerows available at the study areas, in addition to walking within the cultivated land and the grasslands. When walking alongside the hedgerows, a distance of 2 m was maintained to minimise disturbance to encountered snakes. Snakes were individually marked by ventral scale-clipping and temporarily dorsally painted with a white number enabling the surveyors to identify individual snakes that were already marked, thus avoiding further pointless recaptures that may increase wariness in the snakes (Fig. 2). Each identified snake was sexed (by examination of the shape of the cloacal region and the tail), and measured for snout-vent-length (SVL), to ± 0.5 cm precision.

The results are derived from data using mark, release and recapture (MRR) commencing at VEJO during 1995, 1999, 2012 and 2019. At TBM, the MRR was carried



Figure 2. Examples of marked *H. viridiflavus* additionally painted with a white number on the back to minimise capturing of already marked individuals.

Table 1. List of the potential prey species available for snakes (small mammals, saurians and amphibians) recorded at the two study areas (bibliographic and personal data being pooled).

	Tor Bella Monaca	Vejo
Amphibians		
<i>Bufo bufo</i>	0	1
"green frogs"	0	1
<i>Rana italica</i>	0	1
Saurians		
<i>Chalcides chalcides</i>	1	1
<i>Anguis veronensis</i>	0	1
<i>Podarcis muralis</i>	0	1
<i>Podarcis siculus</i>	1	1
<i>Lacerta bilineata</i>	1	1
<i>Tarentola mauritanica</i>	1	1
<i>Hemidactylus turcicus</i>	1	1
Small mammals		
<i>Suncus etruscus</i>	1	1
<i>Crocidura</i> spp.	1	1
<i>Talpa romana</i>	1	1
<i>Microtus savii</i>	1	1
<i>Myodes glareolus</i>	0	1
<i>Apodemus</i> spp.	1	1
<i>Rattus norvegicus</i>	1	0
<i>Rattus rattus</i>	0	1
<i>Mus musculus</i>	1	1
<i>Muscardinus avellanarius</i>	0	1
TOTAL	12	19

out during 1998, 1999, 2000, 2012 and 2019. Logistical constraints impeded us to monitor the snake populations at the two study areas without temporal interruptions.

Statistical analyses.

Snake numbers at each site and recapture frequencies were evaluated using z-scores for differences between proportions p with the null hypothesis H_0 ; $p - p = 0$ at $\alpha = 0.05$. To compare snake abundance G -test goodness of fit tests were applied to the actual numbers caught and marked with the null hypothesis of equality of numbers the expected proportions at $\alpha = 0.05$. Snout to vent lengths (SVL) were used to compare population parameters at different sites by ANOVA with post-hoc tests Tukey HSD for unequal sample sizes. This compares all possible pairs of mean values within and between sites with $\alpha = 0.05$. Before analysis, normality tests were made using Anderson-Darling (a^2) for all data, male, female and pooled. This indicated most male and female data sets fitted a normal distribution (a^2 values from 0.17-0.54, P from 0.09 – 0.87) except in two of the four pooled data sets that were non-normally distributed, TBM 1998 ($a^2 = 0.85$, $P = 0.02$) and Vejo 2012 ($a^2 = 0.79$, $P = 0.03$). Hence, these data sets were \log_e transformed prior to analysis.

Capture-mark-recapture analyses for estimating the abundance of the snake population at the two study areas were performed using methods for closed populations, with each sampling year treated as a distinct entity in the

analyses ($n = 4$ years at VEJO and $n = 5$ years at TBM). A population is defined as "closed" when it has a fixed size over the period of study, with no death, birth, immigration or emigration within the study area (Jolly, 1965; 1982). This assumption offers advantages, but can only be justified if the study is undertaken over a brief period of time. Considering the relatively long life of Mediterranean snakes (> 10 years; Fornasiero et al., 2016), we consider that temporal segments of 1 year are sufficiently short to justify the use of closed population models. In the analyses, we assumed constant natality and conflation of survival and emigration (sensu Rosewell & Shorrocks, 1987; Gilroy et al., 2012) for all snake species, at both study areas.

We used five different demographic models:

(1) 'Equal Catchability (M_0)' (Pollock et al., 1990), or null model. This demographic model assumes that the probability of capture during the course of the study is the same for all individuals of the population.

(2) the Petersen-Schnabel method (Krebs, 1999). This method is based on the Petersen method (or Lincoln index), a simple mark-and-recapture method based on a single episode of marking animals and a second single episode of recapturing individuals. Schnabel (1938) extended the Petersen method to a series of samples.

(3) 'Chao temporal change in capture probabilities (M_t)' (Chao, 1988). This demographic model assumes that the probability of capture of each individual is influenced by temporal parameters.

(4) 'Heterogeneity Model (M_h)' (Chao, 1988). In this demographic model, every individual of the sampled population has a different chance of being captured that is constant for all capture sessions (Pollock et al., 1990), that is determined by parameters such as sex and age.

(5) 'Both individual and temporal differences in capture probability (M_{th})'. This demographic model assumes that the probability of capture varies depending on the temporal parameters and individual parameters (Chao et al., 1992).

Demographic analyses were carried out by Simply Tagging software (version 1.31; see Henderson & Seaby, 2002).

Diversity metrics of the snake assemblages between study areas were assessed by calculating various distinct univariate measures of community diversity for each site (Magurran, 1988):

1. Species richness; the total number of species recorded in each study area;

2. Dominance:

$$D = \frac{\sum_i n_i(n_i - 1)}{n(n - 1)}$$

where n_i is the number of individuals of the taxon i and n is the total number of snakes that were recorded at each study area;

3. Simpson index: $S = 1 - D$, where diversity D lies between 1 and 0.

4. Shannon entropy index (Shannon & Weaver, 1963):

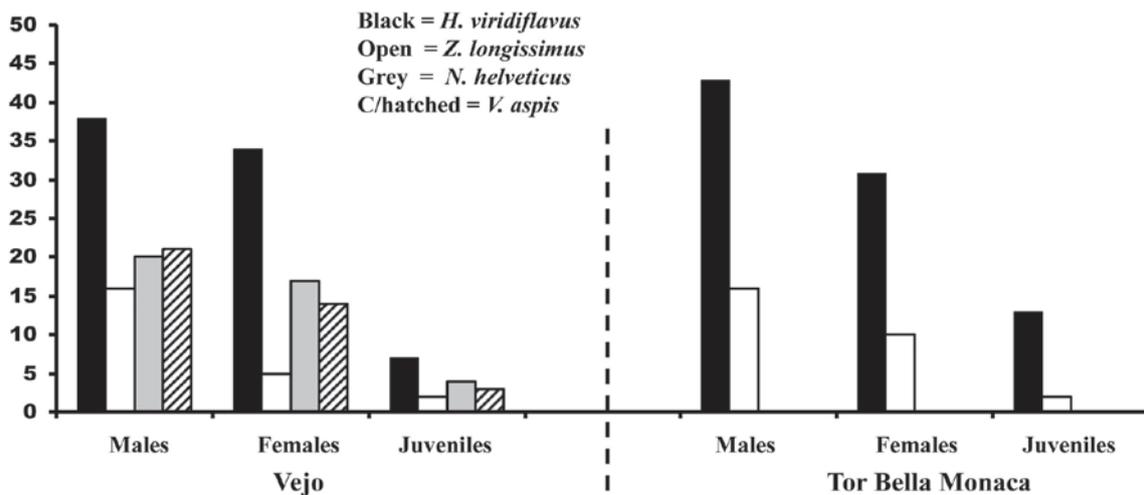


Figure 3. Histograms of total numbers of marked male, female and juvenile snakes found at both study localities.

$$H = -\sum_i \frac{n_i}{n} \ln \frac{n_i}{n}$$

where n_i is the number of individuals of each species at each site and n is the total number of snakes that were recorded at each study area.

5. Evenness, calculated by Buzan and Gibson's formula:

$$e^{H/S}$$

with H' representing Shannon's index, and S the total number of snake species observed at each study area (Magurran, 1988).

6. Chao-1 index, indicating the theoretical number of species at each study area that can be expected on the basis of the sampling regime. The formula of the unbiased Chao-1 estimates is,

$$\text{Chao-1} = S + F_1 (F_1 - 1) / [2 (F_2 + 1)]$$

where F_1 is the number of singleton species and F_2 is the number of doubleton species at each study area.

Bootstrap analysis was applied to generate upper and lower confidence intervals of all indices based on 9,999 random samples, each with the same total number of individuals as in each original sample being generated (Harper, 1999). Diversity metrics analyses were carried out by Past 3.0 software. Apart from the demographic and diversity indices analyses, all other statistical tests were performed with Minitab (V17) software and use of various websites. All tests were two-tailed with alpha set at 5%.

RESULTS

Sample sizes and community metrics

In total 298 snakes were captured and marked during the study period, 115 at TBM and 183 at VEJO. Three species

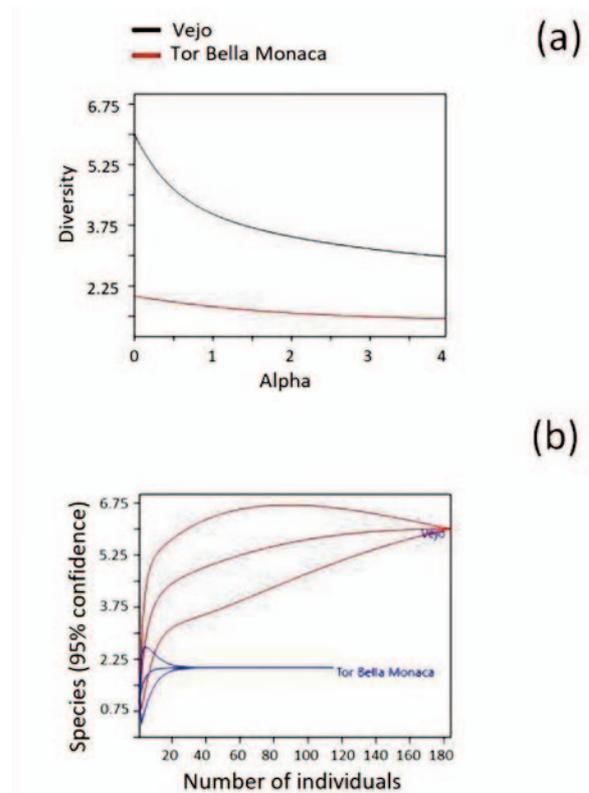
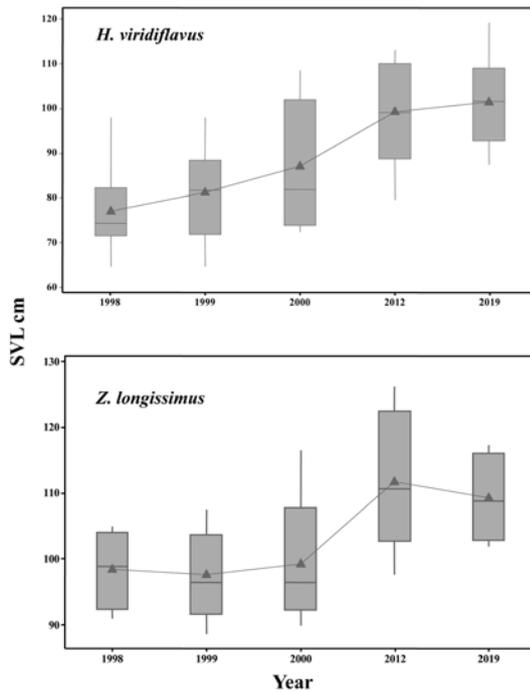


Figure 4. Diversity profiles (a) and saturation curves (with 95 % confidence intervals after 9,999 bootstraps) (b) for community diversity of snakes at the two study localities in suburban Rome.

were found and marked at TBM (*Hierophis viridiflavus* ($n = 87$), *Zamenis longissimus* ($n = 28$)) with *Natrix helvetica* also recorded but not used in the analysis due to low sample size. At VEJO we found and marked *Vipera aspis* ($n = 40$), *H. viridiflavus* ($n = 79$), *Z. longissimus* ($n = 23$) and *N. helvetica* ($n = 41$). *Elaphe quatuorlineata* and *Coronella austriaca* were also found but not used due to low sample size. Therefore adequate sample sizes for analyses were only possible for *H. viridiflavus* and *Z. longissimus* at TBM, and *V. aspis*, *H. viridiflavus*, *Z.*

Table 2. Diversity metrics for the snake communities at the two study areas. Upper = 95 % upper confidence limits; Lower = 95 % lower confidence limits. Confidence limits are calculated after 10,000 random Monte Carlo permutations

	Vejo	Lower	Upper	TBM	Lower	Upper
Species richness	6	5	6	3	2	3
Individuals	184	184	184	115	115	115
Dominance	0.288	0.2587	0.3325	0.6316	0.5636	0.7242
Simpson (1-D)	0.712	0.6674	0.7413	0.3684	0.2758	0.4364
Shannon (H)	1.394	1.284	1.473	0.5551	0.4482	0.6282
Evenness (e ^H /S)	0.6715	0.6099	0.7611	0.871	0.7828	0.9371
Chao-1	6	5	7	3	2	3

**Figure 5.** Boxplots of SVL's of marked snakes during different study periods at Tor Bella Monaca showing increases in SVL's of both *H. viridiflavus* and *Z. longissimus*. Top graphic shows *H. viridiflavus* and lower *Z. longissimus*. Triangles are mean SVL's, boxes represent the interquartile ranges with the ranges the vertical lines either side of the interquartile ranges.

longissimus and *N. helvetica* at VEJO.

Pooled data for all years indicated *H. viridiflavus* was the most abundant species at both sites; 76.6 % of the total at TBM ($G = 32.5$, $p < 0.0001$) and 44.1 % of the total of the four species at VEJO ($G = 36.1$, $p < 0.0001$). Among the species with adequate sample sizes, the least found was *Z. longissimus*, which formed 12.8 % of the samples at VEJO and 23.9 % at TBM.

Pooled data from all years was used to evaluate sex ratios (Fig. 3) and indicated males were consistently found in greater numbers than females in all species and at both sites. Closest to sex ratio equivalence was *N. helvetica* (1.17:1) and *H. viridiflavus* (1.44:1 at TBM & 1.12:1 at VEJO) with greater male bias in *V. aspis* (1.5:1) and especially *Z. longissimus* (1.75:1 TBM & 3.2:1 VEJO). As expected, due to greater difficulty in detection, juveniles were found in fewer numbers than either

males or females in all species and at both sites. Highest juvenile to adult ratios were found in *H. viridiflavus* (15 % at TBM and 8.9 % at VEJO) compared with juvenile *Z. longissimus* 4.3 % at TBM and 8.7 % at VEJO. However, generally adult/juvenile ratios were similar; at VEJO, juveniles formed 10.8 % of the *N. helvetica* sample and 7.3 % of *V. aspis*.

Analysis of community metrics indicated TBM had lower species richness, evenness, Shannon and Simpson diversity indices than VEJO, but a higher dominance index value (Table 2). Diversity profiles revealed that the VEJO snake community was significantly different from the TBM snake community (Fig. 4a). Saturation curves revealed that both communities were satisfactorily sampled throughout our field study (Fig. 4b).

Population SVLs

Adult snake SVLs at the two study areas, by year and by sex, are presented in Table 3 (TBM) and Table 4 (VEJO). Inter-site comparison of adult snake SVL was only possible in *H. viridiflavus* and *Z. longissimus*, which were present at both sites. The results indicated both species had greater SVL's at VEJO (ANOVA and Tukey post hoc; *H. viridiflavus* $F_{(9, 136)} = 12.03$, $P < 0.0001$, *Z. longissimus* $F_{(8, 33)} = 4.5$, $P = 0.001$).

Temporal variation in the mean SVL are given in Fig. 5 (TBM) and Fig. 6 (VEJO). At TBM, *H. viridiflavus* SVL increased by a total of 24.8 % and 11 % in *Z. longissimus*. Over the longer term, SVL's at VEJO were relatively stable between 1995 & 2019 in both *H. viridiflavus* (3.1 % decrease) and *Z. longissimus* (7.2 % decrease) with maximum differences of 8.2 % in *H. viridiflavus* (between 1995 and 2012) and 10.2 % in *Z. longissimus* (between a 1999 maximum SVL and 2019 minimum). In *V. aspis* there was an overall decline in SVL during the same sampling periods (mean SVL's from 67.5 to 58.9 cm; 12.7 % decrease) and in *N. helvetica* from 92.1 to 80.2 cm (12.9 % decrease).

Adult SVLs of *H. viridiflavus* were significantly different during the period of study at TBM ($F_{(4, 69)} = 16.48$, $P < 0.0001$). Tukey post hoc tests indicated SVLs during 2012 and 2019 were significantly greater than 1998, 1999 and 2000. SVLs of *Z. longissimus* also increased significantly during the same time period ($F_{(4, 24)} = 3.74$, $P = 0.02$) with the groupings from the Tukey analysis indicating SVLs were significantly higher in 2012 and lower in 1999.

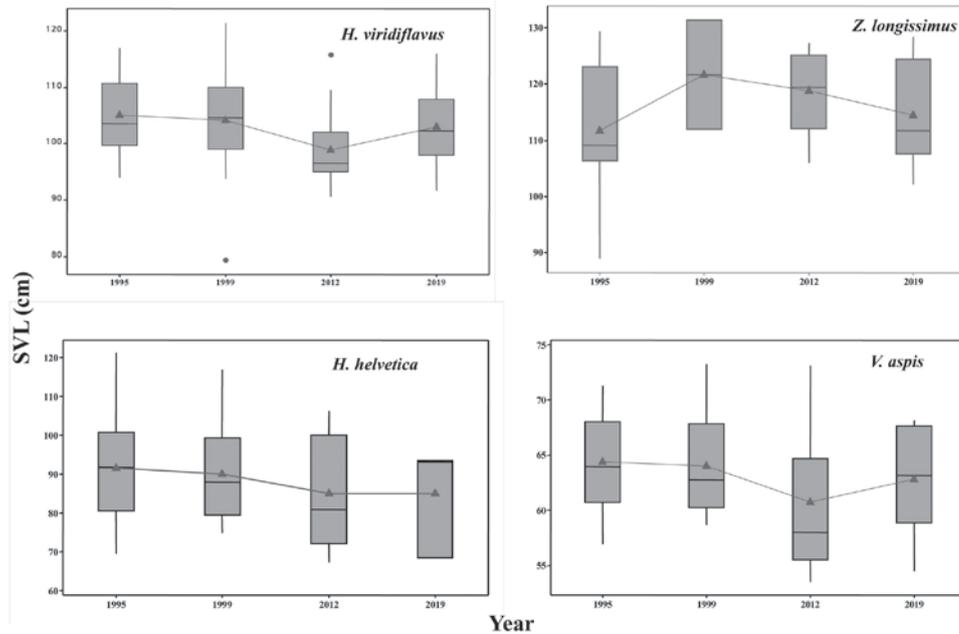


Figure 6. Boxplots of SVL's of marked snakes during different study periods at Vejo showing general SVL stability in all four species. Triangles are mean SVL's, boxes represent the interquartile ranges with the ranges the vertical lines either side of the interquartile ranges, horizontal lines are medians. Circles in the graph for *H. viridiflavus* are outliers. See text for further details.

Recapture frequencies and population size estimates

Recapture frequencies (Figs. 7 for TBM & 8 for VEJO) were in general greater at VEJO (66.3±8.5 %; 4 species) and highest in *V. aspis* (75 %) and *N. helvetica* (73 %). At TBM mean recapture rate was 25±12 % (2 species) with *H. viridiflavus* more frequently recaptured than *Z. longissimus* (37 % versus 13 %; $z = 3.09$, $P = 0.002$; Fig. 7). Comparisons of pooled recapture data from both sites indicated the differences between VEJO and TBM were significant ($z = 5.8$, $P < 0.001$). Intra-specific comparisons indicated multiple recaptures (snakes that were recaptured more than one time) were also higher at VEJO in both *H. viridiflavus* (32 versus 12.5 %; 2-tailed P , $z = 3.1$, $P = 0.002$) and *Z. longissimus* (38.1 versus 3.3.1 %; $z = 3.06$, $P = 0.001$; Fig. 8).

Short-term analysis at TBM.

In 3 consecutive years of sampling at TBM (1998, 1999 & 2000) initially 8 males, 4 females and 3 juvenile *H. viridiflavus* were marked during 1998 (111.8 hours searching). In 1999, 4 of these (3 males, 1 female (27 %) were recaptured along with 10 previously unmarked snakes (3 males, 5 females, 2 juveniles; 101.65 hours search). Of 25 snakes now marked (1998+1999), none were recaptured during 2000 (73.1 hours search effort) but 12 (6 males, 5 females, 1 juvenile) new individuals were captured. During the same time period (and hours of search effort) at TBM, 4 (3 males, 1 female) *Z. longissimus* were marked in 1998. Two of the males were recaptured in 1999 along with 6 new captures (3 males, 2 females, 1 juvenile). None of the marked snakes from 1998/1999 were found in 2000 although 5 (2 males, 3 females) new snakes were found along with one deceased individual. Recaptures at TBM showed similar within-year recapture rates for male (38.1 %) and female

(33.3 %) *H. viridiflavus* ($z = 0.293$, $p = 0.38$). Female *Z. longissimus* were recaptured more frequently than males (33 versus 20 %) but the sample size was small and the result not significant ($z = 0.60$, $p = 0.27$).

Population density estimates.

Tables 5A & B show the full results for the demographic models applied to snake captures and recaptures in the two study localities; Figure 9 shows graphical summaries. Population density and standard error estimates from the 5 models were in remarkably good agreement at VEJO. In general, the highest densities were for *H. viridiflavus* (mean±std dev of 5 model estimates: 25.4±9.7 snakes in the 55 ha study locality). Numbers of *V. aspis* and *N. helvetica* were similar, 12.1±4.9 and 12.1±4.8 respectively. Lowest estimates were found in *Z. longissimus* (8.1±0.7). However, we believe this may in part be due to the very low capture rates in this species due to its more secretive behaviour, which rendered 1999 data insufficient to make a robust analysis. The trends in densities showed a 65 % reduction in *V. aspis* and 73 % in *N. helvetica* between the 1995 and 2019. Smaller declines were found in *Z. longissimus* (12.2 %) and *H. viridiflavus* (5.4 %) but these are what might be expected under natural population fluctuation.

