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*Front cover: Golden mantella photographed at Mangabe protected area, Madagascar (R. A. Griffiths).
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Emerging infectious disease threats to European herpetofauna

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In the past decade, infectious disease threats to European herpetofauna have become better understood. Since the 1990s, three major emerging infections in amphibians have been identified (*Batrachochytrium dendrobatidis*, *B. salamandrivorans*, and ranaviruses) as well as at least one of unknown status (herpesviruses), while two major emerging infections of reptiles (*Ophidiomyces ophiodiicola* and ranaviruses) have been identified in wild European populations. The effects of emerging infections on populations have ranged from non-existent to local extirpation. In this article, we review these major infectious disease threats to European herpetofauna, including descriptions of key mortality and/or morbidity events in Europe of their emergence, and address both the distribution and the host diversity of the agent. Additionally, we direct the reader to newly developed resources that facilitate the study of infectious agents in herpetofauna and again stress the importance of an interdisciplinary approach to examining these infectious diseases.

Keywords: *Batrachochytrium dendrobatidis*, *B. salamandrivorans*, herpesviruses, ophidiomycosis, ranaviruses, amphibians, reptiles

INTRODUCTION

Vertebrates are currently experiencing an ongoing mass extinction event (Ceballos et al., 2017). Infectious diseases are contributing to these declines as they are able to cause major population declines, and can contribute to both local and global extirpations (de Castro & Bolker, 2005). Emerging infections have long been known to pose a serious threat to biodiversity (Scott, 1988; Daszak et al., 2000). The link between the decline and extinction of many amphibian populations with the emergence of infectious agents was drawn approximately 20 years ago (Berger et al., 1998; Daszak et al., 1999). While the link between declines in some reptile populations and emerging infections have been less clear, in the past decade growing evidence shows emerging infectious diseases (e.g. snake fungal disease, Lorch et al., 2016) that are clearly able to decimate populations of these animals as well. Infectious disease related declines can even occur in large, seemingly robust populations of wildlife (Daszak et al., 2003). Therefore, the effects in smaller, more fragile, populations are likely to be even more extreme. Unfortunately, populations of both amphibians and reptiles are shrinking around the globe (Gibbons et al., 2000; Sodhi et al., 2008; Stuart et al., 2004; Todd et al., 2010). This will be exacerbated by other factors such as anthropogenic environmental stressors and infectious disease, which negatively affect wildlife health (Acevedo-Whitehouse & Duffus, 2009).

Duffus and Cunningham (2010) reviewed two major disease threats to European amphibians, *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses. Since the publication of that review, other emerging infectious diseases in European amphibian populations have been identified, *B. salamandrivorans* (*Bsal*, Martel et al., 2013), and the potentially emerging amphibian herpesviruses (Franklinos et al., 2018; Oraggi et al., 2017). In reptiles, the emergence of two troubling infectious diseases has also been documented in wild populations, snake fungal disease (*Ophidiomyces ophiodiicola*, Franklinos et al., 2017) and ranaviruses (Alves de Matos et al., 2011).

EMERGING INFECTIOUS DISEASE THREATS TO AMPHIBIANS

BATRACHOCHYTRIUM DENDROBATIDIS

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, hereafter *Bd*) is a non-hyphal zoosporic chytridiomycete fungus implicated in the decline of amphibian species globally (Skerratt et al., 2007). *Batrachochytrium dendrobatidis* is the causative agent of amphibian chytridiomycosis (Berger et al., 1998) that was first linked to declines in amphibian communities in Central America and Australia. Since then, *Bd* has been found all over the globe and has been associated with the extinction of at least 90 amphibian species, and the declines of several hundred others (Wake & Vredenburg, 2008; Scheele et al., 2019). *Bd* is considered

to be a pandemic pathogen in amphibians (Pasmans et al., 2006) with low genetic diversity among different isolates indicating a rapid spread of the pathogen. Of these strains, the global panzootic lineage (BdGPL) has been attributed to most amphibian declines having likely emerged as a hybrid between less virulent strains (Farrer et al., 2011). For a long time, there was speculation as to where *Bd* had originated, with evidence of America or Africa (Weldon et al., 2004), but recent research shows that the infection originated in east Asia (O’Hanlon et al., 2018).

The standard detection method for *Bd* is to swab an individual with a sterile swab and then test for the presence of *Bd* DNA using real-time PCR (also known as qPCR) after processing the sample (Boyle et al., 2004). Gross clinical signs of *Bd* infection in adult amphibians include, but are not limited to: anorexia, lethargy, unusual skin shedding, reddening of the skin (especially in the vent and upper thigh regions), convulsions and loss of the righting reflex (Fig. 1; Nichols et al., 2011). However, the clinical signs of *Bd* vary among species, their life history stages, and other factors such as environmental conditions. In larval stages, clinical signs of chytridiomycosis are also variable. They include but are not limited to: depigmentation of mouthparts, swimming difficulties, and lethargy (reviewed in van Rooij et al., 2015). Therefore, to properly diagnose chytridiomycosis, histology should be used alongside the standard method of qPCR. The pathogen only infects the keratinized tissues of the body, such as the skin in metamorphosed animals and the mouthparts in larvae (Daszak et al., 1999; Marantelli et al., 2004). There are limited studies on the effects *Bd* has on life history, but Garner et al. (2009a) were able to show that experimental infection decreased the time it took tadpoles (of the common toad, *Bufo bufo*) to reach metamorphosis, but also decreased their weight. At higher doses, there were also higher levels of mortality, but these surprising results show that there may be advantageous effects when tadpoles are infected with *Bd*. For more information on host-fungal interactions of *Bd*, please see Van Rooij et al. (2015), and for further



details on the immune response of amphibians to *Bd*, please refer to Grogan et al. (2018).

Currently the potential impacts of *Bd* on European amphibian communities are not fully understood, but within the past decade multiple studies have started to help illuminate the scientific community (e.g. Bielby et al., 2015; Rosa et al., 2013; Tobler & Schmidt, 2010). The amphibian chytrid fungus has both a wide

Figure 1. A common midwife toad (*A. obstetricans*) that died because of *Bd* caused chytridiomycosis. Note the strange position of the legs, due to muscle spasms and the reddish hue of the vent and thighs. Photo by Frank Pasmans.



Figure 2. The distribution of *Batrachochytrium dendrobatidis* (*Bd*) in wild European amphibian populations.

geographic range and a wide host range within Europe (Table 1 and Fig. 2), with infection first being identified in wild populations in 2001 (Bosch et al., 2001). So far, the susceptibility of species across Europe is not fully understood, but *Bd* has been attributed to declines seen in species including the common midwife toad (*Alytes obstetricans*), common toad (*B. bufo*) and European fire salamander (*Salamandra salamandra*) across the Iberian Peninsula (Bosch et al., 2001; Bosch & Martínez-Solano, 2006). Elsewhere in Europe, infection does not always lead to the development of disease, which may be why we have not seen the mass die-offs of amphibians such as those elsewhere in the world (Berger et al., 1998). Experimentally, some anurans species have been shown to have a limited immunity to *Bd*, whilst others have exhibited tolerance, such as the water frogs (*Pelophylax* spp.), which may be an important factor for host-disease dynamics (Daum et al., 2012; Woodhams et al., 2012).

The apparent lack of mass die-offs within many areas Europe may be attributed to the fact that *Bd* has multiple strains of varying virulence (Fisher et al., 2009). Research in the Netherlands has shown over a seven-year time period, populations of the yellow-bellied toad (*Bombina variegata*) are able to coexist with *Bd* (Spitzen-van der Sluijs et al., 2017). Although for now the toads seem to be safe, this may change in the future due to the effects of climate change and other such environmental effects. For more information on the environmental impact of host-pathogen interactions, which can be hugely deterministic in some cases, see Blaustein et al. (2018) for an in-depth discussion. Experiments have also shown that some salamanders such as those in the genus *Speleomantes* also have some immunity to *Bd* (Pasmans et al., 2013). This combined effect of peptide defence and varying *Bd* strains show that at least for now, some species of European amphibians are protected against the potentially negative effects of *Bd* outbreaks (Fisher et al., 2009).

BATRACHOCHYTRIUM SALAMANDRIVORANS

Table 1. Summary of the countries and amphibian species affected by *Batrachochytrium dendrobatidis* infections in wild European populations.