The TBM (45ha) estimates showed less agreement between models with error rates on combined estimated mean values varying from 2.5 to 30.6 % and incomplete in three of 5-year estimates for *Z. longissimus*. Again, data for the latter species is probably due to low initial number of marked snakes and especially recaptures. The estimates for *H. viridiflavus* varied between years with mean estimates of the five models ranging from 6.2 snakes in 2012 to 29.4 in 2000. We could find no evidence of declines in density in *H. viridiflavus* at TBM.

Table 3. Population parameters of marked adult snakes (juveniles omitted) at Tor Bella Monaca given as snout to vent lengths (SVL) in cm. Statistical tests are one-way ANOVAs for comparisons of male versus female size differences (where possible). Values with * attached indicate a single specimen.

Year	Species	Sex	Mean	Std Dev	Max	Min	n	F	P
1998	<i>H. viridiflavus</i>	males	79.5	9.2	90.8	70.5	8		
1998	<i>H. viridiflavus</i>	females	72.3	5.9	79.3	64.8	4	2.04	0.18
1999	<i>H. viridiflavus</i>	males	83.4	10.2	98.0	90.5	11		
1999	<i>H. viridiflavus</i>	females	78.5	8.0	98.5	64.8	8	1.29	0.27
2000	<i>H. viridiflavus</i>	males	92.5	15.2	108.5	74.0	6		
2000	<i>H. viridiflavus</i>	females	80.6	7.4	89.0	72.5	5	2.25	0.15
2012	<i>H. viridiflavus</i>	males	102.4	10.3	113.0	88.4	7		
2012	<i>H. viridiflavus</i>	females	95.8	11.2	111.3	79.6	6	1.23	0.29
2019	<i>H. viridiflavus</i>	males	105.2	10.5	119.3	92.8	10		
2019	<i>H. viridiflavus</i>	females	96.9	9.3	109.1	87.4	7	2.28	0.11
1998	<i>Z. longissimus</i>	males	100.9	4.3	105.0	96.5	3		
1998	<i>Z. longissimus</i>	females	91*	NA	NA	NA	1	NA	NA
1999	<i>Z. longissimus</i>	males	100.3	6.8	107.5	88.7	6		
1999	<i>Z. longissimus</i>	female	92.4	1.4	93.8	91.0	3	3.7	0.1
2000	<i>Z. longissimus</i>	male	99.3	10.2	116.5	90.0	5		
2012	<i>Z. longissimus</i>	male	114.4	12.5	126.2	97.6	4		
2012	<i>Z. longissimus</i>	female	106.5	2.9	108.6	104.5	2	0.69	0.45
2019	<i>Z. longissimus</i>	male	108.3	8.1	117.4	102.0	3		
2019	<i>Z. longissimus</i>	female	112.2*	NA	NA	NA	1	NA	NA

Table 4. Population parameters of marked adult snakes (juveniles omitted) at Vejo given as snout to vent lengths (SVL) in cm. Statistical tests are one-way ANOVAs for comparisons of male versus female size differences (where possible). Values with * attached indicate single or no specimens, p values in grey indicate significant results.

Year	Species	Sex	Mean	Std Dev	Max	Min	n	F	P
1995	<i>H. viridiflavus</i>	males	107.4	6.7	117.0	94	13		
1995	<i>H. viridiflavus</i>	females	100.7	2.9	103.6	96.0	7	6.02	0.02
1999	<i>H. viridiflavus</i>	males	108.4	7.5	121.5	98.0	10		
1999	<i>H. viridiflavus</i>	females	101.4	8.0	112.4	79.4	15	4.87	0.04
2012	<i>H. viridiflavus</i>	males	101.1	9.4	115.8	91.6	6		
2012	<i>H. viridiflavus</i>	females	96.9	4.2	103.2	90.7	6	1.0	0.34
2019	<i>H. viridiflavus</i>	males	106.2	7.3	116.0	91.7	7		
2019	<i>H. viridiflavus</i>	females	100.3	3.0	104.8	97.4	7	2.62	0.13
1995	<i>Z. longissimus</i>	male	119.7	10.1	129.4	106.0	4		
1995	<i>Z. longissimus</i>	female	103.7	9.8	109.6	89.0	4	5.32	0.06
1999	<i>Z. longissimus</i>	male	121.7	13.7	131.4	112.0	2		
1999	<i>Z. longissimus</i>	female	NA	NA	NA	NA	NA	NA	NA
2012	<i>Z. longissimus</i>	male	123.3	4.0	127.4	119.5	3		
2012	<i>Z. longissimus</i>	female	112.3	8.3	118.2	106.5	2	4.3	0.13
2019	<i>Z. longissimus</i>	male	115.1	10.6	128.4	102.2	5		
2019	<i>Z. longissimus</i>	female	111.4*	NA	NA	NA	1	NA	NA
1995	<i>N. helvetica</i>	male	80.8	8.1	81.5	69.6	9		
1995	<i>N. helvetica</i>	female	101.2	9.0	121.4	92.2	8	24.25	<0.0001
1999	<i>N. helvetica</i>	male	82.5	6.0	88.0	75.0	6		
1999	<i>N. helvetica</i>	female	105.3	10.7	117.0	96.0	3	17.72	0.004
2012	<i>N. helvetica</i>	male	75.2	7.1	81.0	67.3	3		
2012	<i>N. helvetica</i>	female	100.3	8.8	106.6	94.1	2	12.8	0.04
2019	<i>N. helvetica</i>	male	66.9	2.3	68.5	64.3	2		
2019	<i>N. helvetica</i>	female	93.4	0.21	93.6	93.3	2	272.9	0.004
1995	<i>V. aspis</i>	male	65.3	5.4	71.3	58.5	6		
1995	<i>V. aspis</i>	female	61.8	3.7	65.0	56.9	4	1.23	0.30
1999	<i>V. aspis</i>	male	65.9	5.7	73.2	58.7	5		
1999	<i>V. aspis</i>	female	62.1	2.1	65.0	59.6	5	2.0	0.19
2012	<i>V. aspis</i>	male	61.6	7.4	73.1	55.5	5		
2012	<i>V. aspis</i>	female	63.7	0.2	63.8	63.1	2	0.14	0.72
2019	<i>V. aspis</i>	male	58.2	13.8	68.1	35.6	5		
2019	<i>V. aspis</i>	female	60.6	0.5	61.0	60.3	2	0.06	0.82

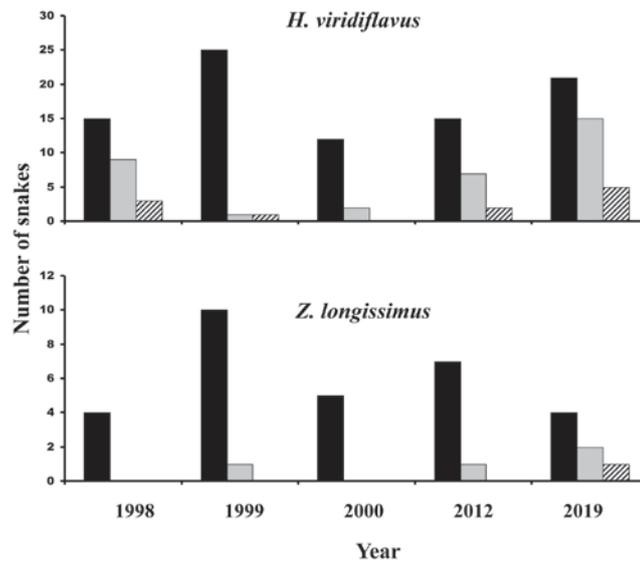


Figure 7. Recapture frequencies at Tor Bella Monaca for *H. viridiflavus* and *Z. longissimus*. Cells shown in black in all graphs represent snakes that were caught, marked and released, grey cells those that were recaptured once and cross hatched cells snakes captured more than once in a single season. See text for further details.

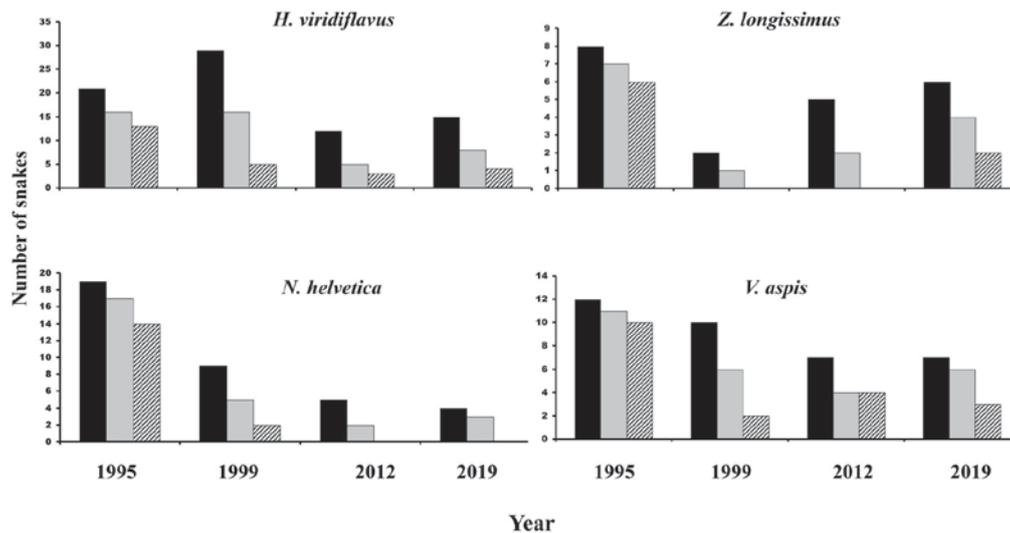


Figure 8. Recapture frequencies at Vejo for *H. viridiflavus*, *Z. longissimus*, *N. helvetica* and *V. aspis*. Cell markings are those in Fig 7. See text for further details.

The incomplete estimates for *Z. longissimus* prevented an evaluation.

DISCUSSION

General considerations

The results of this study revealed an imbalance of species presence, size (SVL), recapture frequencies and population densities between the two study areas. However, in general, we observed two similarities between the study sites: a male-skewed adult sex ratio and a relative scarcity of captured juveniles. The bias in males in all four species is not unusual in snake populations (Parker & Plummer, 1987) and may result from greater female mortality and the costs of reproduction (e.g., Madsen & Shine, 1993; Luiselli et al., 1996; Shine, 2003). Absence of juveniles in the sampling is typical of snake field studies (Pike et al., 2008; Sewell et al., 2016; Cayuela

et al., 2019) and unfortunately this limits knowledge of juvenile sex ratios and other aspects of their biology (Pike et al., 2008). Additionally, the absence of any recaptures during 2000 in the short-term analysis at TBM illustrates the difficulties separating dispersal and mortality in MRR studies. For example, age estimates of *H. viridiflavus* show relatively long live spans potentially reaching 20+ years (Fornasiero et al., 2016), which suggests that at least some 1998/1999 marked snakes were likely present but not found. A possibility is that three of the species in the present study *H. viridiflavus*, *Z. longissimus* and *N. helvetica* are wide foragers moving extensively over the landscape, which may reduce frequency of re-sighting during field searches. Sewell et al., (2016) indicated that very high population estimates for *N. helvetica* in England was possibly due to temporal residence of some individuals in a patch of high-quality habitat within a larger home range. Furthermore Gregory (2013)

Table 5. Results for the various demographic models applied to snake captures and recaptures in the two study areas by year. (A) Tor Bella Monaca; (B) Vejo. Population density estimates given as means and standard deviations (\pm) derived from the 5 population models at Tor Bella Monaca. Maximum- minimum values are the range of standard errors on each of the model means generated by the 5 models. Number of marked snakes (n) in each sampling period is also shown. Low sample size and recaptures in *Z. longissimus* negated analysis for 1998, 2012 and 2019 at TBM (A) and during 1999 at Vejo.

A) Tor Bella Monaca (TBM) 45 ha study area

Year	Species	Population Size (mean)	\pm	Max- min \pm	n
1998	<i>H. viridiflavus</i>	15.8	0.4	1.094-1.846	15
1999	<i>H. viridiflavus</i>	20.4	4.0	0- 5.958	25
2000	<i>H. viridiflavus</i>	29.4	9.0	1.797-13.42	12
2012	<i>H. viridiflavus</i>	6.2	0.4	0.487-1.115	13
2019	<i>H. viridiflavus</i>	22.8	1.7	1.09-2.503	21
1998	<i>Z. longissimus</i>	NA	NA	NA	4
1999	<i>Z. longissimus</i>	14.8	3.5	1.399-10.17	8
2000	<i>Z. longissimus</i>	18.0	1.1	0.689-1.448	5
2012	<i>Z. longissimus</i>	NA	NA	NA	6
2019	<i>Z. longissimus</i>	NA	NA	NA	4

B) Vejo 55 ha study area

Year	Species	Population Size (mean)	\pm	Max- min \pm	n
1995	<i>H. viridiflavus</i>	20.3	0.43	0.055–1.305	23
1999	<i>H. viridiflavus</i>	42.2	2.4	5.589-8.169	29
2012	<i>H. viridiflavus</i>	20.0	6.29	1.106-16.49	13
2019	<i>H. viridiflavus</i>	19.2	1.72	1.159-4.84	16
1995	<i>V. aspis</i>	20	0	0.008 – 0.396	12
1999	<i>V. aspis</i>	11.8	0.74	0.111-2.646	11
2012	<i>V. aspis</i>	9.4	4.32	0.001-0.408	8
2019	<i>V. aspis</i>	7	0	0.0-0.551	7
1995	<i>Z. longissimus</i>	8.2	0.4	0.008-1.323	8
1999	<i>Z. longissimus</i>	NA	NA	NA	2
2012	<i>Z. longissimus</i>	9	1.54	1.268-5.292	6
2019	<i>Z. longissimus</i>	7.2	0.4	0.593-1.305	7
1995	<i>N. helvetica</i>	18.5	0.5	0.019 – 1.871	19
1999	<i>N. helvetica</i>	13.2	1.17	1.178 -3.658	11
2012	<i>N. helvetica</i>	11.6	2.33	1.308-7.552	6
2019	<i>N. helvetica</i>	5	0	0-0.936	5

observed increased wariness in *N. helvetica* after first detection compared to first-time captures. It is therefore possible that repeated capture could result in some individual snakes moving elsewhere suggesting that the use of a secondary mark to enable identification at a distance, as employed here, has value in reducing this effect. These results illustrate just some of the difficulties in calculating snake population densities, that sampling area and home ranges of certain species may not be synonymous and highlight the value of long term MRR studies in understanding snake population ecology.

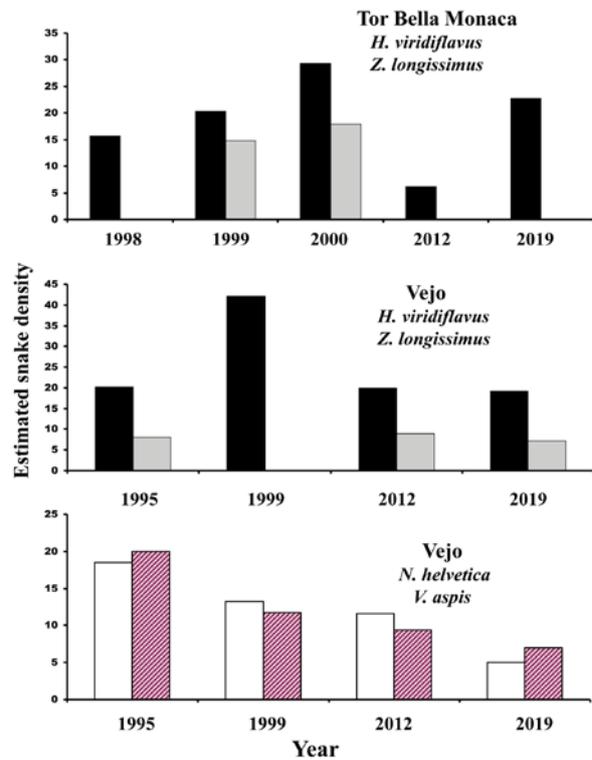


Figure 9. Histograms of estimated population densities of snakes at both 45ha and 55ha sites during the various study periods. More detailed information is given in the text and in Table 5.

Were community metrics (diversity and dominance) different between the two study areas?

Our prediction that community evenness should be higher in the well-preserved area (VEJO) and species dominance in the degraded site (TBM) was confirmed. This supports the notion that viable snake populations likely require the protection of large, contiguous sections of unfragmented habitat (Dodd & Barichivich, 2007), with some Mediterranean species at least particularly affected by habitat fragmentation processes (Luiselli & Capizzi, 1997). Indeed high quality habitat was suggested as one of the key factors involved in very high densities of *N. helvetica* in England (Sewell et al., 2016). The dominant/most abundant species at both our sites, in terms of number of individuals and probably also biomass was *H. viridiflavus*, a wide-ranging forager with a relatively broad-based diet including mammals, birds, reptiles and amphibians (Capizzi et al., 2008; Lelièvre et al., 2012). These dietary characteristics likely explain *H. viridiflavus* greater abundance, which has been found in other snakes with a similar lifestyle (e.g. Fitch, 1999). A wide foraging lifestyle increases contact with prey species and could enhance adaptation to changing environments compared to a sentinel (ambush-foraging) lifestyle (e.g. *V. aspis*). In general, *H. viridiflavus* is almost invariably the most abundant snake species in terrestrial Mediterranean environments in Italy and the least affected by habitat fragmentation processes (Luiselli & Capizzi, 1997 although see Storniolo et al., 2019). Thus,

our data in this regard support the findings of earlier studies.

Were body sizes of snakes greater in the well-preserved site than in the degraded site?

Two species, *H. viridiflavus* and *Z. longissimus*, were present in both study localities but SVL's were significantly greater in the higher quality habitat at VEJO where a much higher diversity of potential prey species was observed. This confirms our a-priori prediction. Unfortunately, there are no data in the literature concerning the variation of the body sizes of snakes in relation to habitat fragmentation for any meaningful comparison. However, there is evidence that maximum body size co-varies with prey density in squamate reptiles (e.g. Jessop et al., 2006).

Was the inter-annual variation in mean body size lower in the well-preserved area than in the altered area?

The body sizes of all species were relatively stable across years at VEJO, but snake numbers fluctuated and showed increased size in the latter years in the two species at TBM. This confirms Hypothesis 3. The diet composition of *H. viridiflavus* and *Z. longissimus* is relatively similar and includes small mammals (Capizzi et al., 2008; Lelièvre et al., 2012). It is possible that increases in body size were driven by increases in small mammal density at TBM during recent years as a consequence of changes in waste management in the area, for example increases in rat numbers due to inadequate waste disposal (Traweger et al., 2006). In addition, higher numbers of rats may have impact on smaller-sized mammal numbers (Capizzi & Santini, 2007; Harris & McDonald, 2007). These factors could favour selection of larger snake body size due to the need to forage upon and subdue, larger prey.

Were population densities declines with time greater in the altered site than in the well-preserved site?

Our data here gave conflicting results indicating relatively stable densities in *H. viridiflavus* and *Z. longissimus* at both sites, thus contradicting our a-priori prediction. The slight downward shift in numbers in the former we tentatively attribute to natural population fluctuations but it is worth noting that declines in *H. viridiflavus* numbers have recently been reported in central Italy (Storniolo et al., 2019).

However, notable declines were observed in population densities of both *V. aspis* and *N. helvetica* at VEJO, although the underlying causes of these apparent declines are not immediately obvious. Rome has experienced annual temperature increases between 1999 and 2011 of approximately around 3° C (Capula et al., 2016). The temperature increases associated with long term phenology and population decline of *V. aspis* north of Rome (Luiselli et al., 2018) could similarly be impacting on populations in the city by influencing offspring fitness (Bonnet et al., 2002) and reproductive effort. This is especially likely in *V. aspis*, a typical capital breeder that delays reproduction until large energy reserves have been amassed resulting in females producing only a single litter during their lifetime (e.g.

Bonnet et al., 1999; Bonnet et al., 2002). In theory, a prolonged season should allow for increased feeding, and thus energy uptake for reproduction (Rugiero et al., 2013) but increased temperatures and subsequent shorter hibernation periods may increase metabolic costs as a consequence of shorter hibernation periods delaying energy uptake and reproduction. A direct consequence of this is a requirement for females to survive for longer periods before reproduction can occur which increases exposure to predation risk before reproduction can take place. Additionally, feeding opportunity relates to prey density, which depends on habitat quality and climate, in particular rain for increased plant growth, and populations of mice/invertebrates, lizards/snakes (Rugiero et al., 2012). However, a warmer climate does not guarantee increased rainfall and resources; there was no correlation between rainfall patterns and climate warming in the Rome area throughout the study period (Rugiero et al., 2012, 2013).

Thermal influences on offspring fitness and survivorship have also been found in *N. helvetica* in northern Europe where optimum temperatures during embryonic development result in larger fitter offspring capable of enhanced locomotor performance (Löwenborg et al., 2011; Hagman et al., 2012). Deviations from optimum temperatures during embryonic development have been associated with low survivorship in adults (Löwenborg et al., 2012). There is also evidence that in natural environments female *N. helvetica* communally select nests with optimum thermal properties in good agreement with the findings of laboratory studies (e.g. Löwenborg et al., 2011; Hagman et al., 2012; Meek, 2017). To access nesting areas involves travelling across the landscape (Meek, 2017) which increases mortality risk (Bonnet et al., 1999) suggesting that the thermal benefits derived from migrating to nesting areas outweigh the risk of movement. The key role temperature plays in snake ecology therefore cannot be underestimated and it is perfectly feasible that global warming may be impacting on snake numbers in many ways, even at the higher quality habitat at VEJO as a consequence of impacts on their thermal ecology. Local selection should favour individuals that are better at selecting the best possible nesting sites, especially across generations, and it cannot be excluded that snakes from VEJO and TBM may be constrained to adjust behaviour similar to that found in more southern conspecific populations that are experiencing greater climate warming pressures.

Population densities (number of individuals per ha) of all species averaged from 0.3 to 0.7 individuals per ha per year. The low density observed at the two study areas may possibly be related to low prey and shelter (predation cover, oviposition, hibernation) densities, as a consequence of reduced microhabitat complexities. Nonetheless, our density estimates are in good agreement with estimates for conspecifics reported in earlier studies (e.g., see Storniolo et al., 2019, and references therein).

At a local level, the densities of snakes in suburban areas of Rome are not generally lower than in more natural areas, and are able to persist if habitats are suitable with abundant food resources. Nevertheless,

when populations are < 30 individuals there may be a risk of inbreeding, genetic drift or population collapse through increased mortality in certain years (by severe winters, drought, low prey density, disease etc., Madsen & Shine, 1992; Madsen et al., 1996; Guicking et al., 2004). It is of interest and surprising that such populations have persisted for over two decades (and much longer) without any apparent immigration. However, we cannot be certain of the latter despite our inability to detect immigration at either localities but it is possible that some individuals may have immigrated into the localities by way of hedgerows bordering the extended agricultural lands surrounding the study areas (especially at VEJO). This is potentially most likely to be found in the wide foraging *H. viridiflavus*, *Z. longissimus* and *N. helvetica* but also in *V. aspis*, which although an ambush-foraging predator, is known to move to and from spring and summer home ranges using hedgerows as ecological pathways (Saint Girons, 1996). Therefore, as a conservation management practice, it is imperative that the network of hedgerows is preserved, indeed increased, in order to conserve the historic remnant isolated snake populations that inhabit the suburban areas of Rome and the Mediterranean region as a whole.

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Dominance and aggression in captive gidgee skinks (*Egernia stokesii*)

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Dominance is a key component of behaviour in many animal species and is central to social system dynamics, resource acquisition, individual fitness and ultimately reproductive success. We investigated dominance interactions and social behaviours in a group of captive juvenile gidgee skinks (*Egernia stokesii*). We hypothesised that a dominance hierarchy existed within the group, and that aggressive behaviours would be used to secure limited resources, especially high-value resources. We also hypothesised that body weight would be positively correlated with dominance and aggressive behaviours. We filmed the lizards at 1200 hours for six days a week over the course of eight weeks. We exposed the lizards to three different diets, which consisted of an animal-based diet (crickets), plant-based diet (plants), and a non-feeding control (no food offered). The relative value of these resources to the skinks was established through preference tests. We identified a dominance hierarchy, with dominant individuals exhibiting more aggressive behaviours than subordinates. We found that the frequency of aggressive behaviours was significantly higher in trials where high-valued resources (crickets) were at stake. Furthermore, we found a significant positive correlation between body weight and dominance, bite and chase; larger individuals were ranked higher in the social hierarchy compared to smaller individuals. Our results demonstrate the importance of morphological and behavioural traits in determining a dominance hierarchy in *E. stokesii* and how dominance can have ecological advantages.

Keywords: dominance, aggression, body weight, behaviour, interaction, lizards

INTRODUCTION

Dominance relationships define the social structure of numerous animal species (Bush et al., 2016; Chase et al., 2002; Drews, 1993). Conflicts occur when animals compete for limited resources such as food, refuges and mates, and the outcome of these conflicts can have major ecological consequences (Huyghe et al., 2005; Wong & Candolin, 2005). For example, where a clear hierarchy exists, higher-ranking individuals tend to deny subordinates access to limited resources and as a result, increase their own reproductive success (Wroblewski et al., 2009). Thus, identifying the traits associated with dominance is important to our understanding of the phenotypic evolution of animal species (Huyghe et al., 2005). For instance, a link between bite force and dominance explains the evolution of larger heads in some species of lizards (Bull & Pamula, 1996; Huyghe et al., 2005; Pratt et al., 1992).

Morphological traits such as body weight play a crucial role in dominance (Smith & Parker, 1976; Carpenter, 1995), but behavioural traits, such as aggression, can also have a major influence (Huang et al., 2011; Wilson et al., 2011). For example, aggressive individuals often out-compete less aggressive individuals for limited resources (Duckworth, 2006; Arnott & Elwood, 2009; Herrel et al.,

2009). It has been found that aggressive behaviour can correlate both positively (Payne & Swanson, 1970) and negatively (McEvoy et al., 2013) with morphological characters such as body weight. Additionally, aggressive behaviours and interactions are often most frequent when resources are of high value. For instance, wasps avoid low-value resources guarded by aggressive rivals but become more likely to challenge the rival when the value of the resource increases (Tibbetts, 2008). Therefore, resource-dependent aggression is likely to be an important mechanism underlying the evolution of social costs.

Dominance hierarchies have been identified in several lizard species (Robson & Miles, 2000; Henningsen & Irschick, 2012; Bush et al., 2016), but lizards have not been thoroughly studied in the dominance literature. The conspicuous displays of iguanids have stimulated a wealth of research that has moulded herpetology's knowledge of social and dominant behaviours in lizards (Stamps, 1977; Ord et al. 2002). By contrast, the largest and most speciose family of lizards, the Scincidae, has received far less attention. This is despite the fact both aggression (Cooper & Vitt, 1987; Torr & Shine, 1996; Jennings & Thompson, 1999; Stapley, 2006; Myers & Paulissen, 2017) and social hierarchies have been identified in various skink species (Done & Heatwole, 1977; Whittier & Martin,

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1992). For example, Myers & Paulissen (2017) found that little brown skinks (*Scincella lateralis*) aggressively interact with each other when they are in close proximity and always establish dominance relationships during paired encounters. Riley et al. (2017) found that dominant tree skinks (*Egernia striolata*) displayed invariable aggression and that dominance relationships had profound effects on an individual's behaviour and fitness. Additionally, rainbow skinks (*Carlia rostralis*) have been found to significantly increase the behaviour frequencies when paired with another skink and express dominance in a linear fashion (Whittier & Martin, 1992).

Lizards are increasingly being recognised as excellent model systems for studying questions regarding the evolution of complex sociality (Whiting & While, 2017). However, few studies have recorded fine scale information on social interactions within a group of juveniles and how this may mediate social group processes, such as the emergence of dominance hierarchies. Dominance hierarchies and territorial behaviours have been previously observed in juvenile lizards (Stamps & Tanaka, 1981; Stamps, 1983a, 1983b; Stamps & Krishnan, 1997), but this is yet to be tested in *Egernia* or other skink species.

The gidgee spiny-tailed skink (*Egernia stokesii*) is a medium sized viviparous lizard endemic to Australia. They typically live in low open shrub land and inhabit the crevices of limestone rocks; the spines on their tails are adapted to prevent predators from extracting them from these shelters (Chapple, 2003). They are active predators and opportunistic omnivores, with their diet comprising of both insects and plant material (Cogger, 1996). Little is known about the natural history of *E. stokesii*, however, they exhibit a high level of social complexity (Greer, 1989; Stow et al., 2001; Duffield & Bull, 2002; Chapple, 2003). A number of 'sophisticated' social behaviours have been observed in *Egernia* spp. and other lizards, such as mate choice (Cooper & Vitt, 1993), monogamy (Bull, 2000; Gardner et al., 2002; O'Connor & Shine, 2003), mate guarding (Olsson & Shine, 1998), kin recognition (Bull et al., 2000, 2001), parental care (While et al., 2009) and aggressive territory protection (Jennings & Thompson, 1999; Chapple, 2003). Whilst the available literature highlights the social behaviours of *Egernia* spp., it is not yet known how such behaviours determine their social structure. However, the existence of stable social groups in nature provides a context in which dominance hierarchies are likely to evolve.

The foregoing illustrates the significant gaps in the understanding of *E. stokesii* social interactions and its impact on this species' ecology. Considering that *E. stokesii* are gregarious (Gardner et al., 2001) and the dearth of information that exists on their social system dynamics, we sought to investigate the possibility of a dominance hierarchy in captive, juvenile *E. stokesii* housed at ZSL London Zoo, and the social behaviours associated with dominance. We tested the hypothesis that *E. stokesii* will demonstrate some degree of dominance hierarchy and use aggressive behaviours to acquire limited food resources; and that the strength of pattern would be dependent on the value of resources over which lizards compete. In addition, we hypothesised that there would be a significant relationship between dominance and

body weight. We addressed these questions by firstly observing the interactions within the group to measure dominance, and then measuring the effect of diet, body weight and dominance on the frequency of aggressive behaviours observed. From this, we can explain what type of structure, if any, exists within the group and the outcome of interactions can allow for a hierarchy to be identified (Valderrábano-Ibarra et al., 2007).

METHODS

Ethics statement

The study design and methods were assessed and approved by the Ethics Committee at ZSL (Zoological Project Database number WAB59). They established that all experiments were non-invasive, and the welfare of the lizards was not risked or compromised. Lizards were fed using a central food presentation which is the zoo's usual feeding method, as this allows keepers to count all lizards, ensure they are all feeding and in good health. In nature *E. stokesii* are social and no lizards had previously been harmed whilst being fed in this manner. Their condition was monitored closely and aggression between individuals would have been stopped if it had escalated to levels of potential harm. For ease of identification, the lizards were marked with a small dot of non-toxic paint, which is the standard procedure at ZSL London Zoo; these markings were in place before the study and remain in use after its completion. All lizards remained in the living collection at ZSL after the end of the study.

Study animals

The study was conducted using seven juvenile gidgee spiny-tailed skinks (*Egernia stokesii*) housed in the reptile house at ZSL London Zoo, England. All lizards were captive bred and produced by the same breeding group of five unsexed animals. The lizards were unsexed and 25 (n=4), 24 (n=2) and 23 (n=1) months of age. Lizards had individual markings (a pink dot) on different areas of the body; the dorsal aspect of the back-left foot (lizard 1), back-right foot (lizard 2), pelvic area (lizard 3), front-right foot (lizard 4), front-left foot (lizard 5), back-right and front-left feet (lizard 6) or front-right and back-left feet (lizard 7), and will be referred to by their number from here on after (e.g. 'L2' is lizard 2, bearing a marking on the hind right foot). The trials were completed between the 21st of June and the 16th of August 2017.

Enclosure and husbandry

The trials were carried out in the lizard's usual enclosure, with dimensions 152 x 75 x 90 cm (length x width x height). The enclosure was made out of wood, mesh and glass, and had a substrate of 70:30 mix of Breedon amber gravel (Breedon Special Aggregates, UK) to peat free compost (Amenity and Horticulture Supplies, UK). It contained a small water dish, rocks, bark, logs and branches in order to provide crevice like shelters (Fig. 1). Lighting switched on at 07.00 h and was set on a 12 hour cycle. Their enclosure was lit using two Arcadia 100W mercury vapour lamps (Arcadia Reptile, UK) and a set of four 60 cm UVB-emitting T5 lamp (12 % UVB) (Arcadia Reptile, UK) mounted within a hydroponic lighting unit (Growth Technology, UK). This

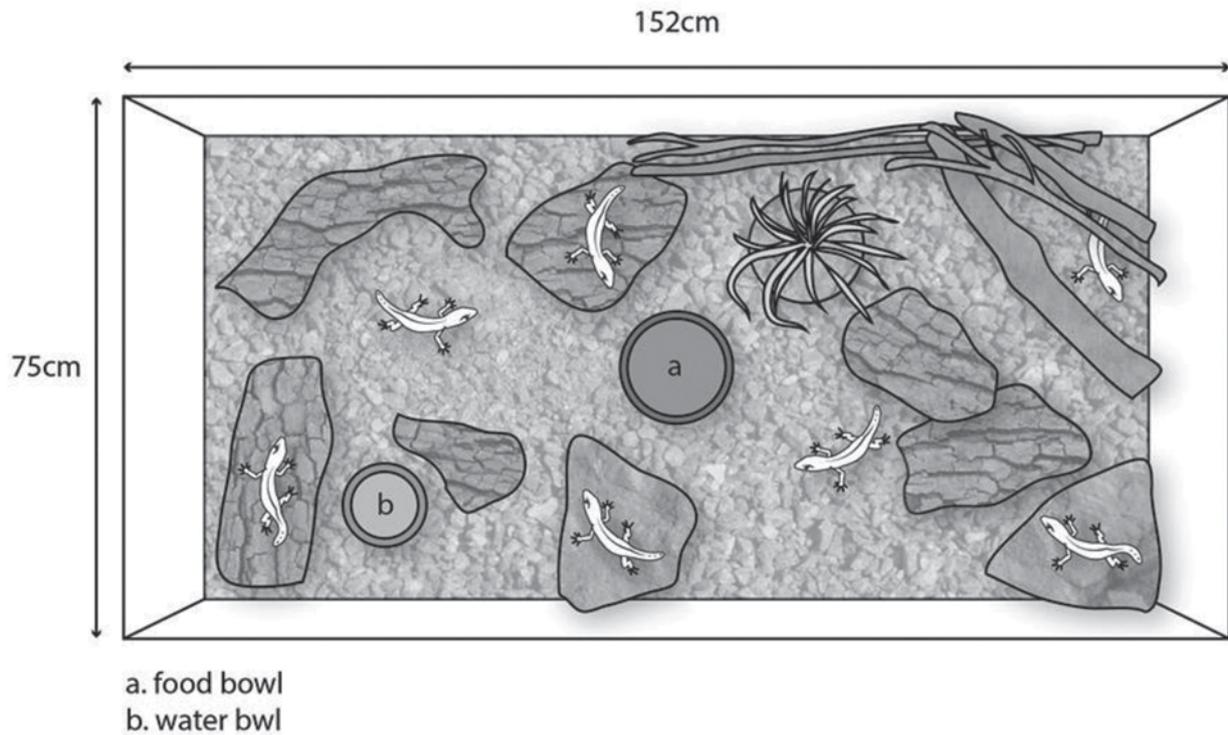


Figure 1. A diagram of the lizards’ enclosure consisting of gravel and peat free compost, rocks, bark, logs and branches. **(a)** The bowl used to present food on throughout test trials. **(b)** A small water dish available to the skinks at all times.

Table 1. An ethogram devised for the gidgee skinks (*E. stokesii*), consisting of state and event behaviours followed by their definitions.

Type	Behaviour	Definition
	Bite	Lizards grabs or attempts to grab another lizard with its jaws.
	Lunge	Rapid jumping movement by one lizard towards a conspecific
Event	Nudge	Lizard moves so that its body comes into contact with a conspecific and then pushes forward and displaces that lizard from the food bowl.
	Chase	Lizard rapidly follows another fleeing lizard.
	Flee	Lizard rapidly retreats from another chasing lizard.
State	Other	Lizard displays any other behaviour that is not listed.
	Out of sight	Lizard is not visible.

combination of lighting provided two bask zones, with UVB gradient between 0-3.3 across the entire enclosure and 1.7-3 within bask zones, and a thermal gradient of 26 °C throughout the day and 22-25 °C during the night and bask zone temperatures of 35 °C. Faeces were removed, clean water provided, and their enclosure was misted daily. The lizards were fed on Mondays, Wednesdays, Fridays and Sundays. The enclosure and set up for test trials remained the same throughout.

Food preference trials

Prior to feeding trials, a choice test was conducted to establish whether crickets or plants were considered more favourable by the lizards. Each lizard received four

trials whereby they were presented with two food bowls, one containing dead crickets (*Acheta domestica*) and the other plants. The bowl they approached first was scored as either a 1 (crickets) or 0 (plants) and then the means were calculated. We used a one-tailed one-sample sign test to test for a significant difference in food choice.

Behavioural assessment

Based on observation of the lizards, an ethogram was devised with a timeline recording sheet with state and event behaviours recorded (Table 1). Behavioural observations were based on video footage and we used focal sampling to record what each lizard was doing at each 1-minute interval and tally any event behaviours observed during each test period. As the lizards are fast moving and behaviours are instantaneous, short observation windows were used. This approach has been established for species with similar activity patterns (Januszczak et al., 2016). All observations were conducted by the same observer (HB).

Behavioural observations

On test days, the lizards were filmed for 15 minutes at 12.00 h. This time ensured that they had sufficient basking time to reach optimum temperature for activity before trials. The lizards received three types of diet; animal-based consisting of three live crickets each, plant-based consisting of 15 g of plant material (dandelions and clovers) between them, and a control where no food was offered. The goal of the control was to determine the baseline behaviour and dominance interactions of the lizards when they received no food. The purpose of the animal-based diet was to see how the lizards interact

with one another when presented with a high-energy and palatable food item. The purpose of the plant-based diet was to see if the interactions observed were similar when presented with lower-energy and less palatable food items. From a husbandry aspect, it was important for the keepers to know when and what the lizards were being fed and thus the diets could not be randomised or reversed in this case. However, we conducted 48 trials (16 of each diet) and so it is unlikely that sequencing or events on specific days had an effect. Animal-based trials were conducted on Mondays and Wednesdays and plant-based trials were conducted on Fridays and Sundays. The control trials were carried out on non-feeding days (Tuesdays and Thursdays). A camera was placed on a tripod above the lizards' enclosure, capturing as much of the enclosure as possible.

All dyadic dominance interactions were recorded using the Altmann (1974) sampling method, that is, all agonistic interactions where there was a clear winner and loser. It is important to highlight the distinction between 'aggressive' and 'dominant' animals; the former describes animals displaying a higher frequency of aggressive behaviours, while the latter describes animals that tend to win in agonistic encounters. In each encounter, all participating lizards were recorded, and we documented the initiating individual, the target, winner and loser. A loss was determined by the displacement of a lizard in control trials, and displacement of a lizard from the food bowl in animal and plant-based trials, whilst the winner did not retreat or stole the conspecific's food.

Statistical analysis

SPSS 22 was used to conduct statistical analyses, with a significance level set at $p \leq 0.05$. Due to the limited sample size we decided that non-parametric analysis was appropriate (Siegel & Castellan, 1988).

To examine the possibility of a hierarchy, dominance ranks were calculated using the Elo rating method (Albers & de Vries, 2001; Neumann et al., 2011), initially developed to rank chess players in tournaments (Elo 1961, 1978) and subsequently widely used to assess dominance within groups (Albers & de Vries, 2001). Elo ratings are based on the sequence in which dyadic dominance interactions occur, rather than the outcome of interactions alone. For instance, after each interaction, the winner gains points and the loser loses points. At the start of the Elo rating process, each lizard started with a predefined rating of 1000. The amount chosen had no impact on the difference between lizard ratings (Albers & de Vries, 2001). After each interaction, each lizard's rating was updated according to the expected outcome: the probability of that individual winning (and losing). For example, a high-rating lizard (L1) winning over a low-rating lizard (L2) would increase L1's rating by a small amount and decrease L2's rating by the same amount. If, however, L2 won the interaction, this would increase L2's rating by a larger amount and decrease L1's by the same amount, as this was an unexpected outcome. The amount of points gained and lost during an interaction (k) was set at 100 (see Appendix 1). The value of k makes little difference to results; when using three different values of k , Albers & de Vries (2001) found dominance

hierarchies to be unidirectional and significantly linear for all values of k .

Generalised estimating equations (GEE) with exchangeable working correlation structure, to account for repeated measures from the individual, and Poisson link function was used to examine the effect of diet (AB, PB, C), dominance and body weight on the frequency of aggressive behaviours. The behaviours assessed were bite, lunge, nudge, chase, flee and other. Results are presented as rate ratio (95 % confidence intervals). For significant effects detected by the GEEs, Wald chi-square post hoc tests were then conducted to compare means. Spearman's rank correlation was used to assess the relationship between dominance and body weight.

RESULTS

Food preference trials

The results from the choice tests revealed that the lizards showed a significant preference for crickets compared to plants ($Z_6 = 2.65$; $p = 0.004$). In the behavioural observations, we found that the frequency of aggressive behaviours varied with diet. Aggressive behaviour frequencies differed significantly between individuals and these behaviours increased in the presence of food (crickets and plants) compared to a non-feeding control.

Behavioural observations

Overall, 183 dyadic dominance interactions were observed. Using the Elo rating method to calculate dominance ratings, we found that L1 was the most dominant individual with an Elo rating of 1200, followed by L2 (1128), L3 (1112), L6 (968), L4 (949), L5 (828) and L7 (656) (see Tables 2 and 3). As observations were conducted over a nine-week period, we were unable to quantify stability of dominance over time; a general overview is displayed in Figure 2.

We found a significant overall effect of diet on the frequency of bite ($X^2 = 8.111$; $p = 0.004$) lunge ($X^2 = 23.704$; $p < 0.001$), nudge ($X^2 = 25.225$; $p < 0.001$), chase ($X^2 = 8.782$; $p = 0.012$) and flee ($X^2 = 62.316$; $p < 0.001$). Animal and plant-based diets increased the frequency of aggressive behaviours compared to the non-feeding control (Figure 3). Open-mouth threats, push downs and tail-wagging were never observed throughout the trials. Using post hoc pairwise comparisons, it was revealed that the animal-based diet caused a significant increase in lunge ($p < 0.001$), nudge ($p < 0.001$), chase ($p = 0.010$) and flee ($p < 0.001$) compared to the control, and caused a significant increase in bite ($p = 0.004$), lunge ($p < 0.001$), nudge ($p < 0.001$) and flee ($p < 0.001$) compared to plant-based. The plant-based diet caused a significant increase in lunge ($p = 0.008$) and nudge ($p = 0.004$) compared to the control. We found no significant increase in behaviour frequencies in the control compared to animal and plant-based diets (see Table 4 for summary of the GEE results).