Location	Common Name	Species	First Report
Albania	Macedonian crested newt	<i>Triturus macedonicus</i>	Vojar et al., 2017
	Yellow-bellied toad	<i>Bombina variegata</i>	Vojar et al., 2017
Austria	Alpine newt	<i>Ichthyosaura alpestris</i>	Sztatecsny & Glaser, 2011
	Smooth newt	<i>Lissotriton vulgaris</i>	Sztatecsny & Glaser, 2011
	European water frog complex	<i>Pelophylax</i> spp.	Sztatecsny & Glaser, 2011
	Fire-bellied toad	<i>B. bombina</i>	Sztatecsny & Glaser, 2011
	Great crested newt	<i>T. cristatus</i>	Sztatecsny & Glaser, 2011
	Italian crested newt	<i>T. carnifex</i>	Sztatecsny & Glaser, 2011
	Yellow-bellied toad	<i>B. variegata</i>	Sztatecsny & Glaser, 2011
Belgium	African clawed frog	<i>Xenopus laevis</i>	Spitzen-van der Sluijs et al., 2014
	American bullfrog	<i>Lithobates catesbeianus</i>	Spitzen-van der Sluijs et al., 2014
	Common midwife toad	<i>Alytes obstetricans</i>	Pasmans et al., 2010
	Common toad	<i>Bufo bufo</i>	Martel et al., 2012
	Marbled newt	<i>T. marmoratus</i>	Spitzen-van der Sluijs et al., 2014
Czech Republic	Alpine newt	<i>Ichthyosaura alpestris</i>	Civiš et al., 2012
	Eurasian toads	<i>Bufo</i> sp.	Baláž et al., 2014
	European water frog complex	<i>Pelophylax</i> spp.	Baláž et al., 2014
	Fire-bellied toad	<i>B. bombina</i>	Civiš et al., 2012
	Great crested newt	<i>T. cristatus</i>	Civiš et al., 2012
	Yellow-bellied toad	<i>B. variegata</i>	Civiš et al., 2012
Denmark	Common frog	<i>Rana temporaria</i>	Scalera et al., 2008
	Edible frog	<i>P. esculentus</i>	Scalera et al., 2008
Finland	Common frog	<i>R. temporaria</i>	Patrelle et al., 2012
France	American bullfrog	<i>L. catesbeianus</i>	Garner et al., 2006
	Common midwife toad	<i>A. obstetricans</i>	Garner et al., 2005
	European water frog complex	<i>Pelophylax</i> spp.	Ouellet et al., 2012
Germany	Agile frog	<i>R. dalmatina</i>	Ohst et al., 2011
	Alpine newt	<i>I. alpestris</i>	Ohst et al., 2011
	Common frog	<i>R. temporaria</i>	Ohst et al., 2011
	Common midwife toad	<i>A. obstetricans</i>	Ohst et al., 2011
	Smooth newt	<i>L. vulgaris</i>	Ohst et al., 2011
	Common toad	<i>B. bufo</i>	Ohst et al., 2011
	European water frog complex	<i>Pelophylax</i> spp.	Ohst et al., 2011
	European spadefoot toad	<i>Pelobates fuscus</i>	Ohst et al., 2011
	European tree frog	<i>Hyla arborea</i>	Ohst et al., 2011
	Fire-bellied toad	<i>B. bombina</i>	Ohst et al., 2011
	Fire salamander	<i>Salamandra salamandra</i>	Ohst et al., 2011
	Great crested newt	<i>T. cristatus</i>	Ohst et al., 2011
	Green toad	<i>Bufo viridis</i>	Ohst et al., 2013
	Moor frog	<i>R. arvalis</i>	Ohst et al., 2011
	Natterjack toad	<i>Epidalea calamita</i>	Ohst et al., 2011
	Palmate newt	<i>L. helveticus</i>	Ohst et al., 2011
Yellow-bellied toad	<i>B. variegata</i>	Ohst et al., 2011	
Greece	Epirus water frog	<i>P. epiroticus</i>	Azmanis et al., 2016
	Green frog	<i>B. viridis</i>	Azmanis et al., 2016
	Marsh frog	<i>P. ridibundus</i>	Azmanis et al., 2016
Hungary	Alpine newt	<i>I. alpestris</i>	Vörös et al., 2018
	Edible frog	<i>P. esculentus</i>	Vörös et al., 2018
	European water frog complex	<i>Pelophylax</i> spp.	Tünde et al., 2012
	Fire-bellied toad	<i>B. bombina</i>	Vörös et al., 2018
	Green toad	<i>B. viridis</i>	Vörös et al., 2018
	Marsh frog	<i>P. ridibundus</i>	Vörös et al., 2018
	Yellow-bellied toad	<i>B. variegata</i>	Tünde et al., 2012
Italy	American bullfrog	<i>Lithobates catesbeianus</i>	Garner et al., 2006
	Apennine toad	<i>B. pachypus</i>	Stagni et al., 2004
	Edible frog	<i>P. esculentus</i>	Adams et al., 2008
	Edible frog complex	<i>Pelophylax</i> spp.	Simoncelli et al., 2005
	Fire-bellied toad	<i>B. variegata</i>	Stagni et al., 2002 (in Bovero et al., 2008)
	Italian agile frog	<i>R. latastei</i>	Garner et al., 2004 (in Bovero et al., 2008)
	Italian alpine newt	<i>I. alpestris apuanus</i>	Zampiglia et al., 2013
	Italian crested newt	<i>T. carnifex</i>	Grasseli et al., 2019
	Italian fire salamander	<i>S. salamandra gigliolii</i>	Zampiglia et al., 2013
	Italian stream frog	<i>R. italica</i>	Zampiglia et al., 2013
	Sardinian brook salamander	<i>Euproctus platycephalus</i>	Bovero et al., 2008
Tyrrhenian painted frog	<i>Discoglossus sardus</i>	Bielby et al., 2009	
Luxembourg	Alpine newt	<i>I. alpestris</i>	Wood et al., 2009
	Edible frog complex	<i>Pelophylax</i> spp.	Wood et al., 2009
	Palmate newt	<i>L. helveticus</i>	Wood et al., 2009
Macedonia	European water frog complex	<i>Pelophylax</i> spp.	Vojar et al., 2017
Montenegro	Common or smooth newt	<i>L. vulgaris</i>	Vojar et al., 2017
	Edible frog complex	<i>Pelophylax</i> spp.	Vojar et al., 2017
	Great crested newt	<i>T. cristatus</i>	González et al., 2019

Location	Common Name	Species	First Report
Netherlands	Alpine newt	<i>I. alpestris</i>	Spitzen-van der Sluijs et al., 2014
	Common midwife toad	<i>A. obstetricans</i>	Spitzen-van der Sluijs et al., 2014
	Common frog	<i>R. temporaria</i>	Spitzen-van der Sluijs et al., 2014
	Common or smooth newt	<i>L. vulgaris</i>	Spitzen-van der Sluijs et al., 2014
	Common toad	<i>B. bufo</i>	Spitzen-van der Sluijs et al., 2014
	Edible frog	<i>P. esculentus</i>	Spitzen-van der Sluijs et al., 2014
	European water frog complex	<i>Pelophylax</i> sp.	Spitzen-van der Sluijs et al., 2014
	European tree frog	<i>Hyla arborea</i>	Spitzen-van der Sluijs et al., 2014
	Pool frog	<i>P. lessonae</i>	Spitzen-van der Sluijs et al., 2014
	Natterjack toad	<i>Epidalea calamita</i>	Spitzen-van der Sluijs et al., 2014
	Yellow-bellied toad	<i>B. variegata</i>	Spitzen-van der Sluijs et al., 2014
Poland	Edible frog	<i>P. esculentus</i>	Kolenda et al., 2017
	European water frog complex	<i>Pelophylax</i> sp.	Sura et al., 2010
	Pool frog	<i>P. lessonae</i>	Kolenda et al., 2017
	Yellow-bellied toad	<i>B. variegata</i>	Kolenda et al., 2017
Portugal	Bosca's newt	<i>L. boscai</i>	Rosa et al., 2017
	Common midwife toad	<i>A. obstetricans</i>	Rosa et al., 2013
	Fire salamander	<i>S. salamandra</i>	Rosa et al., 2017
	Iberian green frog	<i>P. perezii</i>	Rosa et al., 2017
	Iberian tree frog	<i>Hyla molleri</i>	Rosa et al., 2017
	Marbled newt	<i>Triturus marmoratus</i>	Rosa et al., 2017
	Spiny toad	<i>B. spinosus</i>	Rosa et al., 2017
Romania	Common Frog	<i>R. temporaria</i>	Vörös et al., 2013
	Smooth newt	<i>L. vulgaris</i>	Vörös et al., 2013
	Yellow-bellied toad	<i>B. variegata</i>	Vörös et al., 2013
Serbia	Edible frog	<i>P. esculentus</i>	Mali et al., 2017
	Marsh frog	<i>P. ridibundus</i>	Mali et al., 2017
	Pool frog	<i>P. lessonae</i>	Mali et al., 2017
Spain	Betic midwife toad	<i>A. dickhilleni</i>	Bosch et al., 2013
	Bosca's newt	<i>L. boscai</i>	Hidalgo-Vila et al., 2012
	Common midwife toad	<i>A. obstetricans</i>	Bosch et al., 2001
	Common toad	<i>B. bufo</i>	Bosch & Martínez-Solano, 2006
	Fire salamander	<i>S. salamandra</i>	Bosch & Martínez-Solano, 2006
	Iberian green frog	<i>P. perezii</i>	Hidalgo-Vila et al., 2012
	Iberian painted frog	<i>Discoglossus galganoi</i>	Hidalgo-Vila et al., 2012
	Majorcan midwife toad	<i>A. muletensis</i>	Garner et al., 2009a
	Mediterranean tree frog	<i>H. meridionalis</i>	Hidalgo-Vila et al., 2012
	Natterjack toad	<i>E. calamita</i>	Martínez-Solano et al., 2003
	Spanish ribbed newt	<i>Pleurodeles waltl</i>	Hidalgo-Vila et al., 2012
	Southern marbled newt	<i>T. pygmaeus</i>	Hidalgo-Vila et al., 2012
	Western spadefoot toad	<i>Pelobates cultripes</i>	Hidalgo-Vila et al., 2012
Sweden	Common frog	<i>R. temporaria</i>	Kärverno et al., 2018
	Common toad	<i>B. bufo</i>	Kärverno et al., 2018
	Fire-bellied toad	<i>B. bombina</i>	Kärverno et al., 2018
	Moor frog	<i>R. arvalis</i>	Kärverno et al., 2018
	Natterjack toad	<i>E. calamita</i>	Kärverno et al., 2018
	Variable toad	<i>Bufoles variabilis</i>	Kärverno et al., 2018
Switzerland	Alpine newt	<i>I. alpestris</i>	Tobler et al., 2012
	Common midwife toad	<i>A. obstetricans</i>	Tobler et al., 2012
	European water frog complex	<i>Pelophylax</i> sp.	Tobler et al., 2012
	Palmate newt	<i>L. helveticus</i>	Tobler et al., 2012
UK	African clawed frog	<i>Xenopus laevis</i>	Tinsley et al., 2015
	American bullfrog	<i>Lithobates catesbeianus</i>	Garner et al., 2005
	Natterjack toad	<i>E. calamita</i>	May et al., 2011

Table 2. Summary of the countries and amphibian species affected by *Batrachochytrium salamandrivorans* infections in wild European populations.

Location	Common Name	Species	First Report
Belgium	Alpine newt	<i>Ichthyosaura alpestris</i>	Spitzen-van der Sluijs et al., 2016a
	Fire salamander	<i>Salamandra salamandra</i>	Spitzen-van der Sluijs et al., 2016a
Germany	Fire salamander	<i>S. salamandra</i>	Spitzen-van der Sluijs et al., 2016a
Netherlands	Alpine newt	<i>I. alpestris</i>	Spitzen-van der Sluijs et al., 2016a
	Smooth newt	<i>Lissotriton vulgaris</i>	Spitzen-van der Sluijs et al., 2016a
Spain	Fire salamander	<i>S. salamandra</i>	Martel et al., 2013
	Palmate newt	<i>L. helveticus</i>	González et al., 2019

Table 3. Summary of countries and amphibian species affected by herpesvirus infections in wild European populations.

Location	Common Name	Species	First Report
Germany	Common frog	<i>Rana temporaria</i>	Mutschmann & Scheenweiss, 2008
	Moor frog	<i>R. arvalis</i>	Mutschmann & Scheenweiss, 2008
	European spadefoot toad	<i>Pelobates fuscus</i>	Mutschmann & Scheenweiss, 2008
Italy	Agile frog	<i>R. dalmatina</i>	Bennati et al., 1994
Switzerland	Common frog	<i>R. temporaria</i>	Origgi et al., 2017
	Common toad	<i>Bufo bufo</i>	Origgi et al., 2018
UK	Common frog	<i>R. temporaria</i>	Franklinos et al., 2018

The salamander chytrid fungus (*Batrachochytrium salamandrivorans*, hereafter *Bsal*) was first identified in 2013 after a dramatic loss of European fire salamanders (*Salamandra salamandra*) in the Netherlands (Martel et al., 2013; Spitzen-van der Sluijs et al., 2013). The fungus itself is closely related to *B. dendrobatidis*, having diverged sometime in the late Cretaceous or early Paleogene (Martel et al., 2014). The introduction pathway of the pathogen to Europe is believed to have been via the trade in Asian newt species, with *Bsal* being the predominant chytrid fungus affecting Vietnamese salamanders (Laking et al., 2017). There is also alternative evidence to support the introduction of *Bsal* to Europe through the trade in small-webbed fire-bellied toads (*B. microdeladigitora*) from Vietnam (Nguyen et al., 2017). In lab experiments, Asian newts (*Cynops cyanurus*, *C. pyrrhogaster* & *Paramesotriton deloustali*) were discovered to be potential reservoirs of *Bsal*, and worryingly, most European newts died shortly after infection with *Bsal* (Martel et al., 2014). Recent evidence suggests that one of the factors that makes *Bsal* such a threat to urodelean diversity is the fact that infection does not elicit immunity and anurans, such as the common midwife toad (*A. obstetricans*), may act as intermediary hosts (Stegen et al., 2017). More worryingly, alpine newts (*Ichthyosaura alpestris*) can survive for extended periods of time whilst infected with low doses of *Bsal*, with the ability to even clear infection. This is a concern as alpine newts co-occur with *S. salamandra* whilst *A. obstetricans* does not (Stegen et al., 2017).

The clinical signs of *Bsal* are varied (like those of *Bd*) but are usually seen as anorexia, lethargy, ataxia, and skin lesions (Martel et al., 2013). The skin lesions (Fig. 3) are quite diagnostic and easily identified on adult individuals (although this varies by species); however, they are not always present and tend to occur at the end of pathogenesis when the animal is nearly dead (Martel et al., 2013). This means that detailed histological and genetic analyses are needed to confirm the presence of *Bsal* in a suspected infected individual. Again, the standard technique for detection, like *Bd*, is the use of swabs and qPCR.

In the wild, *Bsal* has been only found in a small number of European countries (Table 2) although the presence of the fungus is known to be more widespread in private collections (Fitzpatrick et al., 2018; Sabino-Pinto et al., 2015). It is vital that private collectors take the necessary

biosecurity protocols when disinfecting wastewater and enclosures. More information can be found in Van Rooij et al. (2017). Despite the potentially devastating effects of *Bsal* on naive populations, *Bsal* has a poor dispersal potential which allows some sub-populations to persist in areas where the pathogen is known to be present (Spitzen-van der Sluijs et al., 2018). As highlighted by Spitzen-van der Sluijs et al. (2018), this provides potential for in-the-field mitigation strategies and also shows the importance of biosecurity to ensure that researchers are not accidentally transferring *Bsal* zoospores between sites.

At this time, *Bsal* is limited to wild populations in

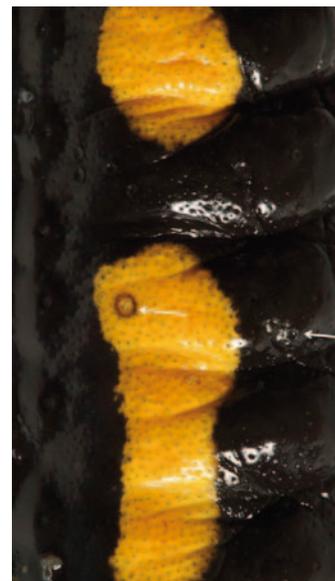


Figure 3. Close up of the skin of a fire salamander (*S. salamandra*) that died because of *Bsal* caused chytridiomycosis. Note the skin erosion with black margins indicated by the white arrows. Photo By: Frank Pasmans.