There was a significant effect of dominance on the frequency of lunge ($X^2 = 8.111$; $p = 0.004$), nudge ($X^2 = 34.630$; $p < 0.001$) and chase ($X^2 = 21.870$; $p < 0.001$), but not for bite ($X^2 = 21.852$; $p = 0.174$) and flee ($X^2 = 2.026$; $p = 0.155$). There was a significant effect of body weight on the frequency of bite ($X^2 = 23.745$; $p = 0.053$) and flee

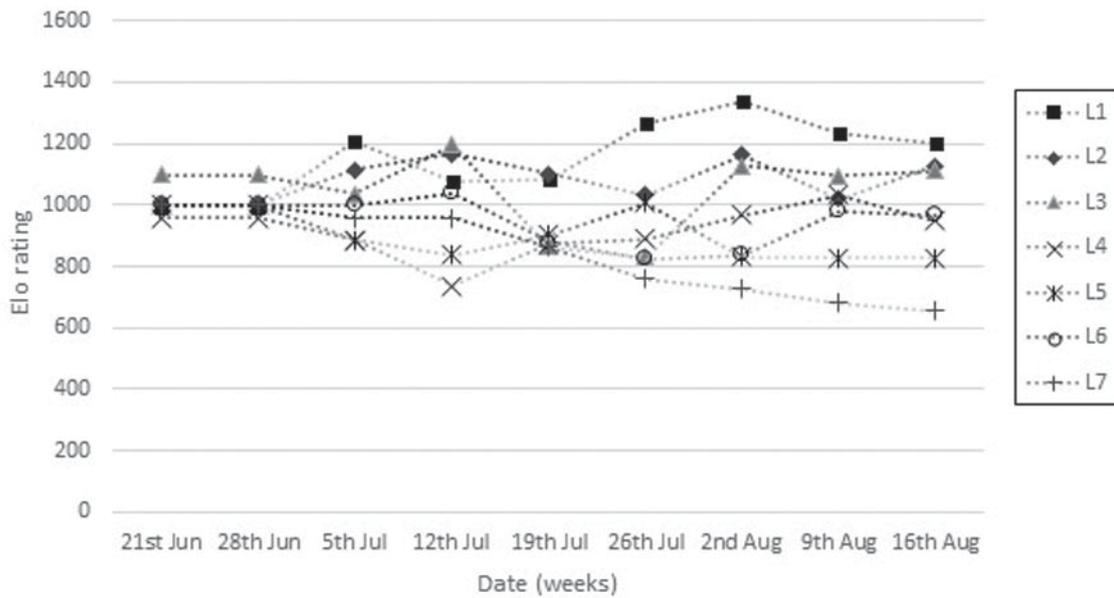


Figure 2. A general overview of dominance stability over time. Individuals are displayed on the right hand-side legend, with L1 being lizard one.

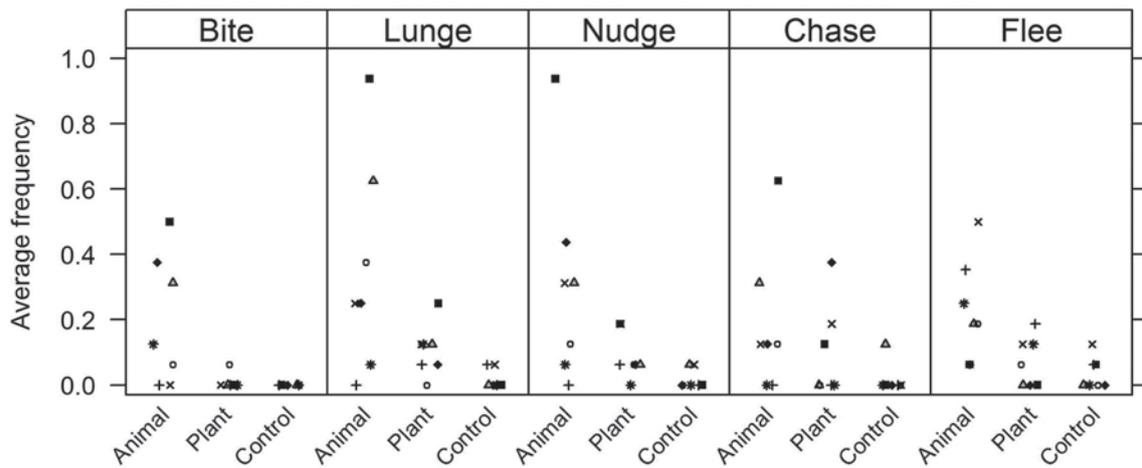


Figure 3. Shows the average frequency of behaviours for each individual in the animal-based, plant-based and control trials.

Table 2. The total frequency of interactions, wins and losses, and dominance rating for each lizard, with the most dominant individual ranked as 1 and the least dominant as 7.

ID	Weight (g)	Total Interactions	Total wins	Total Losses	Elo rating	Dominance
L1	91	92	55	37	1200	1
L2	85	73	50	23	1128	2
L3	86	62	35	27	1112	3
L6	72	38	11	27	968	4
L4	61	58	18	40	949	5
L5	58	39	12	27	828	6
L7	52	22	2	20	656	7

Table 3. The number of wins and losses between lizards in dyadic dominance interactions.

ID	L1	L2	L3	L6	L4	L5	L7
L1	-	8	13	5	17	4	4
L2	10	-	8	10	10	9	7
L3	7	2	-	8	9	5	4
L6	1	2	3	-	2	1	2
L4	2	4	1	4	-	6	1
L5	2	4	2	0	2	-	2
L7	0	0	0	0	0	2	-

Table 4. The number of wins and losses between lizards in dyadic dominance interactions.

Behaviour	Overall effect	Treatment			Dominance		Body weight	
		AB v C	PB v C	AB v PB	p-value	Rate ratio	p-value	Rate ratio
Bite	=0.004	-	-	21.8(2.6,18.3)	=0.174	1.9(0.9,1.5)	=0.053	1.2(0.9,1.5)
Lunge	<0.001	19.9(4.1,96.8)	6.0(1.6,22.6)	3.3(2.0,5.4)	=0.004	1.3(1.1,1.7)	=0.712	1.0(1.0,1.0)
Nudge	<0.001	17.5(4.5,68.2)	5.0(1.7,15.2)	3.5(2.1,5.8)	<0.001	1.7(1.4,2.0)	=0.266	1.0(0.9,1.0)
Chase	=0.012	10.9(1.7,62.8)	5.5(0.5,56.1)	1.9(0.6,6.4)	<0.001	1.5(1.2,1.7)	=0.445	1.0(1.0,1.1)
Flee	<0.001	6.4(3.1,13.1)	2.0(0.8,5.0)	3.2(2.0,5.0)	=0.155	1.2(0.9,1.5)	<0.001	0.9(0.9,1.0)

($X^2 = 14.843$; $p < 0.001$), but not for lunge ($X^2 = 0.136$; $p = 0.712$), nudge ($X^2 = 1.235$; $p = 0.266$) and chase ($X^2 = 0.583$; $p = 0.445$; Table 4). A Spearman's rank correlation found a positive correlation between bodyweight and dominance ($r_s = 0.788$; $p < 0.001$), such that heavier skinks tended to be more dominant. We found a significant positive correlation between body weight and bite ($r_s = 0.800$; $p < 0.031$) and chase ($r_s = 0.847$; $p < 0.016$), but not for lunge ($r_s = 0.571$; $p < 0.180$) or nudge ($r_s = 0.595$; $p < 0.159$). A significant negative correlation was found between bodyweight and flee ($r_s = 0.857$; $p < 0.014$).

DISCUSSION

The purpose of this study was to investigate the possibility of a dominance hierarchy in a group of captive juvenile *E. stokesii*. We hypothesised that *E. stokesii* would display a dominance hierarchy and use aggression behaviours to obtain limited resources, and that the frequency of these behaviours will be highest when competing for high value resources (crickets) compared to lower value resources (plants). The existence of dominance hierarchies was suspected in this species due to their group-living biology (Chapple, 2003) and the existence of complex social dynamics on congeners (see above). Our analyses provide three main results. First, we identified a dominance hierarchy, with dominant individuals (i.e. those that won the most confrontations) tending to exhibit more aggressive behaviours than subordinates.

This is interesting as it indicates that fighting aggressively is necessary to establish dominance; in some other taxa, the most dominant individuals engage in very few fights as other individuals don't even attempt to challenge them. Second, we found that the frequency of aggressive behaviours was highest in diets with high-valued resources (crickets). Finally, we found a significant positive correlation between body weight and dominance, bite and chase, and a significant negative correlation between body weight and flee.

Agonistic encounters among conspecifics can be energetically expensive (Neat et al., 1998) and, in some cases, extremely dangerous (Clutton-Brock et al., 1979). The potential cost of engaging in an agonistic encounter must therefore be weighed against the potential pay-off of winning, which will include access to scarce resources. During a choice test, we found that *E. stokesii* showed a significant preference for crickets over plants as a food source. One explanation for this preference is that crickets have a higher energy content compared to the plant species used in our study. For example, per 100 g, adult

house crickets (*Acheta domesticus*), the taxon used in this study, have a mean energy (ME) of 120 kcal (Clayton, 2014; Nowak et al., 2016) compared to dandelions (*Taraxacum*) ME = 45 kcal (Tan et al., 2017) and clovers (*Trifolium*) ME = 12 kcal (Johansen et al., 2017), which comprised the plant-based diet. Additionally, we found that the frequency of lunge, nudge, chase and flee was significantly higher in animal-based trials (crickets) compared to control (no food offered), the frequency of bite, lunge, nudge and flee was significantly higher in animal-based compared to plant-based, and lunge and nudge was significantly higher in plant-based compared to control. This is supported by previous studies which revealed that the frequency of aggressive behaviours in juvenile lizards increases in the presence of limited, high-value resources (Stamps, 1978).

Dominance scores using the Elo rating method revealed a hierarchy in the lizards, with more dominant animals winning more agonistic encounters. However, as observations were conducted over a nine-week period, we were unable to statistically test for stability of dominance over time (see Figure 2 for general overview). Our results revealed that the frequency of lunge, nudge and chase was significantly higher in dominant lizards compared to subordinates. In support of these findings, Myers & Paulissen (2017) found that, when observing little brown skinks (*Scincella lateralis*), aggressive behaviours such as 'chasing' were only ever exhibited by dominant lizards and these individuals also displayed 'lunging' significantly more times than subordinates. In some cases, aggression has been found to override body size; Schult-Hostedde & Millar (2002) found that smaller aggressive individuals were more dominant over larger, but less aggressive, individuals. In our study, winners gained primary access to food whilst losers were often submissive and consequently displaced from the food bowl. Thus, dominance interactions leading to disparate access to resources between individuals may have evolutionary implications for skinks, as well as ecological implications for the individuals involved (Greer, 1989; Langkilde et al., 2005).

In addition to aggression, other social behaviours can be used to determine resource distribution within species, without the need for aggressive interactions. For example, *E. stokesii* display 'open-mouth threats', 'push downs' and 'tail-wagging' as a way of emphasising their size, strength or quality to warn others and compete for dominance over their opponents (Chapple, 2003). However, these display behaviours were never observed in the trials. This may be because all the animals were of relatively similar size and age, and therefore may

not have been able to use posturing before engaging in aggression. Posturing can be important for showing off size as a proxy for likelihood of winning, so lizards may not bother with this if they perceive that their opponent is so similar that they will have to resort to aggression. Displacement behaviour can also show competitive ability without aggression. When investigating competition in two closely related skinks (*Niveoscincus greeni* and *N. microlepidotus*) that have overlapping geographical distributions and similar habitat preferences, Melville (2002) found that they use displacement to access limited basking sites. Similarly, Dame & Petren (2006) found that house geckos have been displacing indo-pacific geckos on a global scale because of habitat competition. While theoretically, it should be possible for familiar animals to outcompete one another without fighting, our sample predominantly used aggressive behaviours to determine and maintain social dominance and it is for this reason we measured aggression only.

Body weight is a key determinant of dominance in various lizard species and skinks are no exception. It has been found that body weight is significantly correlated with dominance in various skinks, with larger individuals dominating smaller lizards (Whittier & Martin 1992; Torr & Shine 1996; Melville, 2002). Similarly, we identified a significant positive correlation between body weight and dominance, bite and chase, with heavier individuals being ranked higher in the social hierarchy. It is possible that *E. stokesii* assess some correlate of body width or muscle size to parse dominance relationships. In some instances, when *E. stokesii* bite and lock jaws, they fight by rolling around on the ground (Chris Michaels, pers. Obs). Therefore, it is likely that the lizard which is physically stronger wins the fight and subsequently emerges as the dominant individual. However, there will be circularity in the development of increased body weight and access to more resources to increase body weight; without data concerning body weight at hatching and subsequent development of dominance hierarchies, it is impossible to say which determines which in this case.

Social dominance, as measured herein, may also correlate with other variables. For example, testosterone levels linked to sex, which have been found to influence the rate of aggression during the formation of social hierarchies in lizard species (Greenberg et al., 1984). In our experiment, the sex of the lizards was unknown as juvenile *E. stokesii* show no sexual dimorphism and thus sexing the lizards before our experiment was not possible. Moore (1987) and Moore & Marler (1987) demonstrated that testosterone influences aggressive behaviours and that castration dramatically reduced, yet did not eliminate, territorial behaviour in free-living *Sceloporus*. The fact that no individuals reached sexual maturity in this case, means the effects of testosterone levels linked to sex are less likely to have had an impact.

Another explanation could be the slight difference in age between the lizards may have influenced social behaviours within the group. For example, Baird et al. (1996) discovered age-related differences in social behaviours in free-living collared lizards (*Crotaphytus collaris*), and Bajer et al. (2015) found that risk-taking and explorative behaviours were dependent on age in a group

of European green lizards (*Lacerta viridis*). However, the oldest and youngest only varied in age by two months (23 versus 25 months). Our experiment lasted 56 days and the rank order of the individuals tested was consistent across the whole study period. This suggests that the slight difference in age was not important in determining social status and aggression in the lizards.

The competitive behaviours observed in the lizards follow the typical pattern for skinks in both captive (Torr & Shine, 1996; Langkilde et al., 2003) and free-living populations (Carpenter & Ferguson, 1977). However, our sample was limited to seven full and half sibling lizards, which was the only sample available at the time. Small sample size is a frequent limiting factor when studying non-model organisms, especially in a zoo, but by doing so we were able to investigate social behaviours in juvenile *E. stokesii*. It may be hard to extrapolate these particular results to all *E. stokesii*. Indeed, free-living populations are faced with different biological factors (e.g. family size, breeding pairs, number of offspring) and environmental stressors (e.g. predators, abnormal weather patterns, habitat destruction) which are likely to have an impact on the social behaviours of juvenile *E. stokesii*, however this is yet to be assessed.

Nevertheless, the underlying principle that juvenile *E. stokesii* are capable of forming a dominance hierarchy is a significant finding, which is supported by previous studies addressing dominance hierarchies in juvenile lizards. For example, it was found that juvenile *Anolis aeneus* are able to form a dominance hierarchy, (Stamps, 1984). High social status has also been found to be linked to ecological advantages. When investigating growth costs of territorial overlap in juvenile *A. aeneus*, Stamps (1984) found that higher status lizards had significantly less overlap in territories than lower status lizards. This demonstrates that one of the benefits of high social status is access to high-valued resources, in this case, a more exclusive territory.

Our results demonstrate that groups of social lizards can develop a dominance hierarchy, and that dominant individuals within this hierarchy had a larger body size. It is therefore possible that dominant individuals were more likely to gain access to food resources. Moreover, the level of aggression maintaining the hierarchy is correlated with the value of the resources over which lizards are competing. Social dominance and aggressive behaviour therefore likely have fitness consequences for lizards within a hierarchy. Our results add to the small but growing field of reptile sociality and, although they are limited to a captive population, suggest that there is much behavioural and social complexity to explore in this group of animals.

Conflicts of interest

None.

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Appendix 1. Table taken from Albers & Vries (2001) to calculate the difference in Elo ratings and expected outcomes. After each interaction, this table was used to give each rating difference between the two individuals and the corresponding expected chance of winning for the individual with the highest score.

Rating difference	Expected chance of winning	Difference	Chance	Difference	Chance
0>=dif<=3	0.50	122>=dif<=129	0.67	279>=dif<=290	0.84
4>=dif<=10	0.51	130>=dif<=137	0.68	291>=dif<=302	0.85
11>=dif<=17	0.52	138>=dif<=145	0.69	303>=dif<=315	0.86
18>=dif<=25	0.53	146>=dif<=153	0.70	316>=dif<=328	0.87
26>=dif<=32	0.54	154>=dif<=162	0.71	329>=dif<=344	0.88
23>=dif<=39	0.55	163>=dif<=170	0.72	345>=dif<=357	0.89
40>=dif<=46	0.56	171>=dif<=179	0.73	358>=dif<=374	0.90
47>=dif<=53	0.57	180>=dif<=188	0.74	375>=dif<=391	0.91
54>=dif<=61	0.58	189>=dif<=197	0.75	392>=dif<=411	0.92
62>=dif<=68	0.59	198>=dif<=206	0.76	412>=dif<=432	0.93
69>=dif<=76	0.60	207>=dif<=215	0.77	433>=dif<=456	0.94
77>=dif<=83	0.61	216>=dif<=225	0.78	457>=dif<=484	0.95
84>=dif<=91	0.62	226>=dif<=235	0.79	485>=dif<=517	0.96
92>=dif<=98	0.63	236>=dif<=245	0.80	518>=dif<=559	0.97
99>=dif<=106	0.64	246>=dif<=256	0.81	560>=dif<=619	0.98
107>=dif<=113	0.65	257>=dif<=267	0.82	620>=dif<=735	0.99
114>=dif<=121	0.66	268>=dif<=278	0.83	dif<=736	1.00

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Aliens in the backyard: Did the American bullfrog conquer the habitat of native frogs in the semi-deciduous Atlantic Forest?

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The American bullfrog *Lithobates catesbeianus* has a natural distribution in North America, but was spread by human activities in different regions around the world. It is listed as the most invasive amphibian species, affecting terrestrial and aquatic ecosystems and the community of native species. In addition, the American bullfrog is extensively associated with lethal pathogens, with high correlation of the presence of this species with population declines and extinctions. Here we test if this alien species has spread through the landscape, establishing populations at new locations. We used diverse methods including georeferencing of satellite images, ethnobiological interviews and field data to evaluate the dispersion and effects of *L. catesbeianus* introduction on amphibian composition, species number, and density of individuals in forest fragments in an Atlantic Forest landscape. We did not find any relationship between density of individuals, number of species or composition of the native anuran assemblages in forest fragments in relation to the presence or proximity of American bullfrog introduction points. Additionally, we found that the dispersion potential of this species in the studied landscape is zero, as it was only found in those fragments where it was specifically introduced 15 years ago. The species has not established new populations in the landscape. Although exotic, *L. catesbeianus* thrives in lentic habitats and has no apparent effect on the structural metrics of the native anuran assemblage. Despite this alien species exhibiting a capacity to adapt and survive at the point of introduction, its potential for propagation is limited probably by the fragmented terrestrial landscape and regional stream network.

Keywords: amphibians; anurans; biological invasions; community ecology; exotic species; fragmented landscape; freshwater biology; landscape ecology.

INTRODUCTION

The American bullfrog, *Lithobates catesbeianus* (Shaw, 1802), has a natural distribution that runs from northern Mexico to southern Canada, but with foci of introduction in different regions around the world (IUCN, 2019), indicating the species' adaptability in different latitudes and biomes. In Brazil, most introduction points are located in the Atlantic Forest biome (Both et al., 2011). Studies for the Atlantic Forest report that *L. catesbeianus* may compete acoustically with native species (Both & Grant, 2012), and prey upon a variety of species ranging from invertebrates to vertebrates, including many native amphibian species from different families (Silva et al., 2011; Boelter et al., 2012; Silva et al., 2016). In addition, bullfrogs were reported to be a reservoir of spores that transmit pathogens such as the fungus *Batrachochytrium dendrobatidis* (*Bd*), which is lethal to anurans (Schloegel et al., 2010; Schloegel et al., 2012; O' Hanlon et al., 2018), especially in highly anthropised biomes, such as the Atlantic Forest.

The Atlantic Rainforest is severely fragmented and has less than of 11 % of its original extent, including the

small forest fragments (Ribeiro et al., 2009). It harbours many endemic and non-endemic amphibian species, representing 7.7 % of amphibian species in the world (Rossa-Feres et al., 2017). Several declines in amphibian populations have been reported for the Atlantic Rainforest (Heyer et al., 1988, Weygoldt et al., 1989; Eterovick et al., 2005). However, the causes of many of these declines are not well understood (Stuart et al., 2004). Recently, studies showed that the fragmentation, disconnection of terrestrial and aquatic habitats, agricultural crops, livestock and the degradation of vegetation quality in the forest remnants have been associated with local extinctions of anurans in the Atlantic Rainforest (Becker et al., 2007; Ferrante et al., 2017). However, there are few studies about the possible impact of *L. catesbeianus* on native amphibian assemblages in the Atlantic Forest (Both et al., 2014). Considering the critical conservation situation of the biome and the wide distribution of this species, it is important to understand the dispersal capacity and the potential impact of this exotic species on the native anurans.