Germany, Belgium, Spain and the Netherlands (Fig. 4). In *Bsal*'s range the species of focus is the European fire salamander (*S. salamandra*) although other species have also tested positive for *Bsal* (Table 2). In order to track the pathogen's spread through Europe, ongoing surveillance needs to be carried out in areas that may be susceptible to spillover, as well as monitoring vulnerable species. Limited screening in the Czech Republic revealed that *Bsal* was not present in wild or captive salamanders (Baláz et al., 2018), although more samples are needed

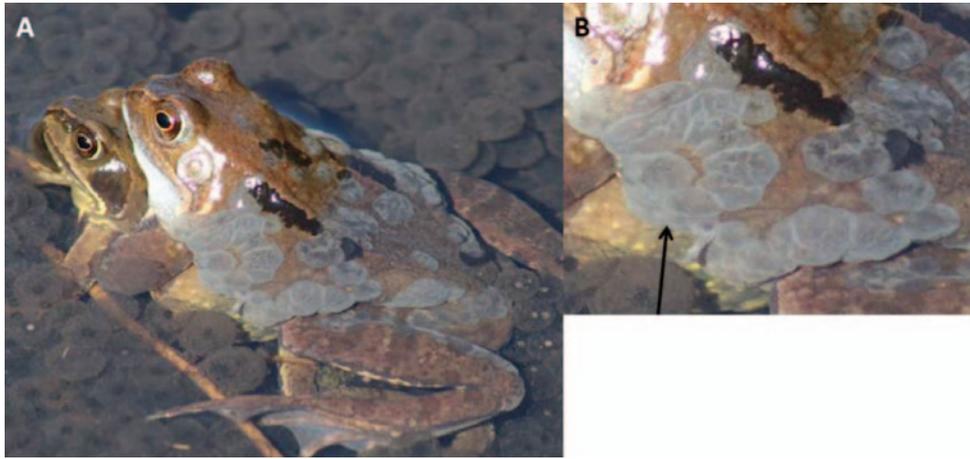


Figure 6. (A) Herpesvirus infection in a male common frog (*R. temporaria*) from the UK. Note the grayish nature of the lesions that are distributed on the dorsal aspect of the frog. **(B)** Close up of the herpesvirus lesions on the dorsum of the same male common frog. Note the ‘fluid filled’ appearance of the lesions. Photo by Matthew Chester.

persistence in populations of affected amphibians. It will be important for interdisciplinary teams to investigate amphibian herpesviruses because of the difficulties that are often encountered with proper identification and diagnosis (see Garner et al., 2013 for further discussion).

RANAVIRUSES

Amphibian ranaviruses are widely distributed in Europe. They are currently known to be present in over ten countries and in over ten amphibian species (see Table 4 and Fig. 7). Amphibian ranaviruses belong to the viral family *Iridoviridae*, which are large, double stranded DNA viruses (Chinchar et al., 2017). In fact, the first documented cases of what were likely to be *Ranavirus* infections were broadly identified as iridovirus-like agents. Fijan et al. (1991) found iridovirus-like particles in edible frogs (*Pelophylax esculentus*) from a population that experienced a mortality event in Croatia. This discovery was quickly followed by the identification of an iridovirus-like agent associated with morbidity and mortality events in UK common frogs (*Rana temporaria*), that was eventually classified as a *Ranavirus* (Cunningham et al., 1993; Cunningham et al., 1996; Drury et al., 1995). Despite the early report of an iridovirus-like agent in Croatia, no subsequent reports of amphibian ranaviruses can be found in the literature for continental Europe until a new *Ranavirus* species emerged in 2007 (see below for further discussion).

The gross clinical signs of ranaviral disease are conserved across species and life history stages. It is important to note that not all ranaviral infections will result in disease, and therefore the absence of gross clinical signs should not be taken as a clean bill of health (see Rijks et al., 2016). Additionally, in most cases, the gross signs of ranaviriosis are considered to be non-specific to the disease, and therefore it is imperative that a full and proper pathological investigation be performed when infection or disease is thought to be a contributing factor to a morbidity and/or mortality event. Gross clinical signs of ranaviriosis of juvenile and adult amphibians include, but are not limited to: lethargy, skin ulcerations, haemorrhages in the skin, reddening of the skin, necrosis

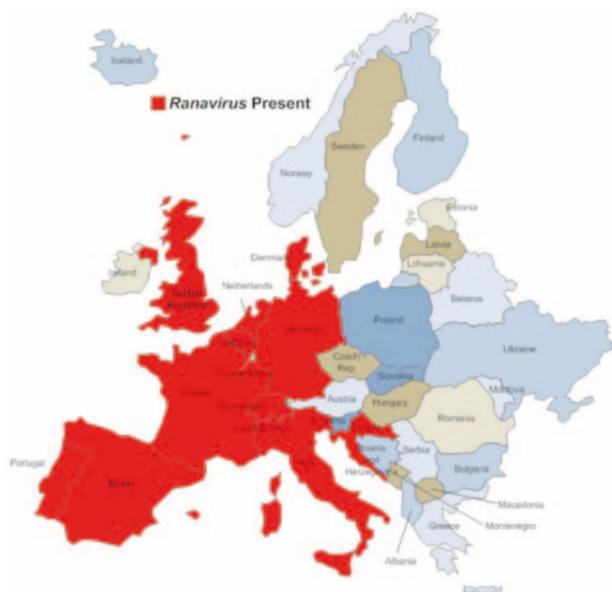
of digits, and internal haemorrhages of multiple organ systems (predominantly the gastrointestinal tract and reproductive tracts; Cunningham et al., 1996; Cunningham et al., 2007a; Cunningham et al., 2007b; Price et al., 2014; see Figure 8 and images in Duffus & Cunningham, 2010). The signs of disease in affected tadpoles/larvae included systemic external and internal haemorrhages (Balseiro et al., 2009; Balseiro et al., 2010). External haemorrhages occur predominantly in the tissues surrounding the eyes of the tadpoles and in the tail, including the musculature (Balseiro et al. 2009; Balseiro et al., 2010) and edema has also been observed (Rosa et al., 2017). Infection, disease, and mortality can occur at any life history stage in amphibians, except for the egg stage. However, the life history stage affected seems to be species specific. In some species, it is the adults that are predominantly affected [e.g. common frogs (*R. temporaria*, Cunningham et al., 1996)] whereas in other species, the larvae/tadpoles are affected [e.g. common midwife toads (*A. obstetricans*) and Alpine newts (*I. alpestris*) (Balseiro et al., 2009; Balseiro et al., 2010)].

There are several different types of *Ranavirus* that are present in Europe. *Frog virus 3* (FV3), *Common midwife toad virus* (CMTV), and their derivatives appear to be the most common types of ranaviruses present in amphibians. FV3 is the type virus of the genus *Ranavirus* (Tan et al., 2004) and is perhaps the most common species of *Ranavirus* around the globe in amphibian populations (see Duffus et al., 2015). However, CMTV-like viruses are known to recombine with FV3-like strains and can create extremely virulent chimeric viruses (Clayton et al., 2017). Experimental infection of smooth newts (*Lissotriton vulgaris*) with different strains of CMTV *Ranavirus* from the Netherlands clearly demonstrates distinct strain-associated pathogenicity within closely related strains of the virus (Saucedo et al., 2019).

The emergence of amphibian ranaviruses and likely misdiagnosis as ‘red-leg syndrome’ in Europe has had a measurable negative effect on some, but not all, populations. The first well-documented morbidity and mortality event in continental Europe occurred in 2007

Table 4. Summary of countries and amphibian species known to harbour *Ranavirus* infections in wild European populations.