It is known that the landscape configuration, such as the arrangement of the fragments and the surrounding

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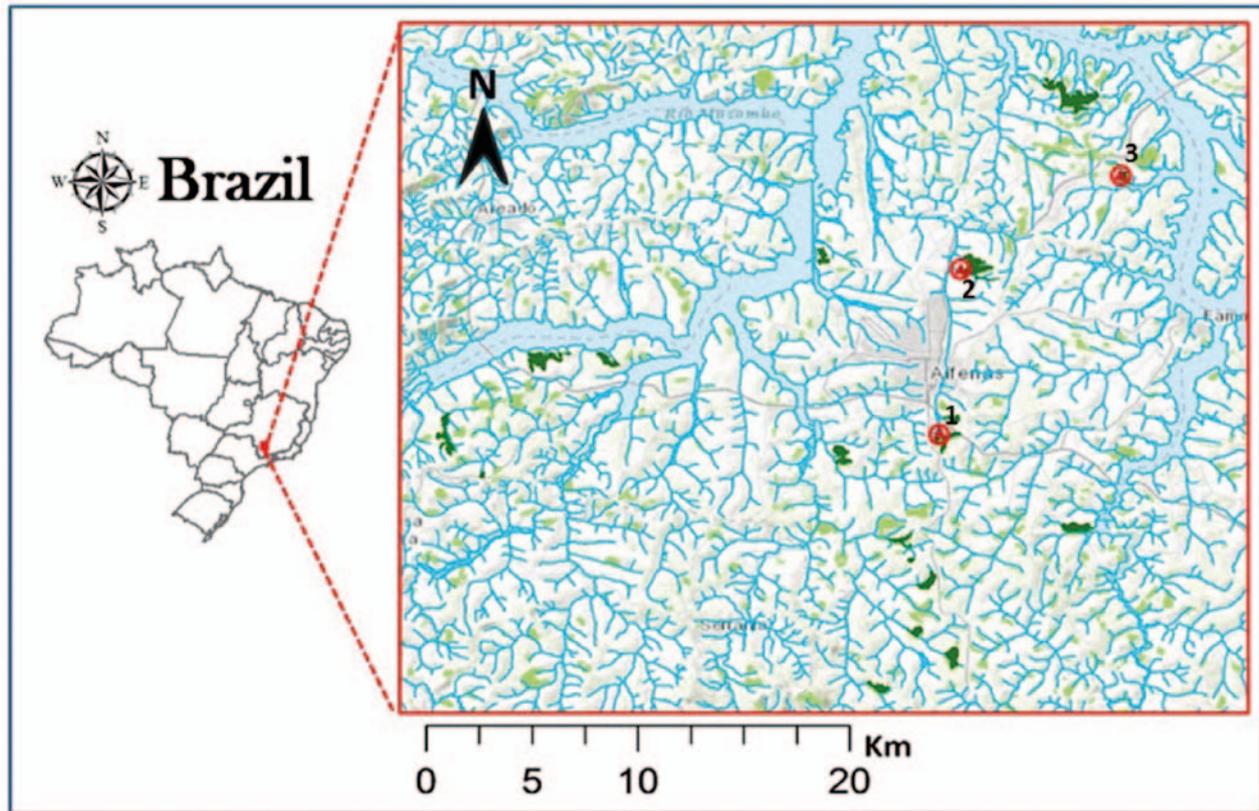


Figure 1. Studied landscape in Minas Gerais state, Brazil. The three introduction points of the exotic species *L. catesbeianus* are represented by circled red triangles, the forest fragments sampled are highlighted in dark green, and the other forest fragments present in the landscape are in light green. The stream network is highlighted in blue.

matrices affect anuran assemblages (Ferrante et al., 2017). The landscape configuration in protected areas, such as the forest-edge-agriculture gradient could facilitate the dispersal of *L. catesbeianus* (Madalozzo et al., 2016). However, little is known about how severely fragmented landscapes might affect the introduced *L. catesbeianus*. In this study, we evaluated 18 forest fragments surrounding three introduction points of the exotic *L. catesbeianus*, in order to test if the alien species has spread through the landscape, so establishing populations at new locations. We also tested the impact of *L. catesbeianus* on native anurans by relating the number of species, density and composition of the native anuran assemblages in these forest fragments with the distance from the *L. catesbeianus* introduction points.

MATERIALS AND METHODS

Study Site

The landscape investigated in this study was in the south of Minas Gerais state, Brazil (21°25'038.42" S; 45°56'053.21" W, Fig. 1.). The region has annual mean precipitation of 1,554 mm (Roldão et al., 2012), and is a typical agricultural area converted from original semi-deciduous Atlantic Forest, with predominance of sun coffee, sugarcane and cattle pasture.

Sampling Design

We mapped the current *L. catesbeianus* occurrence points in the landscape and the time and process of

introduction of this species by means of personal communications with rural workers and land owners. We sampled 18 forest fragments around three known points of bullfrog introduction. The anuran assemblages were sampled within each forest fragment by visual encounter surveys and playbacks of advertisement calls of local species. Each fragment was sampled simultaneously by three researchers along 300-m long and 20-m wide transects parallel to water bodies. Both post-metamorphic juvenile and adult individuals were considered in the sample. All forest fragments were sampled in three nocturnal occasions. The surveys were conducted between December 2011 and March 2012, comprising the local rainy season (Roldão et al., 2012) and the main amphibian breeding season in the Atlantic Forest (Haddad et al., 2013).

Statistical analyses

We compared the number of species between forest fragments with presence of bullfrog, or with bullfrogs at its periphery, and forest fragments with total absence of bullfrogs through rarefaction. We standardised our dataset by the number of individuals, which accounts for sample heterogeneity maintaining the intrinsic abundance relationships between species (Gotelli & Colwell, 2001). We used an analytical method to generate valid confidence intervals (CIs) for the rarefaction curves, which do not converge to zero at the maximum sample size (Colwell & Elsensohn, 2014). We also used analysis of variance (ANOVA) to compare the number of species and

abundance of native species between forest fragments with presence and absence of bullfrogs.

We also used ArcGIS 10 software to measure the shortest linear distance between the points of introduction of the alien species and the distance to each forest fragment surveyed. We used the shapefile of Atlantic forest fragments (SOS Mata Atlântica) and the stream network of the Brazilian ANA (Agência Nacional de Águas). We then, through simple linear regression, related the number of native species and the density of individuals in the forest fragments to the distance in a straight line between the nearest point of *L. catesbeianus* introduction for each fragment. We also used permutational multivariate analysis of variance (PERMANOVA), based on the Bray-Curtis similarity index for abundance and Sorensen data for presence and absence data, to investigate the relationship between the composition of native species and the distance in a straight line between the entry point of *L. catesbeianus* closest to each fragment. The significance values obtained in the analyses were based on 999 permutations. All analyses were performed in the R (R Core Team, 2017) computational environment with the vegan community ecology package (Oksanen et al., 2018).

RESULTS

Local farmers reported that human-caused introductions occurred in three locations at least 15 years ago (Fig. 1). Via interviews with landowners and rural workers in the properties, we received three different explanations for the introduction of the exotic species in the regional landscape: 1 - fugitives, where some individuals escaped from breeding ranches to the environment and established a breeding population in a pond adjacent to the ranch (less than 100 meters); 2 - intentional introduction in an artificial lake at the edges of a forest fragment for consumption; 3 - liberation of individuals with the mistaken intention of promoting local biodiversity. Three populations were observed in artificial ponds, with no water flow, at the edge of forest fragments. Although all the artificial lakes housing *L. catesbeianus* had connections to natural waterbodies running into adjacent forest fragments, bullfrogs were only observed in the fragment corresponding to introduction point 3 (Fig. 1), although the other two bodies of water with the presence of the exotic species were in contact with forest fragments.

In the 18 sampled forest fragments, we found 18 native species of anurans belonging to six families: Bufonidae, Craugastoridae, Hylidae, Leptodactylidae, Odontophrynidae and Phyllomedusidae. The number of anuran species varied from 1 to 7 between forest fragments, and two forest fragments harboured no anuran species. Although depauperate, the accumulation curves were similar between fragments with and without *L. catesbeianus* (Fig. 2). Both the number of species (ANOVA: $F_{1,16} = 0.315$, $p = 0.582$, Fig. 3A) and density of individuals (ANOVA: $F_{1,16} = 0.014$, $p = 0.906$, Fig. 3B) were similar between forest fragments with and without *L. catesbeianus*. We found no relationship between either the distance from the American bullfrog

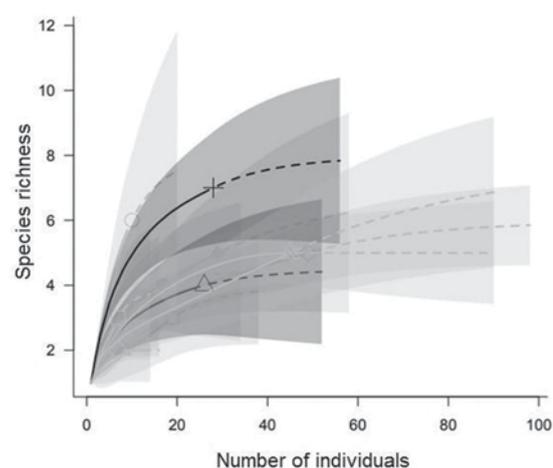


Figure 2. Species rarefaction curves (solid lines) in the invaded (black) and non-invaded (grey) fragments by the bullfrog in Minas Gerais state, Brazil. Dotted lines indicate number of species extrapolation. Shaded grey area represents the 95 % confidence intervals.

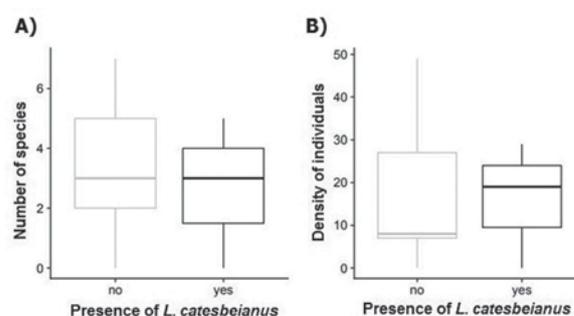


Figure 3. Number of species (A) and density of individuals (B) in the three invaded (black) and 15 non-invaded (grey) fragments by the bullfrog in Minas Gerais state, Brazil.

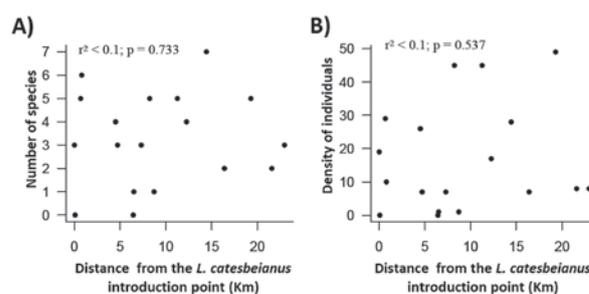


Figure 4. Relationship between the distance to the introduction point of *L. catesbeianus* and metrics related to the native anuran assemblages for each forest fragment.

introduction points and the number of species ($r^2 < 0.1$, $p = 0.733$, Fig. 4A) or density of individuals ($r^2 < 0.1$, $p = 0.537$, Fig. 4B) in the native anuran assemblages of the sampled forest fragments. Based on presence/absence data, NMDS analysis also failed to detect an impact on either structure of the native anuran assemblages within the forest fragments (PERMANOVA: $R^2 = 0.07$, $p = 0.314$, Fig. 5A), or population density data (PERMANOVA: $R^2 = 0.08$, $p = 0.296$, Fig. 5B) in relation to the distance from

the introduction points of this exotic species. Inside the forest fragment with occurrence of *L. catesbeianus*, an individual of this species lacking a hind limb was captured. At the same site, a second individual bearing the same morphological anomaly was seen but not collected.

DISCUSSION

The results of ecological modelling conducted by Giovanelli et al. (2008) and Forti et al. (2017) are the closest to our field-based findings. Although Giovanelli et al. (2008) did not include the introduction points of *L. catesbeianus* in the landscapes we studied, modelling does not suggest the invasion of *L. catesbeianus* to the southern region of the state of Minas Gerais, where the predominant biome is a highly fragmented semi-deciduous Atlantic Forest. Therefore, distribution models based only on the environmental affinities of the species and which ignore whether the species in question has the dispersal capacity to overcome the natural or anthropic geographic barriers present in the studied region should be interpreted with caution (e.g., Loyola et al., 2012). Our results showed that the bullfrog was found only in the areas into which it was originally introduced, and had not dispersed to closely adjacent fragments, even when they were downstream. Thus, in the study region, as well as for most of the semi-deciduous Atlantic Forest, bullfrogs appear to have a null dispersion potential either by aquatic route (due to the water current) or by terrestrial route (due to the impermeability of the anthropic matrix).

However, we recommend that additional landscapes with invasive Bullfrog populations older than 15 years of introduction should also be studied to access the real dispersal potential of this alien species. We do not rule out that some bullfrog individuals may have dispersed

into the landscape in the past, however our results point to a failure to establish new populations.

Forest fragmentation has been shown to be a barrier to forest-dependent anurans, causing inbreeding and genetic erosion in anuran populations in forest fragments in the Atlantic Forest (Dixo et al., 2009). The presence of morphological anomalies reported in this study coincides with the pattern observed in populations of amphibians with strong degrees of inbreeding (Bessa-Silva et al., 2016), which may be indicative of the low genetic diversity of the studied populations. The interpretation of low genetic diversity in *L. catesbeianus* is speculative, as there are no genetic studies on this topic at the current study site. However, the small number of individuals introduced into the sites associated with the strong commercial selection of the breeding individuals suggests inbreeding. Future studies should evaluate the genetic diversity of populations of this exotic species and their possible natural decline induced by genetic factors. Our results suggest that even years after the introduction of the bullfrog, the number of species in forest fragments with and without the bullfrog are similar. We also did not observe a reduction in the density of adult anurans at the sites where the American bullfrog was introduced. Our results echo other studies from southern Brazil where the presence of the invasive *L. catesbeianus* in ponds also did not change the composition of native amphibian assemblages (Both & Melo, 2015). In aquatic vertebrate communities invaded by the American bullfrog in Uruguay, rarefaction curves indicated no changes in species richness patterns (Gobel et al., 2018). However, Gobel et al., (2018) observed that the abundance of tadpoles for some species was significantly lower, although this was not universally the case for the tadpole stages of all species in the sampled anuran assemblage (Gobel et al., 2018). For the Atlantic Forest, the habitat fragmentation

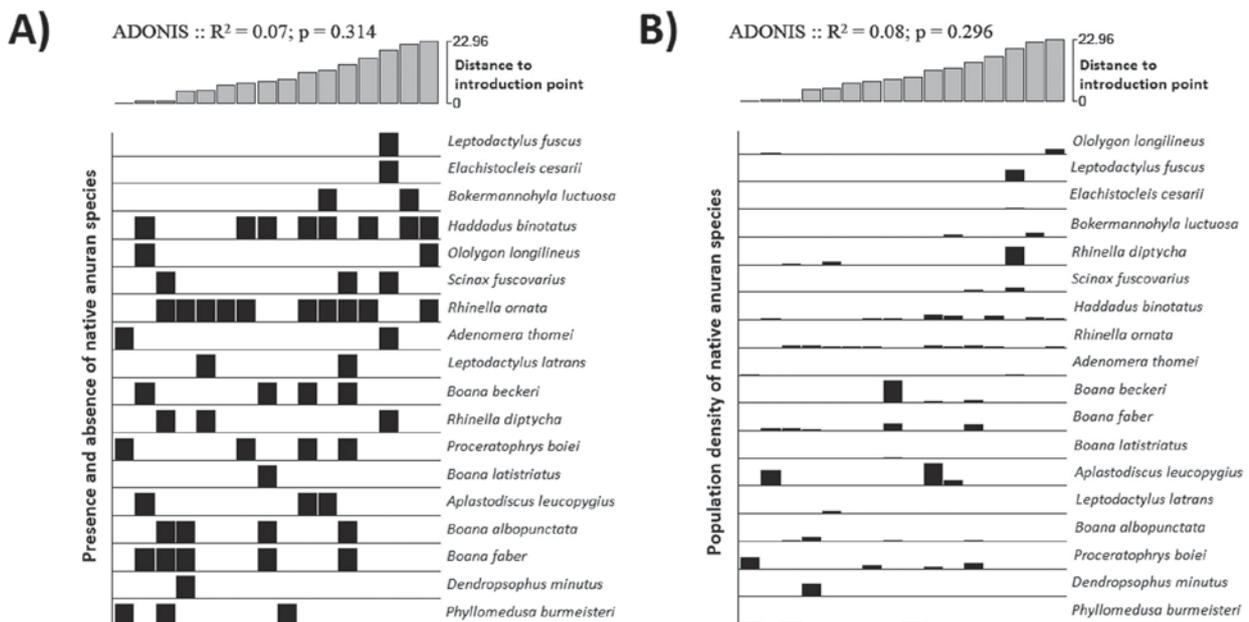


Figure 5. Composition of the native anuran assemblage in relation to distance to introduction point of *L. catesbeianus*. **(A)** Presence/absence of native species and **(B)** density of individuals.

and agricultural crops such as sugarcane and pastures for the livestock (Ferrante et al., 2017) seem to constitute a stronger threat to anuran assemblages by affecting density of individuals as well as richness and composition of species than does *L. catesbeianus* introduction.

In the studied landscape, species such as *Leptodactylus latrans* and *Leptodactylus labyrinthicus* occur (Ferrante et al., 2015). These species can be considered ecologically similar to American bullfrogs because of their equivalent body size, habitat use and reproductive mode (Kaefer et al., 2007, Haddad et al., 2013). The introduction of *L. catesbeianus* in a Cerrado (Brazilian Savanna) landscape has been reported to extirpate *L. latrans* and *L. labyrinthicus* (Batista, 2002). However, *L. latrans* and *L. catesbeianus* do not overlap in their food niche (Silva et al., 2016). *Leptodactylus labyrinthicus* naturally does not occur inside forests (Haddad et al., 2013), it was not found in any of the forest fragments, and no structuring of *L. latrans* presence or density was observed with greater proximity to *L. catesbeianus* introduction points (Fig. 5ab). The fact that *L. latrans* and *L. labyrinthicus* have habitat preferences for open areas (Haddad et al., 2013) may explain the absence of these species in the forest fragments without signifying any decline that can be associated with the presence of the American bullfrog in the landscape.

Even though studies report heavy predation by *L. catesbeianus* on native frogs (Silva et al., 2011, Boelter et al., 2012), it is not known to what extent predation by this exotic species affects recruitment or alters the density of native anuran species populations. In the landscape studied here, our data suggests that the amphibian density was not affected by the presence of the American bullfrog. In addition, juvenile bullfrogs are also reported to be eaten by native anuran species (Silva & Ribeiro-Filho, 2009), indicating that the species now forms part of the food web in the Atlantic Forest, and may therefore be under density-dependent regulation. Accordingly, our results add a new layer of complexity to studies of the introduction of the bullfrog in Brazil, demonstrating both a surprisingly imperceptible effect of this exotic species on the native assemblages at a landscape level.

The exotic species *L. catesbeianus* has been proposed as a reservoir of the *Bd* fungus (Schloegel et al., 2010), as well as being responsible for introducing an Asian *Bd* lineage in Brazil (O' Hanlon et al., 2018). However, in the current study, we found no evidence of native species declines or density reduction of amphibians in relation to the presence and distance of American bullfrog introduction foci, which is contrary to what would be expected if bullfrogs were either dispersing or harbouring some lethal pathogen.

CONCLUSIONS

Although *L. catesbeianus* is considered to be a generalist species due to its ability to survive in disturbed environments (IUCN, 2019), this species tends to establish populations in lentic water bodies (Boelter et al., 2012, Haddad et al., 2013, IUCN, 2019). As a result, the local waterbody network may also act as a natural

barrier in the study landscape, since it is composed mostly of flowing streams. In addition, severe landscape fragmentation and agricultural crops may further limit bullfrog dispersal capacity through land. Our results suggest that, although exotic, *L. catesbeianus* is not invasive at the landscape level and does not exert a negative impact on native anuran assemblages in the severely fragmented landscapes of the Atlantic Forest investigated by this study.

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Chytrid infection in Asia: How much do we know and what else do we need to know?

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We conducted a systematic review to evaluate the knowledge base for amphibian chytrid *Batrachochytrium dendrobatidis* (*Bd*) infection in the continent of Asia. Despite an indication of geographic bias in terms of studying chytrid fungus distribution in Asia, 167 amphibian species (145 spp. native to Asia) from 16 countries have been reported as infected with *Bd*. Our meta-analysis shows that overall prevalence is 8.19 % (out of 28,433 samples), and *Bd*-positive rate in amphibia significantly varies among sampling sources ($\chi^2= 380.57$, $DF= 6$, $P< 0.001$) and age categories ($\chi^2= 22.09$, $DF= 2$, $P< 0.001$). We used Kernel Density analysis to produce a hotspot map for chytrid infection, and Digital Elevation Model to understand the distribution of chytrid positive locations across different elevations. In our meta-analysis, most of the *Bd*-positive sites range between 4.45–27.49 °C, 167–4,353 mm rainfall, 10–40°N, and at lower elevations (<500 m). Using land cover analysis, we did not find a statistically significant difference among six different land cover categories in relation to the prevalence of *Bd* across Asia. Although no mass die-off events have been reported so far, Maximum Entropy modelling shows that *Bd* distribution and infection may potentially occur across a vast region of south-east Asia. In conclusion, we call for more systematic research and monitoring strategies in place for countries with little to no information, but have a moderately higher risk of chytrid distribution and infection.

Keywords: chytrid fungus, *Batrachochytrium dendrobatidis*, chytridiomycosis, emerging infectious disease, amphibian conservation, amphibian disease, Asia

INTRODUCTION

Chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), causes chytridiomycosis disease; one of the greatest threats to global amphibian diversity (Bower et al., 2017; Gascon et al., 2007; Lötters et al., 2009; Skerratt et al., 2007; Vredenburg et al., 2010). This infectious disease, first reported in 1998 (Berger et al., 1998), is known to infect over 700 species and affect at least 501 species from multiple continents (Olson et al., 2013; Scheele et al., 2019).

Evidence supporting the connection between *Bd* infection and frog declines started unfolding from Australia (Woodhams & Alford, 2005), Europe (Mutschmann et al., 2000), North America (Vredenburg et al., 2010), Central America (Lips et al., 2006, 2008; Woodhams et al., 2008) and Asia (Kusrini et al., 2008; Une et al., 2008). Since then, chytridiomycosis has been regarded as one of the most devastating diseases, causing events of mass mortality, population declines, and even extinction of amphibian populations around the world (Lips et al., 2006; Skerratt et al., 2007). Evidence also suggests that some amphibian populations survive beyond the initial outbreaks, and some with dramatically reduced abundances and distributions (Crawford et al., 2010; Vredenburg et al.,

2010). The cause of widespread and severe "enigmatic" declines in about 43 % of all amphibian species around the world (Stuart et al., 2004) has now become obvious, and the fungus *Bd* has become a major conservation concern worldwide.