Location	Common Name	Species	First Report
Belgium	Common toad	<i>Bufo bufo</i>	Martel et al., 2012
	American bullfrog	<i>Lithobates catesbeianus</i>	Sharifian-Fard et al., 2011
Croatia	Edible frog	<i>Pelophylax esculentus</i>	Fijan et al., 1991
Denmark	Edible frog	<i>P. esculentus</i>	Ariel et al., 2009
France	Common frog	<i>Rana temporaria</i>	Miaud et al., 2016
Germany	Edible frog	<i>P. esculentus</i>	Stöhr et al., 2013
Italy	Edible frog	<i>P. esculentus</i>	Holopainen et al., 2009
Netherlands	European water frog complex	<i>Pelophylax spp.</i>	Kik et al., 2011
	Smooth newt	<i>Lissotriton vulgaris</i>	Kik et al., 2011
	Common frog	<i>Rana temporaria</i>	Rijks et al., 2016
	Common toad	<i>B. bufo</i>	Rijks et al., 2016
	European spadefoot toad	<i>Pelobates fuscus</i>	Spitzen-van der Sluijs et al., 2016c
Portugal	Great crested newt	<i>Triturus cristatus</i>	Spitzen-van der Sluijs et al., 2016c
	Common midwife toad	<i>Alytes obstetricans</i>	Stöhr et al., 2015
	Bosca's newt	<i>L. boscai</i>	de Matos et al., 2008
	Spiny toad	<i>B. spinosus</i>	Rosa et al., 2017
	Fire salamander	<i>Salamandra salamandra</i>	Rosa et al., 2017
	Iberian tree frog	<i>Hyla molleri</i>	Rosa et al., 2017
Spain	Marbled newt	<i>T. marmoratus</i>	de Matos et al., 2008
	Common midwife toad	<i>A. obstetricans</i>	Balseiro et al., 2009
	Common frog	<i>R. temporaria</i>	Price et al., 2014
	Alpine newt	<i>Ichthyosaura alpestris</i>	Balseiro et al., 2010
	Bosca's newt	<i>Lissotriton boscai</i>	Price et al., 2014
Switzerland	Marbled newt	<i>T. marmoratus</i>	Price et al., 2014
	Marsh frog	<i>P. ridibunda</i>	Stöhr et al., 2015
UK	Common frog	<i>R. temporaria</i>	Drury et al., 1995
	Common toad	<i>B. bufo</i>	Hyatt et al., 2000
	Common midwife toad	<i>A. obstetricans</i>	Duffus et al., 2014
	Smooth newt	<i>L. vulgaris</i>	Duffus et al., 2014

**Figure 7.** The distribution of *Ranavirus* spp. in wild European amphibian populations.

in 'Picos de Europa' National Park, Spain (Balseiro et al., 2009). The mortality event involved common midwife toad (*A. obstetricans*) tadpoles that showed classic signs

of ranavirosis (Balseiro et al., 2009). Investigations into the cause of the mortality event revealed that it was caused by a novel *Ranavirus*, then tentatively called the common midwife toad virus (CMTV, Balseiro et al., 2009) and CMTV was only recently designated as its own species in the genus *Ranavirus* (Chinchar et al., 2017). The following year, CMTV was responsible for another morbidity and mortality event. This time not only did it affect common midwife toad tadpoles, but also alpine newt (*I. alpestris cyreni*) larvae (Balseiro et al., 2010). Since then, declines and local extirpations of several species have been documented in association with the emergence of ranaviruses in the same park. Price et al. (2014) found that at several locations, common midwife toads (*A. obstetricans*) were extirpated and both alpine newts (*I. alpestris*) and common toads (*B. bufo*) experienced severe declines. Unfortunately, it does not appear that the populations are recovering from the effects the emergence of ranaviral infection and disease at this time (Price et al., 2014). Many more outbreaks of ranavirosis have now been documented across continental Europe. Perhaps the largest scale morbidity and mortality event involving a CMTV-like virus occurred in 2010 in the Netherlands (Kik et al., 2011; van Beurden et al., 2014). The event involved more than 1000 individuals, mostly

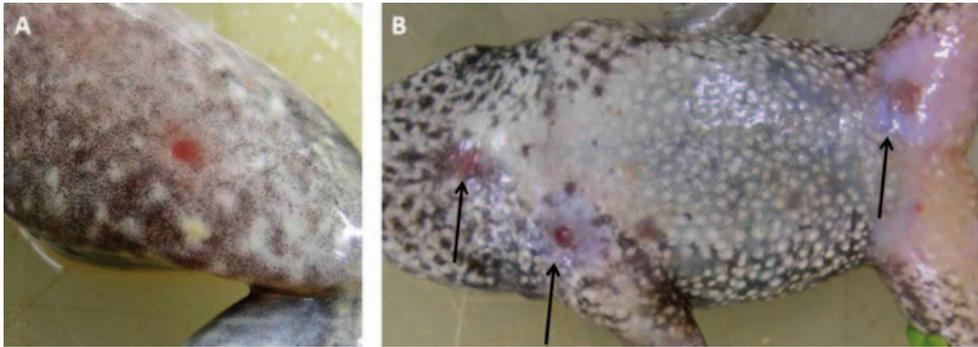


Figure 8. (A) Ranavirus-associated ulceration on the upper portion of the leg of a common frog (*R. temporaria*) from the UK. (B) Ranavirus-associated ulcerations in a common midwife toad (*A. obstetricans*) from the UK. Arrows indicate the lesions. Note the greyish area around the ulcerations. Photos by Zoological Society of London/Amanda L.J. Duffus



Figure 9. A barred grass snake (*N. helvetica*) in from the UK with suspected ophidiomycosis. (A) Arrow indicates the 'crusty' scales that are common in snakes suffering from ophidiomycosis. (B) Arrows indicate some of the deformed scales frequently seen in snakes with ophidiomycosis. (C) The arrow indicates the crusty scales on the snout. Photos by Silviu Petrovan.

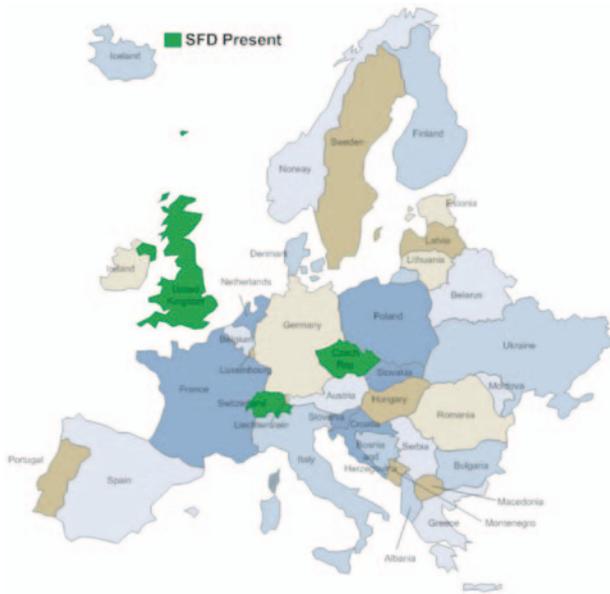
Pelophylax spp. but some common newts (*L. vulgaris*; Kik et al., 2011). Subsequent investigations into amphibian morbidity and mortality events in the Netherlands has revealed that the CMTV-like *Ranavirus* is spreading and is the causative agent of many of the observed events (Rijks et al., 2016; Spitzen-van der Sluijs et al., 2016b; Saucedo et al., 2018). It is also important to note that these outbreaks of disease are affecting the common spadefoot (*P. fuscus*), which is a threatened species (Spitzen-van der Sluijs et al., 2016c). In the UK, common frog populations where ranaviruses have emerged have significantly decreased in size. Teacher et al. (2010) found that on average, common frog populations where *Ranavirus* was found declined by approximately 81 % when compared to populations where the virus was absent. Furthermore, Teacher et al. (2010) report that larger populations were disproportionately affected, with larger populations losing a greater number of animals to disease emergence than those that were initially smaller when *Ranavirus* infections emerged. The emergence of amphibian ranaviruses has also changed the population structure of common frogs in the UK, making them more susceptible to stochastic events (Campbell et al., 2018). This is cause for great concern with the ever-increasing

random events that climate change will cause. Thus, the emergence of ranaviruses in European amphibians is a cause for great conservation concern. They have the potential to be lethal pathogens and are now known to drive populations to decline or even local extinction (e.g. Teacher et al., 2010, Price et al., 2014, Rosa et al., 2017), as a single responsible agent or in co-occurrence with for instance *Bd* and/or *Bsal*. Ranaviruses are persistent pathogens, meaning that they remain on field gear far longer than *Bd* or *Bsal* (Nazir et al., 2012; Van Rooji et al., 2017); therefore, proper disinfection methods should always be used on field gear and enhanced biosecurity in facilities where the pathogen may be found. Investigating amphibian health and disease should be made a priority for many species, even common ones, and include an interdisciplinary approach to ensure that these animals have a future.

The effects of the emergence of ranaviruses may be compounded when another disease, such as *Bd*, has emerged previously in a population. In the 'Serra da Estrela' Natural Park, *Bd* related declines of amphibian populations were occurring for many years before ranavirosis emerged (Rosa et al., 2017). Ranavirosis was first documented in 2011 and is now known to occur in

Table 5. Summary of the countries and snake species affected by snake fungal disease (*Ophidiomyces ophiodiicola*) infections in wild European populations.

Location	Common Name	Species	First Report
Czech Republic	Dice snake	<i>Natrix tessellata</i>	Franklinos et al., 2017
Switzerland	Grass snake	<i>N. natrix</i>	Meier et al., 2018
UK	Barred grass snake	<i>N. helvetica</i>	Franklinos et al., 2017
	Adder	<i>Vipera berus</i>	Franklinos et al., 2017

**Figure 10.** The distribution of ophidiomycosis cases in wild European snake populations.

several species (*T. marmoratus*, *A. obstetricans*, *L. boscai*, *S. salamandra*, and *B. spinus*, Rosa et al., 2017). The emergence of ranaviruses was correlated with sharp declines in two species (*L. boscai* and *A. obstetricans*) at one of the sites within Serra da Estrela Natural Park (Rosa et al., 2017). The ability of *Ranavirus* emergence in amphibians to change the species assemblages at a site and alter host community composition and structure is a huge threat to these animals (Rosa et al., 2017). Therefore, surveillance efforts must not just be centred around one potential pathogen or one potential host; a community-based approach for pathogen surveillance should be used whenever possible.

EMERGING INFECTIOUS DISEASE THREATS TO REPTILES OPHIDIOMYCOSIS/SNAKE FUNGAL DISEASE

In recent years it has become apparent that amphibians are not the only herptiles to suffer from declines caused by emerging fungal pathogens. There are a number of minor fungal diseases that infect reptiles (Paré & Sigler, 2016) but the one that has recently caught international attention is snake fungal disease. The causative agent was first identified in 2009 as *Chrysosporium ophiodiicola* (Rajeev et al., 2009) but subsequent research has seen the fungus moved to its own genus of *Ophidiomyces* (Sigler et al., 2013). Commonly known as snake fungal disease (hereafter SFD) but more correctly known as ophidiomycosis, is troubling for many reasons, one of which is that the clinical signs are not pathognomonic

and so histological evidence, as well as genetic, is needed to confirm infection. SFD was first identified from snakes in the US (Dolinski et al., 2014; Rajeev et al., 2009) and it has recently been found to be present in snake populations in Europe (Franklinos et al., 2017; Meier et al., 2018).

The clinical signs of ophidiomycosis vary slightly between species but include skin lesions, dermatitis, scale deformity and yellow-brown crusty areas (Lorch et al., 2016). Abrasion may create opportunities for infection. Once the stratum corneum has been compromised, *O. ophiodiicola* can then quickly penetrate the epidermis, at which point the host's immune response leads to the aforementioned clinical signs developing (see Fig. 9; Lorch et al., 2015). As a response to the disease, snakes tend to increase their sloughing frequency in order to try to cast off the pathogen. The new skin underneath is clinically normal and this behaviour may lead to snakes successfully curing themselves of ophidiomycosis. However, if old skin is stuck to the new skin during a shed, then there is the potential for reinfection (Lorch et al., 2015). All of this is very worrying for snake species that may already be suffering from the effects of fragmentation, persecution, and inbreeding depression (Madsen et al., 1996; Ursenbacher et al., 2009). The current evidence suggests that *O. ophiodiicola* only has the ability to infect snakes, although this is an area that needs further investigation.

So far, investigations into the presence of ophidiomycosis in Europe are limited, but the presence has been confirmed in a number of species in a small number of countries (Table 5, Fig. 10). The first evidence of ophidiomycosis being present in Europe came from a screening of archived specimens collected by the Garden Wildlife Health project between 2010-2016 (Franklinos et al., 2017). This effort identified that wild European snakes in two countries (the UK and Czech Republic) were positive for *O. ophiodiicola* using a combination of histological and genetic diagnostic techniques. Since then, snakes from Switzerland have also tested positive for the causative agent of ophidiomycosis (Meier et al., 2018). At this stage it is not known how virulent the disease is to European snake species. Franklinos et al. (2017) noted that skin lesions were mild in most of the cases they examined but in some, these lesions were quite severe and would have contributed to the animals' mortality. At this time, we do not have a clear picture of how widespread ophidiomycosis is in Europe or what that means for its snake species. More research is needed to help understand the status of this pathogen. In particular, it is not known whether or not ophidiomycosis is an

Table 6. Summary of countries and reptile species known to harbour *Ranavirus* infections in wild European populations.

Location	Common Name	Species	First Report
Portugal	Iberian mountain lizard	<i>Iberolacerta monticola</i>	Alves de Matos et al., 2011
Spain	Viperine snake	<i>Natrix maura</i>	Price et al., 2014
UK	Slow worm	<i>Anguis fragilis</i>	Price et al., 2017

**Figure 11.** The distribution of *Ranavirus* spp. in wild European reptile species.

endemic disease that has become more virulent due to recent changes or whether it is introduced. Genetic work conducted by Franklinos et al. (2017) demonstrates that the strains of ophidiomycosis in Europe are different to those in the US, but again further research is needed to fully understand this relationship.

RANAVIRUSES

Amphibians are not the only group of poikilothermic vertebrates to be affected by ranaviruses; both fish and reptiles are also susceptible. In Europe, most cases of *Ranavirus* infections in reptiles have been reported in captive populations (see Duffus et al., 2015). There are only three reports of *Ranavirus* infections in wild European reptiles in three different species; however, it is extremely likely that this is not reflective of the actual situation (see Table 6 and Fig. 11).

Signs of ranaviriosis in reptilians are diverse and non-specific, including but not limited to: lethargy, anorexia, upper respiratory tract necrosis, edema (especially in the region of the neck), dermatitis, and nasal discharge (Marschang, 2011). Most reptilian *Ranavirus* infections have actually been reported in testudinids, with comparatively few in squamates (see Duffus et al., 2015). Since the gross signs of ranaviriosis in reptiles are diverse, it is important for a full pathological investigation to be undertaken when disease is thought to be the cause of mortality. The first confirmed report of an iridovirus-like virus was documented in an Iberian mountain lizard (*Lacerta monticola*) from 'Serra da Estrela', Portugal (Alves

de Matos et al., 2011). The animal was asymptomatic and released after a blood sample was taken (Alves de Matos et al., 2011; Stöhr et al., 2015). A *Ranavirus* spp. was isolated from this blood sample and it was shown to be an FV3-like virus (Alves de Matos et al., 2011; Stöhr et al., 2015). A report of a *Ranavirus* infection in a slow worm (*Anguis fragilis*) is made in Price et al. (2017) but very little information about the affected animal was provided. However, in 2014, a more concerning report of a *Ranavirus* infection in a reptile was published. Price et al. (2014) report the first *Ranavirus*-associated mortality in a wild European reptile. A CMTV-like *Ranavirus* was isolated from a dead viperine snake (*Natrix maura*), from 'Picos de Europa' National Park, Spain, that had been scavenging on amphibians from a *Ranavirus*-associated mortality event (Price et al., 2014). This underscores the ability of ranaviruses to infect multiple hosts from different taxa and is a further demonstration of how severe a threat to herpetofauna that this group of pathogens is.

GENERAL RESOURCES FOR STUDYING HERPETOFAUNAL INFECTIOUS DISEASES

In the past decade, there have been a number of important resources published that examine different aspects of the study of herpetofaunal infectious diseases. Gray et al. (2017) provides an in-depth methodology on pathogen surveillance specifically for herpetofauna. They examine how to design a surveillance study, how to collect samples to perform the study, biosecurity, and even intervention strategies for when an emerging infectious disease has been detected. Gray et al. (2017) is a valuable resource for those seeking to improve study design and limit the spread of infectious diseases in herpetofauna. Langwig et al. (2015) provide an excellent resource for examining the emergence of infectious diseases in wildlife. They provide a framework for decision making and necessary actions pre-and post-infection detection.