Despite *Bd*'s global distribution, significant loss of amphibian diversity has occurred within restricted geographic regions (Garner et al., 2005; Lips et al., 2006; Vredenburg et al., 2010). In Asia, *Bd* is a post-2007 phenomenon, with chytrid was first reported in December 2006 (published by Une et al. in 2008). However, a later study finds the presence of chytrid in a museum specimen of *Andrias japonicas* collected in 1902 (Goka et al., 2009). Compared to other continents, reports of chytrid in Asia (Bai et al., 2010; Kusrini et al., 2008; Mendoza et al., 2011; Nair et al., 2011; Parto et al., 2013; Rowley et al., 2013; Savage et al., 2011; Swei et al., 2011; Une et al., 2008; Voros et al., 2012; Yang et al., 2009) are comparatively recent, and severe declines of wild amphibian population have not been reported. This could be due to the lack of adequate and detailed research on *Bd* (Molur et al., 2015) and on amphibian demography in Asia (Lips, 2016). The dearth of information and the insidious nature (i.e. pathogenicity, epidemiology and possible impacts) of potential chytridiomycosis are of concern to amphibian

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conservation in Asia.

Despite no record of a chytridiomycosis epidemic leading to mass die-off of any amphibian population in Asia, *Bd* has been detected both in native and exotic amphibian species at a relatively low prevalence. Several studies (Lötters et al., 2009; Puschendorf et al., 2009; Rödder et al., 2009; Ron, 2005; Swei et al., 2011) have used different environmental factors to model the distribution of *Bd* in Asia. The results suggest that *Bd*-suitable conditions exist across Asia. Suitable environmental conditions, however, do not guarantee the occurrence of *Bd* because dispersal and persistence also play a crucial role. Evidence has been gathered in support of both a 'novel pattern hypothesis', i.e. *Bd* spreads anthropogenically, and is an 'endemic pathogen hypothesis', i.e. *Bd* is endemic (Goka et al., 2009). Ecological factors and the combination of both hypotheses are required to comprehensively explain the known distribution of *Bd* in Asia.

From an ecological perspective, the spatial distribution of a pathogen is important to understand its pathogenicity, prevalence and epidemiology. The applicability of remote sensing and Geographical Information System (GIS) as tools to study species epidemiology have allowed researchers to understand disease occurrence and contributing environmental factors (Rytönen, 2004). Previous reviews on the epidemiology of chytridiomycosis (Fisher, 2009; Skerratt et al., 2007) provide a comprehensive global overview; however, the localised perspective of Asia remains masked owing to a lack of data from Asia, compared to the plethora of information from other continents. However, spatial analysis has often been applied for epidemiological studies in a data-poor context (Graham et al., 2004).

Here we conducted a systematic review and meta-analysis of existing information and presented a comprehensive overview on chytrid infection in Asia. With an aim to evaluate the current knowledge base, we have framed this review to answer the following questions;

- i) How does the research effort on *Bd* vary a spatial and temporal scale within Asia?
- ii) What is the prevalence of chytrid in different amphibian species and Asian countries?
- iii) What is the difference in *Bd* prevalence across different sources (wild, pet store, food market, etc.) and age categories (adult, juvenile and tadpole) of amphibian samples?
- iv) What is the spatial distribution of chytrid hotspots in Asia (based on existing data for *Bd*-positive localities)?
- v) What is the range of environmental and geographical factors (e.g. land cover, temperature, rainfall, elevation and latitude) for *Bd*-positive localities in Asia?
- vi) How could *Bd* potentially spread or distribute in Asia?

METHODS

Literature search protocol and data collection

To compile all the relevant literature published between 2007 and 2018 on chytrid infection in Asia, we made a comprehensive search in Google Scholar, PubMed, PubMed Central and ResearchGate using keywords

such as, 'chytridiomycosis in Asia', '*Batrachochytrium dendrobatidis* in Asia', 'chytrid fungus infection', '*Bd* haplotypes', and other combinations of these keywords. We screened titles, abstracts and keywords of papers that are found from these searches to determine literature relevant to chytrid and its infection within Asia. We also conducted a snowball sampling of the bibliographic section of papers to find new papers that were missing from the search made with the above-mentioned keywords. Our selection protocol resulted in 53 articles for the review, of which 50 were peer-reviewed journal articles and three were conference papers (see Supplementary Material 1).

We have used 'literature', 'paper', and 'publication' interchangeably; however, we made a distinction between these words and 'study'. For example, when a paper deals with samples from several Asian countries, we treated each country as an individual study for the convenience of data handling and analysis. Therefore, one 'paper' or 'publication' may represent one or several 'studies'. The age of each sampled species was considered as adult unless otherwise mentioned in the paper. All data described in Table 1 were collected for each country and amphibian species studied for *Bd* infection across Asia. To conduct the meta-analysis, each publication was carefully read and data were extracted to address the questions asked in this review.

Table 1. Different data extracted from publications to address questions set for the review.

Data types	To address questions posed in Introduction	Outcomes of corresponding analysis
a) Number and corresponding year of peer-reviewed literature relevant to chytridiomycosis in Asia between 2007 and 2018	(i)	a) The trend of <i>Bd</i> related research efforts in Asia both at spatial and temporal scale
b) Asian countries sampled to investigate chytrid infection		
c) Amphibian species sampled to investigate chytrid infection	(ii)	b) Number of native and exotic amphibian species infected by <i>Bd</i> in Asia
d) Volume of samples collected and test results from each country and per species		c) Prevalence of chytrid infection in different species and countries across Asia
e) Total number of amphibian samples collected from different sources and age categories	(iii)	d) Understanding of the trend of chytrid infection in amphibians from different sources and age categories
f) Localities of <i>Bd</i> -positive reports and number of positive and negative samples of that corresponding localities across Asia	(iv)	e) Understanding the hotspots for <i>Bd</i> infection across Asia
g) Environmental factors (temperature and rainfall) form <i>Bd</i> -positive localities within Asia	(v)	f) Suitable range of environmental and geographical factors for <i>Bd</i> infection in Asia
h) Geographical factors (land cover, elevation and latitude) from <i>Bd</i> -positive localities within Asia		
i) Bioclimatic variables (n=19) of <i>Bd</i> -positive localities within Asia	(vi)	g) Future potential distribution of <i>Bd</i> across Asian regions

GIS database preparation and analysis

From the selected literature, we gathered 209 *Bd*-positive locations across Asia. The positive locations are more in number but we did not extract data from papers that did not provide any coordinates. In papers where the coordinates of positive localities were mentioned, we directly extracted those. In papers where positive locations were shown only in a map, we georeferenced the map and extracted the coordinates of *Bd*-positive locations. In few cases, we failed to extract exact locations from the study map without coordinates, and so we contacted the corresponding author of those papers to ask for the positive site locations. We extracted the elevation data for every point location using Global Multi-resolution Terrain Elevation Data 2010 (GMTED2010; downloaded from EROS; <https://earthexplorer.usgs.gov>). From the downloaded GMTED Digital Elevation Model, we used mosaic (ArcGIS software setting the parameter for projection system as WGS_1984_World_Mercator and pixel type as 16-bit signed integer) to obtain a single image. We used "extract multi values to point" tool in ArcGIS software to extract elevation for all point locations. We also employed Kernel Density in ArcGIS software to identify the hotspots of likely *Bd* occurrence (Silverman, 1986). We did not consider *Bd*-positive locations without having exact data on sample size and *Bd*-positive records in that respective location, hence we selected 193 *Bd*-positive locations for Kernel Density analysis. In few papers, amphibian sample size was available without any

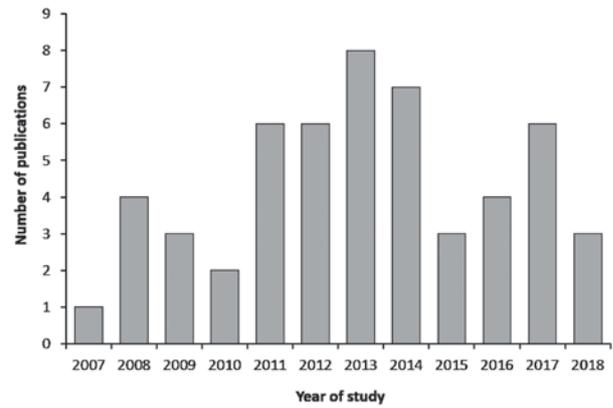


Figure 1. Total number of articles relevant to chytrid infection in Asia published per year between 2007 and 2018. Literature searches resulted into 53 articles, of which 50 were peer-reviewed articles and three were conference papers. The most number of articles were published in 2013.

specific location - just mentioning the name of city or state. For such cases, we set a point where most samples were taken from that corresponding city or state and used that for all respective samples. We performed maximum entropy (Maxent) modelling for *Bd* distribution (Phillips et al., 2006). To perform the distribution modelling, we downloaded 30 arc seconds (~1 km²) resolution bioclimatic variables data in raster format from WorldClim

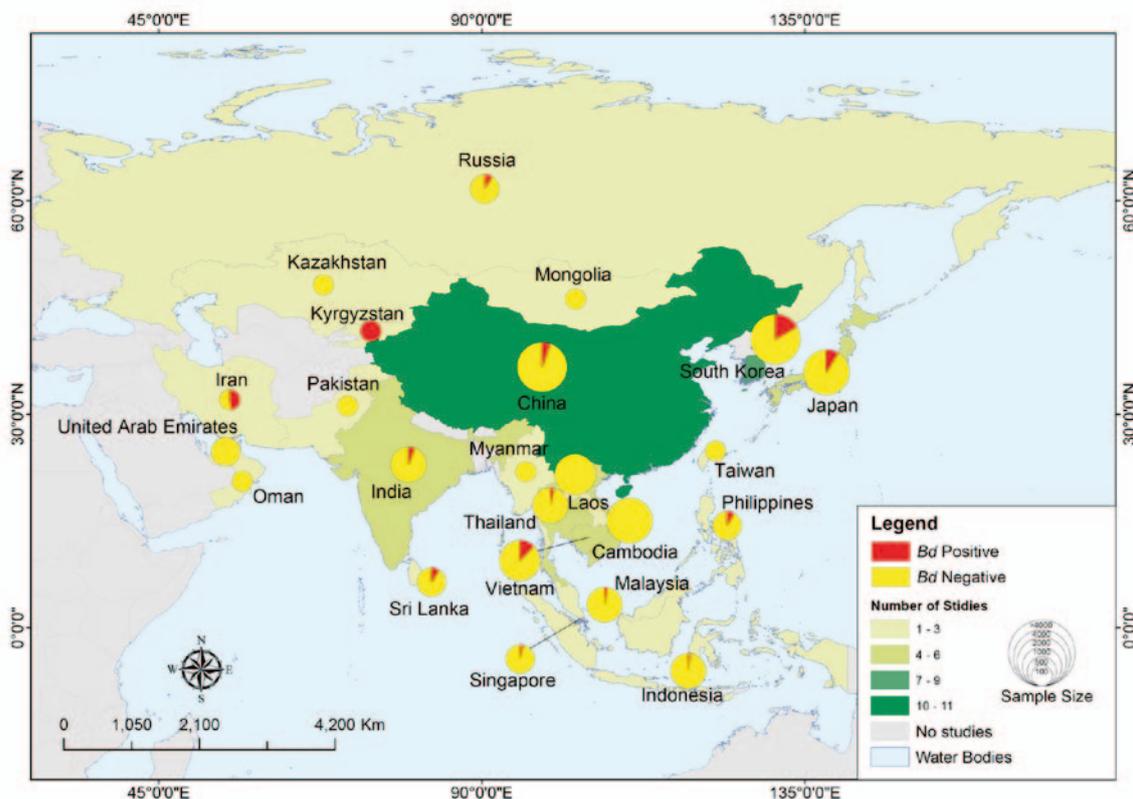


Figure 2. Number of studies conducted in different countries across Asia between 2007 and 2018. When we found a published article dealing with samples from several countries, we treated each country as a separate study for the convenience of data handling and analysis. Chytrid was most studied in China within this period of time. It should be noted that data from Taiwan were treated separately to China. Pie chart to each corresponding country represents the prevalence data of chytrid infection.

version2 (<http://worldclim.org/version2>) (Global Climate Data) (Fick & Hijmans, 2017). Country boundaries were downloaded from the GADM (Database of Global Administrative Areas) website to show the study area boundary. Downloaded raster data were pre-processed in ArcGIS software using SDM toolbox, downloaded from <http://sdmtoolbox.org> (Brown, 2014). Downloaded raster data were clipped by masking from the SDM toolbox using the study area shapefile. The clipped raster was then converted in ASCII format and the projection of converted ASCII raster data were set as WGS84. The occurrence dataset was prepared in csv format using MS Excel software converted to shapefile using csv to shapefile setting the projection as WGS84. The effects of spatial clusters of localities were reduced by spatially rarefying the occurrence dataset. The 30 arc second resolution coordinate bias dataset were downloaded (<http://sdmtoolbox.org>) to fit the model. Maxent modelling software (Version 3.4.1) was downloaded (accessed on 8 May 2019) in jar format from American Museum of Natural History (https://biodiversityinformatics.amnh.org/open_source/maxent).

The Maxent modelling is a probability statistic that is calculated based on the existing location correlated with the bioclimatic variables. However, the probability statistic does not mean that the higher level of probability will surely ensure *Bd* presence; rather it denotes strong possibility of having *Bd* infection, and vice versa. Kernel Density calculation represents the density of the existing occurrence, and Maxent modelling forecasts the possible location of *Bd* occurrence.

We conducted a land cover analysis to see whether *Bd* prevalence varies in different land cover patterns. To do this, we extracted the land cover information of sampling sites from the reviewed articles; if it was not mentioned we extracted manually, placing all the sampling points in Google Earth at a higher resolution (5m zooming scale). We divided land cover into six categories, i.e. agriculture, fallow land, forest, vegetation, water bodies and built-up. We did not consider the positive locations/points for this analysis without having prevalence data on that corresponding point. Therefore, the analysis was conducted on 182 points. The most recent land cover information has been considered because not all the locations have time series data in Google Earth.

We performed Chi-square (χ^2) analysis to understand whether *Bd* prevalence rate varied based on different sources and age categories. A non-parametric Kruskal-Wallis test was used to analyse significant differences in prevalence among different land cover categories. We also used post-hoc tests to compare difference between two categories. We conducted all statistical tests using R version 3.5.1 (R Development Core Team, 2018).

RESULTS

Research efforts on amphibian chytrid in Asia

Amphibian chytrid fungus research in Asian countries has been consistent since its discovery. The number of scientific articles published between 2007 and 2018 does not exceed eight articles in any year (Fig. 1). These studies are spread across 23 countries (out of 49 Asian countries;

Taiwan has been considered as a separate country for the convenience of analysis). It is notable that six countries (i.e. Cambodia, China, Japan, South Korea, Thailand and Vietnam) represent 60 % of the total studies conducted in Asia (Fig. 2). Other Asian countries (except for Cambodia, China, Japan, South Korea, Thailand and Vietnam) have fewer than three or no studies on *Bd* infection on amphibians.

Prevalence of chytrid infection in Asia

A meta-analysis shows that 8.19 % samples (out of 28,433 samples) were *Bd*-positive (Table 2). Regardless of sample size investigated per country, country-wise prevalence rate shows that Kyrgyzstan has the highest rate (100 %), followed by Iran (47.25 %), South Korea (16.39 %), and Cambodia (12.04 %). The rest of the 19 countries have a prevalence rate below 10 %, Laos being the lowest (0.24 %).

Across the published literature, samples were taken from seven different sampling sources (i.e. wild, food market, commercial farm, pet store, zoo and aquarium, preserved, and imported specimens) to evaluate the prevalence of *Bd*. The proportion test suggests that the *Bd*-positive rate for each sampling source significantly differs ($\chi^2= 380.57$, DF= 6, $P < 0.001$) from other sampling sources. Post-hoc pairwise comparison shows that pet store frogs have significantly higher *Bd* prevalence than all other sources ($P < 0.01$ for each comparison). Likewise, statistical test reveals that the *Bd*-positive rate in each age category (i.e. adult, juvenile and tadpole) significantly differs ($\chi^2= 22.09$, DF= 2, $P < 0.001$) from others, though none of pairwise comparison shows significant difference (Table 3).

Impact of *Bd* on amphibian species in Asia

At least 167 amphibian species (146 anurans, 17 urodeles, and four caecilians) belonging to 27 families (19 anurans, seven urodeles, and one caecilian) from 16 Asian countries have been reported to be infected with *Bd*. The number of infected species is subject to change (likely to be higher) because many positive samples came from animals of unresolved taxonomic status (e.g. *Ceratophrys* sp., *Fejervarya* sp., *Limnonectes* sp., *Leptolalax* sp., *Megophrys* sp., *Leptobrachium* sp., *Microhyla* sp., *Xenophrys* sp., *Odorrana* sp., *Gracixalus* sp., *Kurixalus* sp., *Philautus* sp., *Rhacophorus* sp., *Telmatobius* sp., *Ambystoma* sp. (Supplementary Table S1). Of *Bd*-positive amphibian species, 145 species were native and remaining 22 species were either introduced or traded from Africa, Europe and South America for research or commercial purposes. Without accounting for sample size and the number of amphibian species tested for *Bd*, our analysis shows that Japan has the highest number of species (40) infected with *Bd*, followed by China (32), India (23), South Korea (16), Cambodia (16), Malaysia (12) and Indonesia (9) (Supplementary Table S1).

Suitable range of geographical and environmental factors for *Bd* infection

The Digital Elevation Model suggests that *Bd*-positive sites are distributed between 0 and 2,701 m above sea level (Fig. 3); however, with the increasing elevation, the number of

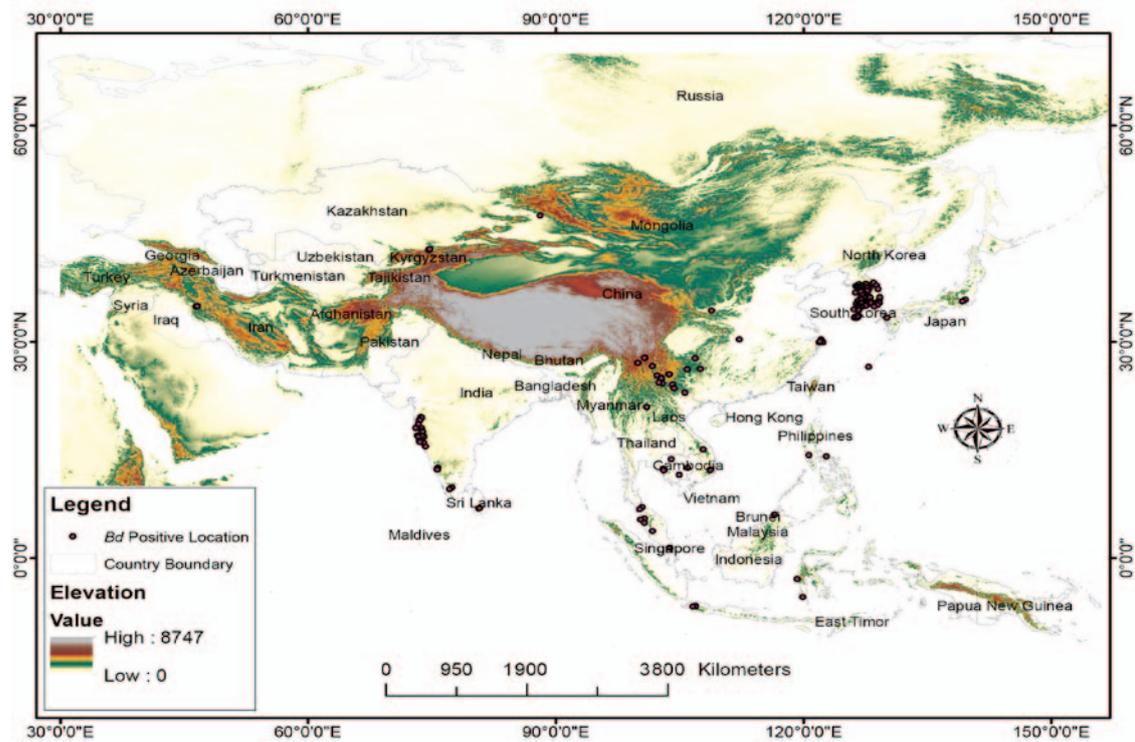


Figure 3. Relationship between elevation and positive localities for *Bd* infections within Asia using Digital Elevation Model. We gathered 209 *Bd*-positive locations across Asia from the selected literatures. Elevation for every point location was extracted from Global Multi-resolution Terrain Elevation Data 2010, downloaded from EROS (<https://earthexplorer.usgs.gov>).

Table 2. Percentage of *B. dendrobatidis* infection in Asian countries (samples were taken from wild or natural habitat, pet shops and food markets, commercial farms, zoos and aquariums, imported individuals, and historical samples preserved in museums). The Clopper Pearson binomial confidence intervals (95 %) were calculated using the R package PropCIs in R 3.5.1 version.