Few resources for wildlife professionals have been developed that deal with necropsy techniques, as most have been written for veterinary professionals. While we encourage multidisciplinary teams that include veterinary pathologists, necropsies of herpetofauna may also be carried out by trained wildlife professionals. Duffus et al. (2017) provides a guide for investigating herpetofaunal mortality events. The manuscript is geared towards wildlife professionals and provides methods for necropsies, tissue sample collection, and sample storage and shipping. There are also publications that specifically deal with the detection and diagnosis of specific pathogens. For example, Thomas et al. (2018) examines the current diagnostic tools available for *Bsal*. They provide guidelines not only for the use of these

tools, but also for interpreting the results that these tools provide (Thomas et al., 2018). It is incredibly important to standardise both the methodologies and the interpretation of their results to ensure that results from different studies can be easily and consistently compared. Unfortunately, not all studies follow these guidelines and this leads problems in comparing results. For example, Dalbeck et al. (2018) uses a significance threshold of 0.05 GE when testing for *Bsal*, whereas a threshold of 0.1 GE is recommended by Thomas et al. (2018), when diluting extracted samples prior to qPCR. This of course, will lead to confusion and a difference in results when comparing studies unless there is a universally agreed standard, such is recommended in the case of *Bd* (Boyle et al., 2004; Blooi et al., 2013).

In addition to print resources, there are online databases that have been developed to track some emerging infections of herpetofauna. The Amphibian Disease Portal (<https://amphibiandisease.org>) is a database that is used to track reports of *Bd* and *Bsal* around the globe. Additionally, there is a *Bsal* reporting system for Europe (www.BsalEurope.com) and an associated early warning system. The *Bsal* reporting system and the early warning system for Europe will be extremely important in tracking infections and mitigating the effects of *Bsal* once it is found in an area. The Global Ranavirus Reporting System (<https://mantle.io/grrs>) is another online database that tracks reports of *Ranavirus* infection around the globe. These are both relatively new online databases and rely on researchers to upload their data into the system. To ensure that these resources are viable, we encourage researchers to upload their published data to these sites. An older resource that has been previously been used to track the global emergence of *Bd* is www.bd-maps.net (Olson et al., 2013). There are over 40,000 entries from over 80 countries in this database.

Ranaviruses, *Bsal*, and *Bd* are all reportable infections in amphibians (see <http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/> for a list of reportable infections). The OIE (<http://www.oie.int/>) has specific guidelines for reporting these infections and they should be reported to the appropriate agency in the country that they are discovered in. Additionally, risk analyses may be performed and there are several sources for guidelines, including Jakob-Hoff et al. (2014) and one jointly published by the OIE and IUCN (OIE and IUCN 2014). These are invaluable resources for anyone who deals with infectious diseases in wildlife.

CONCLUDING REMARKS

With the global declines of both amphibians and reptiles, it is now more important than ever to understand the threats to these unique and important animals. Although we have only focused on five emerging infectious disease threats to these animals in Europe, there are likely more (e.g. *Amphibiocystidium*, identified in Duffus & Cunningham, 2010), that are understudied and poorly understood, or even unknown infections that are taking a toll on the populations of these animals. Unfortunately, as stated above, the emergence of several of these

infectious diseases has resulted in population declines and local extirpations of several species, some of which were rare, but others, more worryingly, were considered to be common.

An important and necessary difference between the infection status of an animal and the appearance of clinical signs of disease is still often overlooked in many studies in herpetofauna. Infection is the presence of the potentially pathogenic or disease-causing agent in an organism or population, whereas disease is a measurable negative effect that the infection process has imposed in an animal (Scott, 1988). The effects of an infection may or may not result in the development of clinical signs of disease (Scott, 1988). Usually, disease is measured by the appearance of clinical signs. However, the presence/absence of these signs are often dependent upon the scale at which one is examining the organism (Scott, 1988).

Recent research has focused on both the imperfect detectability of infectious diseases (such as *Bd*) when using swabs and qPCR (DiRenzo et al., 2018) as well as the costs involved to complete the analysis (Sabino-Pinto et al., 2018). As previously mentioned, qPCR isn't infallible and false-positive or false-negative results are always possible. We therefore recommend that multiple detection techniques be used in order to be certain when screening suspected infected individuals. In order to fully understand the effects that emerging infectious diseases have on populations, an interdisciplinary team, as stressed in Duffus and Cunningham (2010), should be used. Ideally, such a team would include ecologists, herpetologists, population biologists, and veterinary pathologists. The team should investigate the entire situation, sometimes including multiple species in an area, to garner a deeper understanding of the issues that are potentially being caused by the emergence of an infectious agent.

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Microhabitat preference of the critically endangered golden mantella frog in Madagascar

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The golden mantella (*Mantella aurantiaca*) is a critically endangered (CR) frog, endemic to the eastern rainforests of Madagascar. Although the species is very popular in the pet trade and widely bred in captivity, its specific habitat requirements in the wild are poorly understood. Ten forested sites in the Moramanga district of Madagascar were surveyed for microhabitat and environmental variables, and the presence or absence of golden mantellas in quadrats positioned along transects in the vicinity of breeding sites. Mixed models were used to determine which variables best explained microhabitat use by golden mantellas. Sites where golden mantellas were found tended to have surface temperatures of 20–23 °C, UVI units at about 2.9, about 30 % canopy cover, and around 30 % herbaceous cover. Within sites, golden mantellas preferred microhabitats that had 70 % leaf litter coverage and relatively low numbers of tree roots. This information can be used to improve the identification and management of habitats in the wild, as well as to refine captive husbandry needs.

Keywords: mantella, Madagascar, amphibian, montane, rainforest, protected area

INTRODUCTION

Conservation of critically endangered species requires information at different spatial scales. Species distribution models (SDMs) can combine climatic and landscape variables from regional or national sources to provide large-scale pictures of habitat preferences and predicted distribution ranges (Guisan & Thuiller, 2005). However, within the predicted range a species is likely to be patchily and unevenly distributed, with occurrence within a habitat patch dependent on microhabitat and its associated microclimate. Microhabitat variables cannot usually be extracted from remote sensing or landcover maps and need to be measured directly on the ground (Stanton et al., 2012). This can be problematic for small, microhabitat specialist species that are difficult to observe. However, understanding microhabitat preferences is crucial to both providing appropriate habitat management in the field, and for informing captive management conditions in ex situ programmes (Semlitsch et al., 2009; Piludu et al., 2015; Tapley et al., 2015).

The golden mantella (*Mantella aurantiaca*) is a small, montane, diurnal, frog endemic to the eastern rainforests

of Madagascar (Glaw & Vences, 2007). Its extent of occurrence is 699 km² centred in the Moramanga district (Piludu et al., 2015). The known area of occupancy for this species is low at less than 10 km² (Vences & Raxworthy, 2008) with two main population clusters, one to the north of Moramanga at Ambatovy, Torotorofotsy forest and Analabe forest (Piludu et al., 2015). South of Moramanga clusters of breeding ponds are also found within fragments of Mangabe forest (Piludu et al., 2015). Due to a low area of occupancy, fragmented distribution and a decline in both numbers and suitable forest habitat, this species is categorised as critically endangered (CR) B2ab (iii, v) and listed on CITES Appendix II (Vences & Raxworthy, 2008). Current threats to the golden mantella and their rainforest habitat include logging, illegal collection for the pet trade, the destruction of breeding ponds due to mining activity, forest clearance to make way for subsistence agriculture and climate change (Andreone et al., 2008; Vences & Raxworthy, 2008; Piludu et al., 2015). The golden mantella therefore continues to be a prime candidate for in situ and ex situ conservation initiatives, but further research on habitat needs could help fill some knowledge gaps (Randrianavelona et al., 2010).

Most of the forest fragments inhabited by golden

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mantellas are deemed to have protected status (Piludu et al., 2015). In reality, the actual practical protection afforded to these areas is low, and forest clearance, mining and the illegal collection of golden mantellas continues regardless. According to Piludu et al. (2015) there are now more threatened golden mantella populations in forests with protected status than there are in forests without protected status. There is clearly a need to identify and prioritise new sites for future conservation actions such as assisted colonisation (Piludu et al., 2015; Andreone et al., 2016). However, without an in-depth knowledge of specific environmental/habitat requirements for the species, finding, creating, restoring or protecting optimum habitat is difficult. This study was therefore designed to determine the environmental and microhabitat variables that influence the presence of golden mantellas in the wild. The results will help to identify areas where this species is most likely to persist and thrive.