Sub-regions	Country	Year of first publication	Samples tested	<i>Bd</i> positive	Prevalence (%)	95 % Confidence interval
Central Asia	Kazakhstan	2011	4	0	-	0- 60.24
	Kyrgyzstan	2011	9	9	100.00	66.37- 100
East Asia	China	2007	7645	409	5.35	4.86-5.88
	Japan	2008	3640	308	8.46	7.58-9.41
	Mongolia	2011	23	0	-	0-14.82
	South Korea	2009	7411	1215	16.39	15.56-17.26
South Asia	Taiwan	2008	20	0	-	0- 16.84
	India	2011	821	47	5.72	4.24-7.54
	Pakistan	2011	5	0	-	0-52.18
South-east Asia	Sri Lanka	2011	117	10	8.55	4.17-15.16
	Cambodia	2011	1213	146	12.04	10.26-14
	Indonesia	2008	955	17	1.78	1.04-2.83
	Laos	2011	1677	4	0.24	0.06-0.61
	Malaysia	2011	578	13	2.25	1.2-3.81
	Myanmar	2011	62	0	-	0-5.78
	Philippines	2011	412	34	8.25	5.78-11.34
South-west Asia (Middle-east Asia)	Singapore	2013	419	13	3.10	1.66-5.25
	Thailand	2008	785	25	3.18	2.07-4.67
	Vietnam	2011	2133	15	0.70	0.39-1.16
	Iran	2013	91	43	47.25	36.69-57.99
	Oman	2016	60	0	-	0-5.96
North Asia	United Arab Emirates	2012	103	0	-	0-3.52
	Russia	2013	250	22	8.80	5.59-13.02
	Total		28433	2330	8.19	7.88-8.52

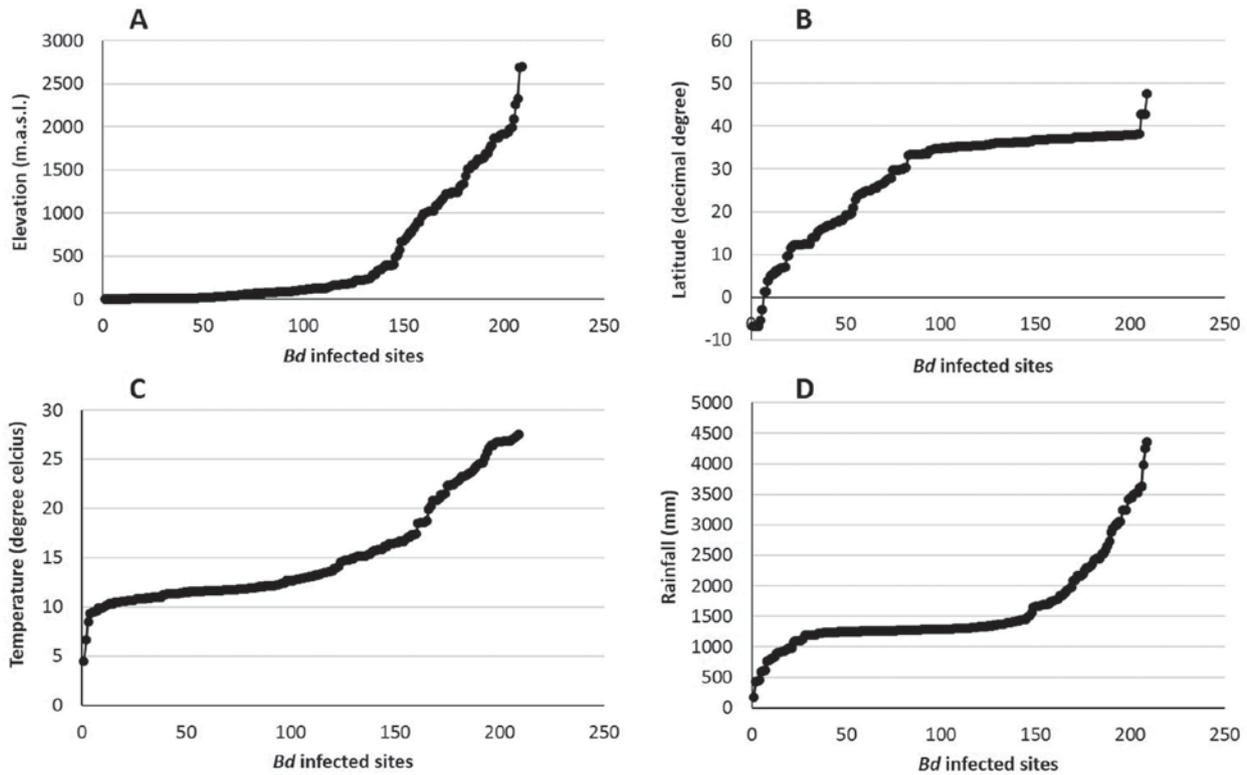


Figure 4. *Bd*-infected sites within Asia in different elevation (A), latitude (B) temperature (C) and rainfall (D).

Bd-positive sites decreases. The data set shows that more than two thirds (146 occurrences) of chytrid infected sites are below 500 m elevation followed by 14 occurrences between 500 and 1,000 m and 44 occurrences between the elevations of 1,001 and 2,000 m (Fig. 4A), suggesting that *Bd* is more widespread at lower elevations (<500 m above sea level). Our analysis also reveals that nearly 90 % (185 occurrences) of *Bd*-positive sites are located between 10°N to 40°N (Fig. 4B), suggesting a high risk area for *Bd* infection. The temperature of *Bd*-positive sites fell between 4.45 °C and 27.49 °C, where about 62 % of infected sites had temperatures of 15 °C or below, and 80 % sites were below 20 °C (Fig. 4C). Annual rainfall of *Bd*-positive sites ranged between 167 mm and 4,353 mm (Fig. 4D). It is notable that 70 % of *Bd* infected sites were located in regions with rainfall between 1,001 and 2,000 mm, and about 83 % infected sites are located in rainfall regions between 1,001 and 3,000 mm (Fig. 4D). Only about 10 % of infected sites were in regions with rainfall below 1,000 mm and 2 % of sites had rainfall of 500 mm or less.

Our analysis of *Bd*-positive sites shows that *Bd* prevalence does not significantly differ among different land cover patterns (Kruskal-Wallis Test, $\chi^2= 5.88$, DF= 5, $P> 0.05$). Post-hoc pair-wise comparison also shows that prevalence does not significantly differ between any two land cover categories. However, prevalence is slightly higher in fallow land than other categories. A maximum of 100 % prevalence was found in every land cover category except for 'vegetation' (Fig. 5).

Hotspot analysis for *Bd* infection in Asia

According to our spatial hotspot analysis, *Bd* prevalence

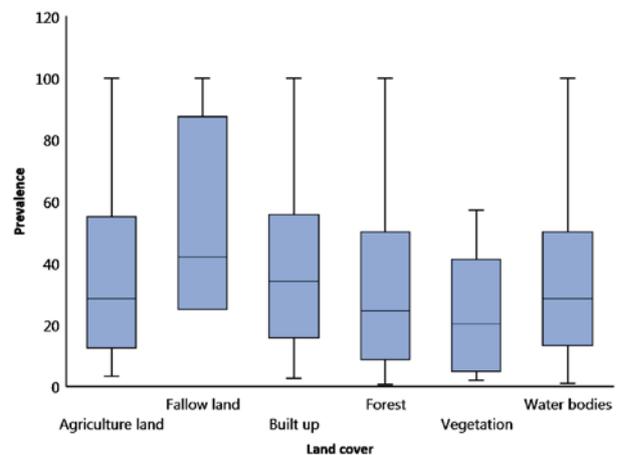


Figure 5. *Bd* prevalence in different land cover categories; quartile 2 and 3 are shaded with the dividing line as median, whiskers indicate quartile 1 and 4. Land cover information was extracted manually from Google Earth at 5m zooming scale when land cover of sampling sites was not mentioned in the respective paper. We only used *Bd*-positive point locations (182 in this case) that have prevalence data on that corresponding point.

rate in *Bd*-positive localities was higher in the Korean peninsula, Japan, Thailand, Vietnam, Cambodia, the southern part of China; and moderately higher infection rates were observed in Sri Lanka, Singapore, Indonesia, southwestern part of India, and the northwestern part of Iran, Philippines and Malaysia (Fig. 6).

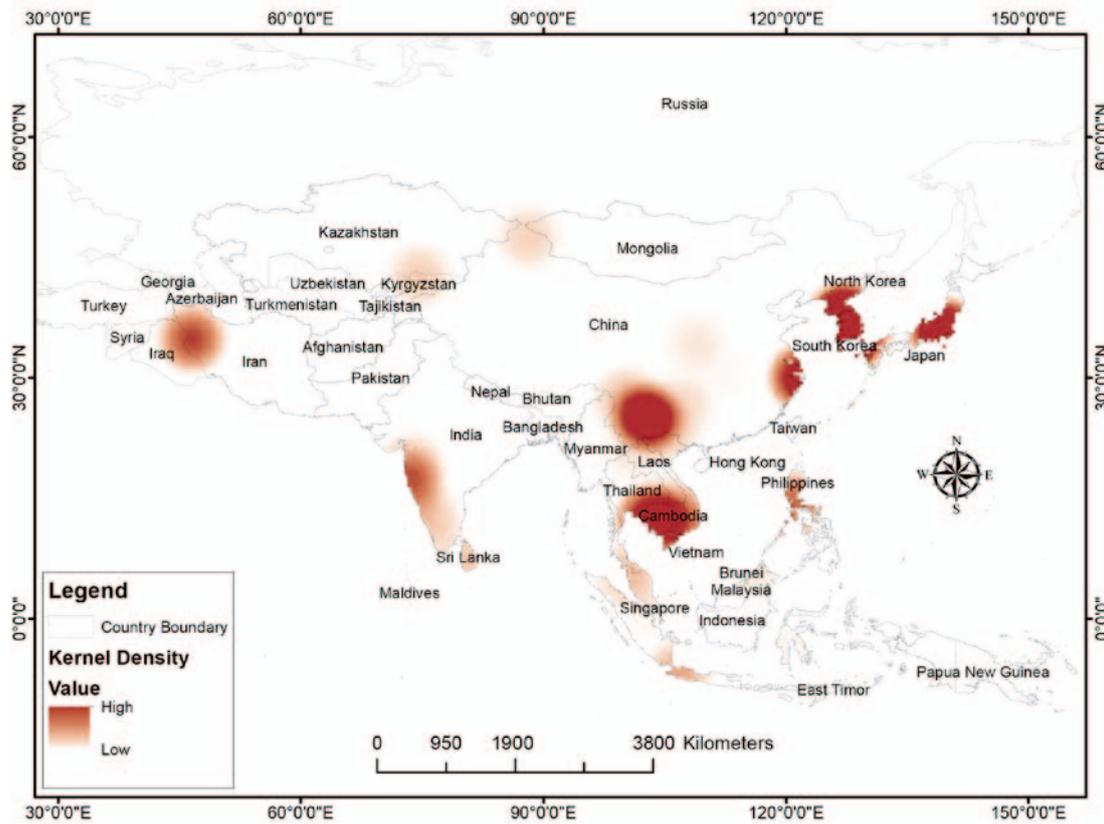


Figure 6. Kernel Density analysis based on *Bd*-positive sites highlights the hotspots for *Bd* infection in Asia. We used *Bd*-positive locations that have confirmed *Bd*-prevalence data, along with some points without specific coordinate locations only mentioning the city or state as the study area. In those cases, we set a point where most samples were taken from that corresponding city or state and used this point for all respective samples, resulting in 193 positive points for this analysis.

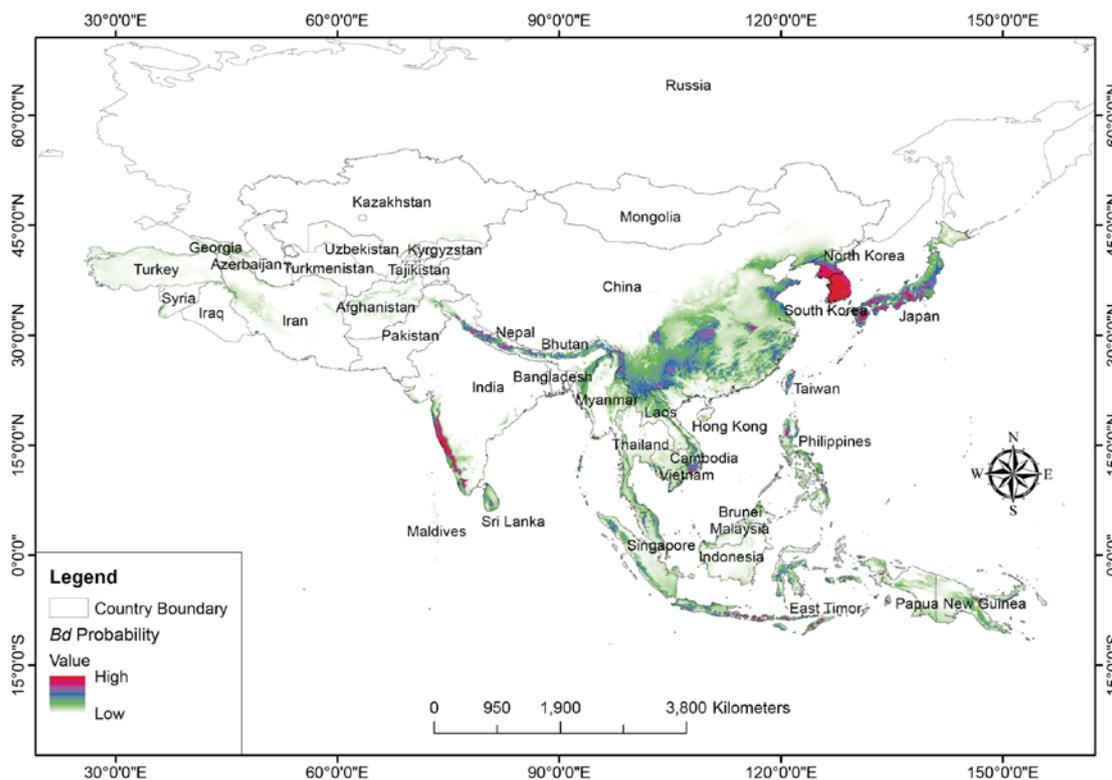


Figure 7. Potential distribution of *Bd* infection in Asia based on Maximum Entropy Modelling. We used 30 seconds (~1 km²) resolution bioclimatic data that was downloaded from WorldClim version2 (<http://worldclim.org/version2>) for the model. 30 arc second resolution coordinate bias dataset was also downloaded from <http://sdmtoolbox.org> to fit the model.

Probability of *Bd* distribution in Asia

The Maxent modelling showed the probability of *Bd* infection based on the existing infected locations (Fig. 7). Korea, Japan, the Western Ghats of India, and southern part of Indonesia had a higher probability of *Bd* infection. The Himalayan mountainous region also showed a moderate level of probability of *Bd* infection. The results showed that the oriental region and the south-eastern part of China have a moderate probability of having *Bd* infection.

DISCUSSION

Research gaps on *Bd* in Asia

The continent of Asia represents a significant portion of global amphibian diversity and a high level of endemism, however lesser focus has been put on the emergence of chytrid in this region compared to Australia, Europe, South and North America (Swei et al., 2011). The number of relevant journal articles (i.e. 53) in Asia are meagre considering the potential threat that chytrid fungus poses to amphibian populations worldwide. The geographic bias in survey effort is clearly evident (Fig. 2) from this review, where countries with no and limited investigation on *Bd* in Asia have been identified. Despite the reported cases of *Bd* infection in wild and captive amphibian populations from different Asian countries (Bai et al., 2010, 2012; Bataille et al., 2013; Dahanukar et al., 2013; Goka et al., 2009; Kusriani et al., 2008; Savage et al., 2011; Swei et al., 2011; Une et al., 2008; Yang et al., 2009; Zhu et al., 2014b), extensive areas of central, northern, and western mainland Asia still lack information on the presence and impacts of *Bd* (Olson et al., 2013; Olson & Ronnenberg, 2014). Consistent with this view, our analysis shows that sites of interest chosen by researchers are geographically sporadic in distribution—highly concentrated in the Korean Peninsula, Japan, south-east Asia, southern part of China and south-western part of India. The sporadic geographic distribution of positive records may result from the lack of search and research efforts rather than a true absence of *Bd* in Asia (Yang et al., 2009).

Although *Bd* research is slowly gaining traction in different countries across Asia, some countries, such as Afghanistan, Bangladesh, Bhutan, Nepal and North Korea lack any studies (Fig. 2, up to June 2018). Geographic bias within countries is also evident. In China, *Bd* has been reported from 10 central and southern provinces of the country (Bai et al., 2010, 2012; Zhu et al., 2014a, b), with northern regions remaining poorly surveyed for the occurrence of this emerging disease. For India, only the south-western part of country has been surveyed, while no investigation has targeted the northern and eastern parts despite high amphibian diversity and abundance. The current lack of *Bd* research has implications for amphibian conservation in Asia. A lack of information limits our ability to resolve the origins of *Bd* and to understand its pathogenicity, and potential threats to native Asian amphibian populations.

We reviewed and expressed concerns about whether sample size, frequency, and timing was sufficient to accurately represent the prevalence of *Bd*. Species-specific responses to *Bd* infection and a lower level of

susceptibility to clinical chytridiomycosis (Retallick et al., 2004) can make the duration of infection longer and prevalence higher (Nelson & Williams, 2014). Therefore, follow-up surveys of species from the same sites are critical to understanding the prevalence of *Bd* infection. A sample size that reflects the amphibian population in question is also crucial in this pursuit; our review shows that more than 60 % of species (out of 167) surveyed for *Bd* in Asia had a sample size equal to or less than 30 (Supplementary Table S1). Our analysis of country-wide prevalence rate shows the need for more investigation with larger sample sizes. For example, Kyrgyzstan has 100 % prevalence rate but only nine samples were tested (Swei et al., 2011). Similarly, in Iran 47 % of samples tested *Bd*-positive where the sample size was 91 (Table 2). Countries with a sample size of 100 or more reveal a prevalence rate lower than 17 % regardless of the number of species sampled (Table 2). Our analysis also shows that the prevalence rate was almost double in tadpoles and juveniles than in adults (Table 3). However, compared to adults the sample size of other two age categories was negligible to draw any conclusion about actual prevalence rate and susceptibility to *Bd* infection.

Table 3. Percentage of *Batrachochytrium dendrobatidis* positive samples from different sources and age categories.

Source of sample	Sample tested	<i>Bd</i> positive	%	X ² Test	DF	P value
Wild	20912	1801	8.61			
Food market	2419	78	3.22			
Farm	1371	99	7.22			
Pet store	578	149	25.78	380.57	6	< 0.001
Zoo & aquarium	542	49	9.04			
Preserved	1785	65	3.64			
Imported	826	89	10.77			
Total	28433	2330	8.19			
Age categories						
Adult	27853	2234	8.02			
Juvenile	80	11	13.75	22.09	2	< 0.001
Tadpole	388	55	14.18			
Total	28433	2330	8.19			

Chytrid has been reported as pathogenic and virulent at temperatures between 12 to 27 °C (Skerratt et al., 2007), and Swei et al. (2011) showed that *Bd* infection occurs at ranges from 330 meters above sea level in Philippines to 1,949 meters above sea level in Indonesia. A study conducted by Dahanukar et al. (2013) also reported *Bd* within the above-mentioned limits of temperature and elevation. These results are consistent with the higher incidence of chytrid in wild amphibians during cooler months that have been widely reported in past studies (Aplin, 2000; Kriger & Hero, 2007; McDonald et al., 2005; Retallick et al., 2004; Woodhams & Alford, 2005). Kusriani et al. (2008) called for a survey in the mountainous areas because declines of amphibian populations at high elevations associated with cooler temperatures have

been linked to *Bd* (Skerratt et al., 2007). The reported prevalence of chytrid among wild Asian amphibians could be underestimated, caused by surveys being conducted at lower elevation sites associated with high temperatures. However, Savage et al. (2011) detected no significant regional or altitudinal difference in *Bd* prevalence for the 127 samples across 11 localities in Peninsular Malaysia that they surveyed. The reported prevalence of chytrid among wild Asian amphibians could not be entirely underestimated and linked to survey bias, i.e. higher number of surveys being conducted at lower elevation sites associated with high temperatures because new evidence from Russell et al. (2019) stresses the critical role of low elevation in the spread and persistence of chytrid. These inconsistencies could possibly be addressed by an increased survey effort with random and representative samples of amphibian species across all habitats in Asia.

Origin of *Bd* in Asia

East Asia has been found to be the geographic hotspot for *Bd* biodiversity and the original source of chytrid infecting amphibians worldwide (O'Hanlon et al., 2018). They also showed that the emergence of the pathogenic *Bd*GPL (Global Panzootic Lineage) in the early 20th century coincides with the global expansion of commercial trade in amphibians. Based on the lower prevalence of *Bd* and the absence of mass die-off events or chytridiomycosis epidemics in Asia, Swei et al. (2011) suggested a thorough historical analysis of museum records. Tests on museum specimens indicate that the *Bd* has been present in Asia for more than 100 years (Fong et al., 2015; Goka et al., 2009; Zhu et al., 2014a), suggesting that chytrid fungus is native in Asia.

Bai et al. (2010) suggested the possibility that native frogs with chytridiomycosis had already died before the survey was conducted. This is unlikely, however, because several studies have found no clinical signs of chytridiomycosis despite the presence of *Bd*. No clinical signs may be due to species-specific susceptibility to chytrid infection (Blaustein et al., 2005; Rollins-Smith et al., 2006), thus some native amphibian species may display innate resistance to chytridiomycosis and act as reservoir hosts. However, studies on horizontal transmission of chytridiomycosis in Asia are still lacking. Analysis conducted by Goka et al. (2009) on the isolates of *Bd* from Asia showed sufficient genetic differentiation to implicate that *Bd* is endemic in Asia. Most of the 26 haplotypes detected by Goka et al. (2009) in Japan have never been reported in other countries. Molur et al. (2015) suggest that there is a single widespread haplotype of *Bd* in the Western Ghats (India), which is identical to the Asian (100 % similar to the strain collected from Japan and China) endemic strain as argued by Dahanukar et al. (2013). Savage et al. (2011) also argued that *Bd* is endemic to Peninsular Malaysia. Bai et al. (2012) hypothesised that the haplotype of *Bd* in Asia belongs to a lineage that has a unique association with Asian amphibians, and differs significantly from the global panzootic lineage, *Bd*GPL (James et al., 2009). A recent study traced the source of *Bd* to the Korean peninsula, where one lineage exhibits the genetic hallmarks of an ancestral population that seeded the panzootic (O'Hanlon et al., 2018).

Our meta-analysis shows that a higher percentage of *Bd*-positive results in the samples collected from pet stores and imported frogs than wild counterparts, suggesting that the international and domestic trades could be responsible for the dispersal and transport of *Bd* (Daszak et al., 1999; Fisher & Garner, 2007; Skerratt et al., 2007; Weldon et al., 2004). A higher percentage of free-ranging American bullfrogs (*Lithobates catesbeianus*) and African clawed frogs (*Xenopus laevis*) with *Bd* infection compared to native amphibians is consistent with the prevalence reported in other countries where they have been introduced (Cunningham et al., 2005; Garner et al., 2005, 2006; Hanselmann et al., 2004; Mazzoni et al., 2003). These two frog species also appear to be relatively resistant to clinical chytridiomycosis (Daszak et al., 2004), and are ideal carriers for introducing *Bd* into uninfected regions in the world. In support of this argument, Borzee et al. (2017) showed that *Bd* prevalence is the highest at sites in Korea where *L. catesbeianus* is found, implying that this exotic species serves as potential reservoir. However, they also found a negative correlation between the presence of this exotic frog species and a native Korean species. Goka et al. (2009) also found that the incidence of the fungus in naturalised individuals of *L. catesbeianus* is much higher than that in native Japanese species. Nevertheless, the authors found no trend suggesting that the presence of the fungus in amphibians increases around habitats where infected individuals of *L. catesbeianus* were detected. On the contrary, the study found that sites where native species were infected were not adjacent to sites of infected *L. catesbeianus*.

Molur et al. (2015) reported slightly higher loads of *Bd* zoospore in samples from Western Ghats (India) than found in previous studies conducted by Nair et al. (2011) and Dahanukar et al. (2013) in the same area, and in Asia (Swei et al., 2011) in general, thereby identifying a widespread *Bd* infection as a plausible threat in the future. Despite the uncertain nature of the pathogenicity of the currently circulating strain of *Bd* in India and East Asia, Molur et al. (2015) called for continuous surveillance of amphibian populations because there are other stressors that trigger *Bd* infection.

Environmental and geographical factors limiting *Bd* distribution

Several studies (Lötters et al., 2009; Puschendorf et al., 2009; Rödder et al., 2009; Ron, 2005; Swei et al., 2011) have modelled the distribution of *Bd* in Asia based on different environmental factors such as temperature and seasonality of precipitation. The species distribution models used in these papers suggests that *Bd*-suitable conditions are widely distributed across Asia. However, suitable environmental conditions do not guarantee the presence of *Bd* because dispersal and persistence abilities of *Bd* also play a crucial role. In an analysis conducted on 3,363 samples from 15 Asian countries, Swei et al. (2011) suggest that *Bd* is much less widespread than predicted by models. Models contain uncertainties; hence we collected data from *Bd*-positive localities across Asia to understand the suitable range of temperature and rainfall. The prevalence of diseases at different elevations is correlated with temperature (Grundler et al., 2012),

and Piotrowski et al., (2004) showed that *Bd* can survive in the temperature range of 4–28 ° C with an optimum growth temperature of 17–25 ° C. Our meta-analysis is consistent with their findings.

We found that fallow land and built-up areas, which are expectedly close to human settlement, have higher prevalence rates than forest and vegetation categories, which are more isolated from human settlements (Fig. 5). Proximity to human habitation and land cover patterns influence amphibian distribution, susceptibility and exposure to diseases (El Mouden et al., 2011; Pauza et al., 2010; Saenz et al., 2015; Thorpe et al., 2018a, b; Van Sluys & Hero, 2009). Our finding is consistent with previous studies that suggest that areas with little human disturbance may have lower *Bd* prevalence compared to areas with high anthropogenic disturbances. However, what factors are responsible for this are still unknown (Becker & Zamudio, 2011; Lane & Burgin, 2008) and beyond the scope of this review, but it would not be overstated to say anthropogenic activities can possibly act as one of the key factors for *Bd* infection.

We have attempted to define the most suitable environmental and geographical range for *Bd* infection in Asia; however, our conclusions could be skewed because of missing information caused by the large geographic bias in the location of chytridiomycosis studies across Asia.

CONCLUSIONS

Within the scope of this review we have attempted to provide an overview of *Bd* infection, its spread, and its impacts on amphibians in Asia, but we did not include *Batrachochytrium salamandrivorans* (*Bsal*) in the review. It should be mentioned that *Bsal* has been present in Asian salamanders both in wild and museum samples for over 150 years (Laking et al., 2017; Martel et al., 2014). To date, the decline of an amphibian population due to *Bd* has not been reported from Asia. However, we advocate for long-term survey and monitoring strategies across countries with a higher potential of *Bd* infection in future. Countries where studies on *Bd* have not been conducted as of yet should focus on assessing its baseline immediately. We also recommend more research on the transmission of *Bd* infection across amphibian populations and between amphibian species to better understand the potential for chytrid epidemics in Asia.

Availability of data and materials

All data extracted and analysed during this review study are available in this published article (and its supplementary information files).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MSI planned the review and initially designed it. MMR extensively contributed to revise the initial design. MMR, MS and MFR reviewed literature, extracted and analysed data, and generated figures. MMR and MKB wrote the manuscript. All authors provided their comments and approved the final manuscript.

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People's perceptions of crocodiles in Nigeria

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Throughout Africa, feelings towards crocodiles vary according to the danger or fear experienced by communities living alongside them. Crocodile conservation programmes must therefore be based on reliable assessments of cultural attitudes towards these reptiles. In this study, we interviewed a random sample of 300 persons in six states in southern Nigeria to determine their perception of crocodiles. Our results revealed that most respondents were very familiar with crocodiles, with animals being regularly sighted but only in small numbers. Most interviewees were aware of just two crocodile types, consistently describing the dwarf crocodile (*Osteolaemus tetraspis*) and the West African Nile crocodile (*Crocodylus [niloticus] suchus*); only a minority of respondents reporting they were aware of the West African slender-snouted crocodile (*Mecistops cataphractus*).

Keywords: *Crocodylus*; *Osteolaemus*; *Mecistops*; local ecological knowledge; conservation; West Africa

In most tropical regions, crocodiles and other reptiles are important as food and traditional medicine, as well as for clothing or ornaments (Alves et al., 2006, 2008, 2009, 2013). Wherever crocodilians occur alongside humans, peoples' attitudes towards these animals may vary from indifference to antagonism. Crocodiles can provide direct benefits through their sustainable use, especially via the skin trade (Webb et al., 1987). However, local communities may be convinced to protect these large predators because they are thought to play an important role in maintaining the productivity and diversity of wetland ecosystems (van der Ploeg et al., 2011). Cultural and intrinsic values for protecting crocodiles can also be strong motivational reasons to be used when developing crocodile management plans (Pooley, 2016). In West Africa, crocodilians, alongside snakes and chelonians, are also central to many cultural beliefs (Ben-Amos, 1976; Fretey et al., 2007). However, depending on the level of conflict between crocodiles

and humans, attitudes towards the conservation of these reptiles may differ, as shown in rural communities in Benin experiencing distinct levels of human-crocodile conflict (Kpéra et al., 2014).

Rural people living in close proximity to crocodiles in West Africa often revere and protect them from harm. In some countries, this is due to their belief that, just as water is essential to crocodiles, crocodiles are crucial for water, since this would permanently disappear if they were not there (Kpéra, 2003; Kpéra et al., 2004). Although there are records of the folklore surrounding crocodiles in West Africa (Kpéra et al., 2014), our knowledge of the nature of the relationship between human communities and crocodiles is still fragmentary (e.g. Anadu & Oates, 1982; Powell, 1993, 1995; Akani et al., 1999; Pooley, 2016). Although knowledge of the ecology of crocodiles in West Africa (Shirley et al., 2009, 2018), and primarily in Nigeria is growing (Luiselli et al., 1999a, 1999b, 2012), there are few studies assessing people's perception of the presence and abundance, or their value as a source of food or income. This information, as Kpéra et al. (2004) have shown, can be used to improve the management of these species, e.g. in turning traditional uses of crocodiles for medicines into a sustainable industry in Benin.

Although in-depth interviews involving intensive individual interviews with a small number of respondents are ideal, here we use shorter interviews applied to a large number of people to determine attitudes and knowledge of crocodilians in southern Nigeria. These interview campaigns were used to gather indirect data of conservation and biological interest (Huntington, 1997).

Between March and May 2014, we interviewed different people in seven states in southern Nigeria (Oyo, Edo, Delta, Bayelsa, Rivers, Akwa-Ibom and Cross River). These states are characterised by a network of rivers, streams and water basins (mostly within the Niger Delta region), most of them inhabited by crocodiles (e.g., Luiselli et al., 2012). The banks of rivers and streams are lined by gallery forests in the freshwater tracts and by mangroves in the brackish water expanses. There are extensive agricultural areas and large urban centres in the region (the largest city being Port Harcourt), and the

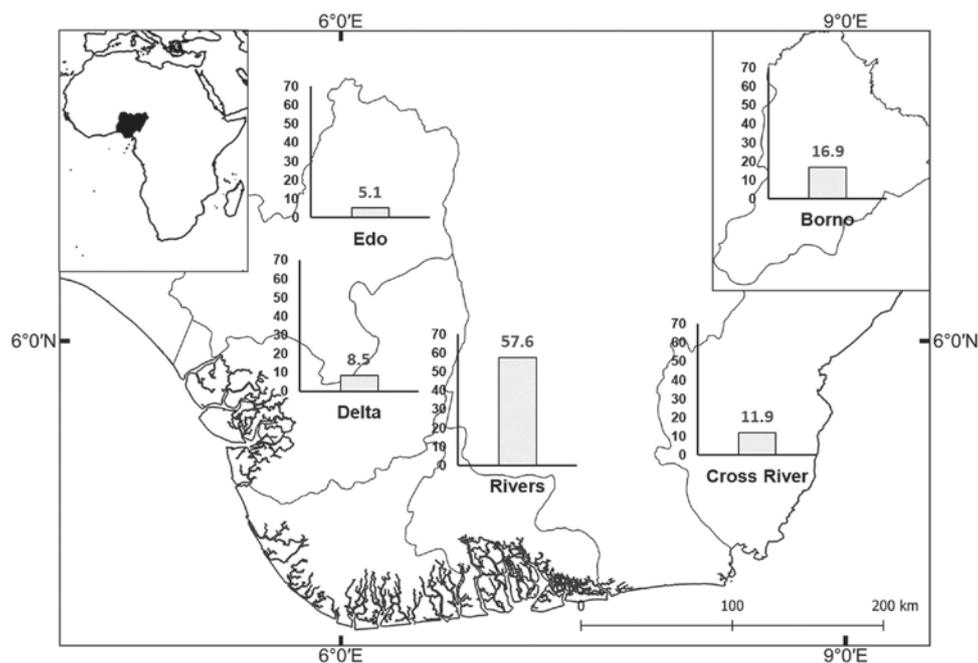


Figure 1. Map of Nigeria showing, by State, the valid percentages of answers on the question: “Do you know any community or area where crocodiles exist but are not hunted or killed?” Valid percent would indicate the percentage calculated, for a given answer, only to the people who gave an answer (thus excluding the “no answer” cases).

overall human population is well over 10 million people (e.g., Luiselli et al., 2012). Based on previous visits in which we collected data on the presence of crocodiles in their surroundings, we selected a sample of villages and towns in each state to interview people (see below for the details). In these localities, we applied semi-structured face-to-face interviews, consisting of eight questions as follows:

- 1) Have you ever seen a crocodile in Nigeria?
- 2) Where did you see crocodiles in Nigeria?
- 3) When was the last time you saw a crocodile in Nigeria?
- 4) How many crocodiles did you see?
- 5) In what condition did you see the crocodiles?
- 6) How many types of crocodiles do you know?
- 7) Do you know any community or area where crocodiles exist but are not hunted or killed?
- 8) Do you know any community or area where crocodiles exist but are hunted or killed?

Interviewees were selected by randomly picking persons in marketplaces, canteens, restaurants, roadsides, hairdressing salons, food shops, and other gathering places. This random selection procedure consisted in stopping the first person met after a given time period (in minutes), with the time interval randomly generated by a random number generator. Local scientists applied all interviews in the local language. Interviewed persons were informed of the aims of the project beforehand and were asked for their verbal consent before proceeding. No minors (<18 years) were approached. All interviews followed the ethical recommendations of the British Sociological Association. Each interview lasted about 15 minutes on average.

During each interview, we noted the interviewee gender (male or female) and age (18 to 25 years, 26-

50 years, ≥ 51 years) but not their names to ensure anonymity (St. John, 2010; Nuno et al., 2014; Luiselli et al., 2017). To avoid non-independence of data, we did not question persons of the same family or those living in the same house, even if they were not relatives (see also Hema et al., 2017). A total of 300 people (241 men; 59 women) were interviewed.

Frequency differences between types of answers were analysed using a χ^2 test, performed by Past 3.0 statistical software, with alpha set at 5 %. All analyses were done with Past v3.2.

Question 1. Valid cases were 299, with 277 “yes” and 22 “no” as answers. There was a statistically significant difference between the frequencies of the two answers ($\chi^2=217.47$, $df = 1$, $P < 0.0001$).

Question 2. The different answers provided by interviewees are summarised in Figure S1. In this case, 275 valid cases were retained for analysis. There was a significantly uneven distribution of the various answers ($\chi^2=109.16$, $df = 4$, $P < 0.0001$), with the majority of answers being “wild” and “park/zoo”. Interestingly, a relatively low percentage of people (7.7 %) answered “market” (Fig. S1a).

Question 3. A total of 255 valid cases were retained for this question. There was a significantly uneven distribution of the various answers ($\chi^2=144.88$, $df = 4$, $P < 0.0001$; Fig. S1b), with a greater majority of answers being 1-5 years.

Question 4. A total of 269 valid answers were retained for this question, providing a statistically uneven distribution of answers ($\chi^2=547.6$, $df = 4$, $P < 0.0001$; Fig. S1c). Almost three quarters of people interviewed have seen less than five crocodiles (Fig. S1c), with no significant frequency differences among the surveyed states of Nigeria ($\chi^2=4.6$, $df = 6$, $P = n.s.$).

Question 5. Out of 267 valid cases retained for

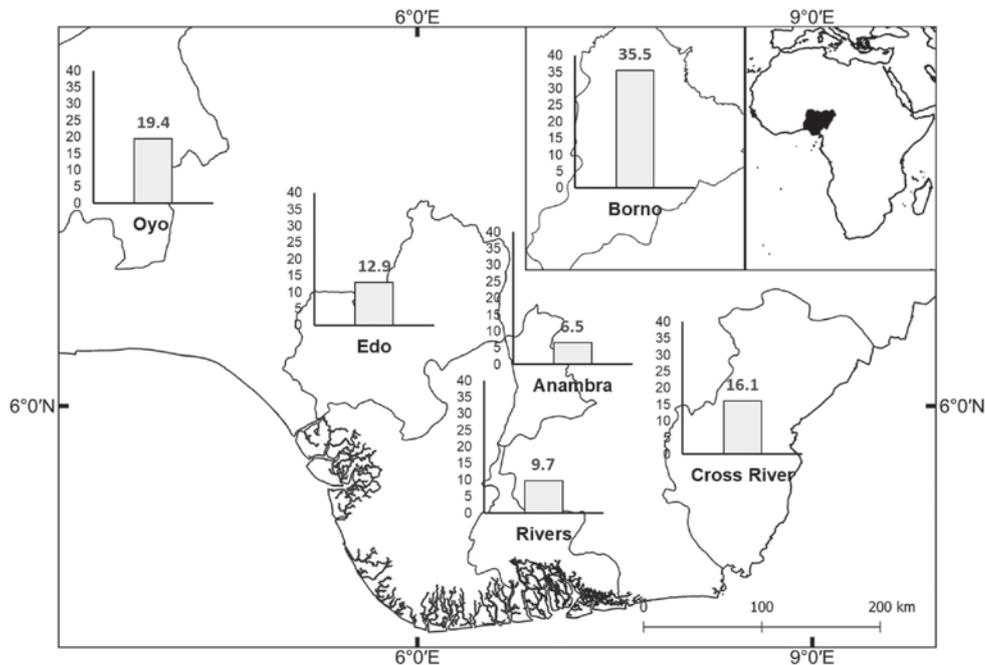


Figure 2. Map of Nigeria showing, by State, the valid percentages of answers on the question: “Do you know any community or area where crocodiles exist but are hunted or killed?” Valid percent would indicate the percentage calculated, for a given answer, only to the people who gave an answer (thus excluding the “no answer” cases).

analysis, 94.4 % of respondents mentioned that they saw crocodiles alive, 2.6 % dead, and 3.0 % as skin.

Question 6. In total, 249 valid cases were retained. Most interviewees answered that they have seen two types of crocodiles (Fig. S1d), and consistently described the dwarf crocodile (*Osteolaemus tetraspis*), locally known as alligator in Pidgin English, and the West African Nile crocodile (*Crocodylus [niloticus] suchus*). Conversely, a small proportion of respondents (< 5 %) reported that they are aware of the West African slender-snouted crocodile (*Mecistops cataphractus*), whereas the fourth type of “crocodile” mentioned is the forest monitor lizard (*Varanus ornatus*); because of its swimming behaviour, this animal is sometimes considered a crocodile.

Question 7. Out of a total of 144 valid cases, 86 people answered “no” and 50 answered “yes”. The location of traditional veneration areas for crocodiles was, according to respondents of our questionnaires, quite widespread, with areas in the south-west (Edo State), south-east (Cross River State) as well as in northern areas of the country (Borno State) (Fig. 1).

Question 8. Out of a total of 115 valid cases, a large majority of people ($n = 79$) people answered “no” and only 36 answered “yes” (Fig. 2).

Our interviews revealed that most people were familiar with crocodiles, with wild and park/zoo animals being the most regularly observed individuals. Interestingly, as the majority of respondents claimed to have seen crocodiles in recent years and in the wild, this suggests that crocodiles are still frequently encountered by people in southern Nigeria. However, pooling the outcomes of question 4) with the results for question 3, it becomes evident that in recent years most interviewees saw crocodiles, but in small numbers, suggesting that crocodiles are still widespread but relatively rare in southern Nigeria.

Most respondents claimed that they saw live crocodiles, not dead specimens. Although the term alive could refer to living animals in different situations, not just in the wild (many crocodiles are traded alive in bushmeat markets, see Fig. 3), it is likely that some specimens reported alive by our interviewees were just ready to be killed, and in any case not going to be released to the wild. Most crocodiles observed were *O. tetraspis* and *C. [niloticus] suchus* but our results indirectly indicate that *M. cataphractus* is extremely rare. The perception that our interviewees had of the three crocodile species mirrors available field data collected during the last twenty years in southern Nigeria. *Mecistops cataphractus* is very rare in the whole of West Africa with very few records for Nigeria (Shirley et al., 2009; Shirley et al., 2018), whereas *O. tetraspis* and *C. [niloticus] suchus* are still widespread and locally abundant, especially in the remote wetlands



Figure 3. Dwarf crocodile (*O. tetraspis*) still alive while being sold at Edumanom market, Bayelsa State (Nigeria).

of the Niger Delta region (Luiselli et al., 2012). In the Niger Delta area, Luiselli et al. (2012) collected 94 records of *C. [niloticus] suchus* and 344 records of *O. tetraspis*. The findings of our interviews reinforce the value of "Local Ecological Knowledge" (LEK, sensu Padmanaba et al., 2013; Turvey et al., 2015) as reliable when contrasted with scientific data collected in the field (Luiselli et al., 2018).

Our study has also shown that traditional veneration of crocodiles is not exceptional in Nigeria, an attribute that can be used to guide potential conservation programmes, as in other parts of the world (e.g. Philippines, van der Ploeg et al., 2011).

We observed that relatively few persons were able to answer with a precise locality where crocodiles are hunted or venerated. We think that this relatively low percent of people assumed that, once arriving to a market for being sold, the provenance of a crocodile is not a matter of interest for customers. Thus, many people did not ask where the animal were hunted and therefore it remains unknown to them where exactly human communities still hunt for wild crocodiles. Overall, the distribution of answers would indicate that hunting areas for crocodiles are still quite widespread, with areas in south-west (Edo State), south-east (Cross River State) as well as in northern areas of the country (Borno State) (Figure 2). It must be noted that there was a wide overlap between areas cited in Figure 1 and Figure 2, thus showing that veneration and hunting may coexist at fine spatial scales. Therefore, for conservation planning, it is necessary to have a fine-scale knowledge of the traditions and culture of local communities if we want to make efficiently the management of the local crocodile populations, and further studies on the cultural attitudes of humans towards crocodiles are strongly needed in this region of West Africa. In particular, since a suite of different variables influences behaviour (attitudes, perceptions, norms, perceived control etc., Marchini & McDonald, 2012), a fuller study of factors influencing human behaviour should be studied if we want to efficiently manage the crocodile populations in the whole region.

From a geographical point of view, our data suggest that the Rivers State is the most important for crocodile protection and long-term survival since interviews from this area not only generated a high number of localities in which at least two species (*C. [niloticus] suchus* and *O. tetraspis*, possibly also *M. cataphractus*) were found but also several sites that included "traditional protection". Conversely, Borno State appears as the area where crocodiles are most hunted and therefore of special conservation concern for crocodiles.

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procedures followed the ethical standards accepted by the British Sociological Association and did not involve any minors.

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Herpetological Journal

Volume 30, Number 2, 2020

Contents

Full papers

Discovering the biogeographic history using predefined areas and explicit geographical data in the South American *Liolaemus elongatus* group (Iguania: Liolaemidae) 53-68
María Soledad Ruiz, Sabrina Noelia Portelli, Thomas Nathaniel Hibbard & Andrés Sebastián Quinteros

Snakes and the Eternal City: Variation in community metrics, body size and population density of snakes in contrasted landscapes of suburban Rome (Italy) 69-82
Lorenzo Rugiero, Luca Luiselli, Massimo Capula, Fabio Petrozzi, Massimiliano Di Vittorio, Nic Pacini, Leonardo Vignoli, Daniele Dendi, Giuliano Milana, Giovanni Amori & Roger Meek

Dominance and aggression in captive gidgee skinks (*Egernia stokesii*) 83-92
Holly Baines, Beatrice Gini, Yu-Mei Chang & Christopher J. Michaels

Aliens in the backyard: Did the American bullfrog conquer the habitat of native frogs in the semi-deciduous Atlantic Forest? 93-98
L. Ferrante, F. B. Baccaro & I. L. Kaefer

Chytrid infection in Asia: How much do we know and what else do we need to know? 99-111
Md. Mokhlesur Rahman, Mahatub Khan Badhon, Md. Salauddin, Md. Fazle Rabbe & Md. Sirajul Islam

Short note

People's perceptions of crocodiles in Nigeria 112-116
Edem A. Eniang, Godfrey C. Akani, Daniele Dendi, John E. Fa & Luca Luiselli