METHODS

Data Collection

Ten sites within the protected area of Mangabe-Ranomena - Sahasarotra, Moramanga District, eastern Madagascar, each containing or bordering known golden mantella breeding ponds, were targeted for surveys. Nine of these sites were surveyed between 28 November 2014 – 12 December 2014, and the tenth earlier on in the year in March 2014. These periods correspond to the main breeding activity periods for this species.

All surveys took place between 0700–1400 hrs each day, one visit per site. The surveys were centred on breeding pools located in shallow valleys. A series of transects were established on the slope running down to each pool. The first transect was positioned at the valley bottom and ran parallel to the pool. Subsequent transects were positioned at 30 m intervals up the slope, each following the contour at that position, with the last transect positioned along the crest of the slope (Fig. 1). The number of transects and the number of associated quadrats surveyed depended on the length, width and topography of the slope accessible to the survey team, i.e. two sites contained five transects, seven sites had three transects and one site had two transects. Where the top of a slope was bordered by a pathway the crest transect was placed 3 m down slope from the pathway, two further transects were then surveyed, one either side of the path. This meant that the two sites with crest paths had five transects in total. Along each transect 1 m x 1 m quadrats were established at 4 m intervals and transects contained between 10–20 quadrats, sites with more transects therefore having more associated quadrats. A two-person research team moved along the transect line stopping, surveying and recording environmental variables (Table 1) and the number of golden mantellas counted in each quadrat. Transect lines at the valley bottom were surveyed first, followed by next nearest transect as the slope was ascended. Golden mantellas observed outside the transects were also recorded and microhabitat variables measured within 1 m² of these locations.

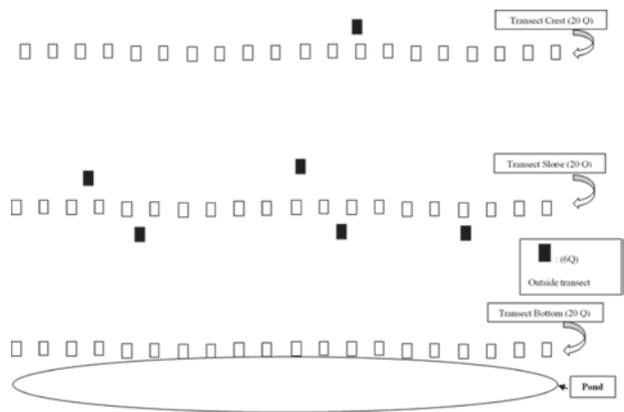


Figure 1. Diagrammatic representation of transect lines of twenty 1 x 1 m quadrats (white boxes) spaced at 30 m intervals running parallel to the breeding pond (white oval). Black boxes indicate where a golden mantella was seen outside of the transect/quadrat line and all environmental and microhabitat data within 1 m² of the individual were recorded. (Courtesy of Rakotondrasoa et al., 2015; unpublished report).

Statistical analysis

Statistical analyses were carried out using the statistical software R (R Core Team, 2017). The quadrats from the ten forests were classified into presence or absence of golden mantella categories and then initially tested for significant differences in microhabitat variables using the Wilcoxon Rank Sum Test. A generalised linear mixed model (GLMM) was then developed using the number of quadrats occupied and unoccupied to determine which independent variables (Table 1) were most likely to influence the microhabitat preference of golden mantellas (Table 2). We then followed Zuur et al. (2009) by removing the independent variable with the highest p value and re-running the GLMM. This procedure was repeated until only significant ($p \leq 0.05$) independent variables were left. Site was used as a random factor in the models, and we assumed a binomial error distribution with a logit link function.

RESULTS

Our analyses showed that for all ten sites combined, two microhabitat variables differed between quadrats with and without mantellas: litter cover and number of tree roots (Wilcoxon tests all $P < 0.001$). The GLMM also identified litter cover, number of tree roots and surface temperature as important predictors of golden mantellas (Table 2). Although not important at the microhabitat selection level, at the time of the surveys the sites where golden mantellas were found tended to have surface temperatures of 20–23 °C, UVI units at about 2.9, and about 30 % canopy cover and 30 % herbaceous cover (Table 3; Figs 2–3).

Within the sites, golden mantellas tended to occupy quadrats with at least 70 % leaf litter coverage and low (mean = 1.73) numbers of tree roots rather than quadrats with no or very low numbers of tree roots (Table 2).

Table 1. Variables, type and method of measurement used to collect data

Variable	Method of collection
Surface temperature (°C)	Rolson™ Infrared thermometer
Ultra-Violet B (UVI units)	Solarmeter 6.5TM Ultra-Violet Index (UVI) meter
Canopy cover (%)	Estimate
Herbaceous cover (%)	Estimate
Moss cover (%)	Estimate
Litter Cover (%)	Estimate
Litter depth (cm)	Tape measure
N° dead trees	Count
N° large trees (diameter < 1 m)	Count
N° small trees (~ 1.5 m height)	Count
N° trees cut	Count
N° trees damaged by cyclone	Count
Canopy height (m)	Estimate
Number of tree roots	Count

Table 2. Generalised Linear Mixed Model results showing potentially important predictor variables associated with golden mantellas (As canopy cover is alphabetically first in the list of variables it is labelled by R software as the Intercept and then used as a reference point). We provide the z-value ($z = x - \bar{x} / s$) and corresponding p-value for testing the null hypothesis that the slope and intercept is equal to 0 (Zuur et al., 2009).

Variable	Estimate	Std Error	z value	p (> z)
Intercept	-0.682018	0.858396	-0.795	0.42689
Surface temperature	-0.085088	0.037610	-2.262	0.02368
Litter cover	0.011037	0.003636	3.035	0.00240
Litter depth	0.038146	0.020607	1.851	0.06415
Tree roots	0.173847	0.050318	3.455	0.00055

However, across the sites, the number of golden mantellas declined in areas with very dense tree roots (Fig 2).

DISCUSSION

Although the relative number of occupied quadrats varied between sites, this may have been a result of environmental conditions on those survey days being particularly propitious for mantella activity, rather than reflecting real differences in abundance between sites. Nevertheless, our results show that at quadrat or transect level, the number of frogs encountered increases as percentage litter cover increased. Golden mantellas are a tropical forest floor species and are dependent on leaf litter to provide cover, create territories, forage, breed, and more easily regulate hydration state and body temperature. Like all frogs, golden mantellas can mitigate for the effects of evaporative water loss via the skin in dryer or warmer conditions by morphological and/or behavioural means (Duellman & Trueb, 1994). Adult frogs take up water via absorption across the skin surfaces when in close contact with moist soils and substrates (Duellman & Trueb, 1994). Granular skin on the ventral surface then facilitates increased capillary action drawing water up from moist soils and provides increased skin surface areas for absorption. However, morphological adaptations such as cutaneous sculpturing or increased permeability and vacuolisation will only

be advantageous in moist microhabitat (Hillyard et al., 1998). Therefore, the frogs must move between, or remain in, microhabitats where they are able to reduce the evaporation gradient of water from the body to the surrounding environment and rehydrate at a rate that offsets the amount of water lost. Blomquist and Hunter (2010) obtained similar results for wood frogs (*Rana sylvatica*), which were more likely to inhabit areas with greater humidity, substrate moisture, canopy cover, leaf litter depth and coverage. Seymour (1972) and Walvoord (2003) found that green toads (*Bufo debilis*) and cricket frogs (*Acris crepitans*) were more likely to select moist habitat when exposed to higher temperatures. Several other amphibian studies have obtained similar results and demonstrated that core temperatures, evaporative water loss and subsequent habitat selection were all highly influenced by ambient temperature and humidity (Tracy, 1975; Tracy, 1976; Pough et al., 1983; Semlitsch et al., 2009; Kohler et al., 2011; Tracy et al., 2013). It is now widely regarded that anuran activity is more limited by the effects of dehydration than by temperature, and as such hydoregulation is more important than thermoregulation (Seymour, 1972; Prest & Pough 1987; Tracy et al., 1993; Prest & Pough, 2003; Tracy et al., 2013).

Our results suggest that golden mantellas prefer sites with about 30 % canopy cover, and there is a tendency for fewer frogs to be observed in areas with

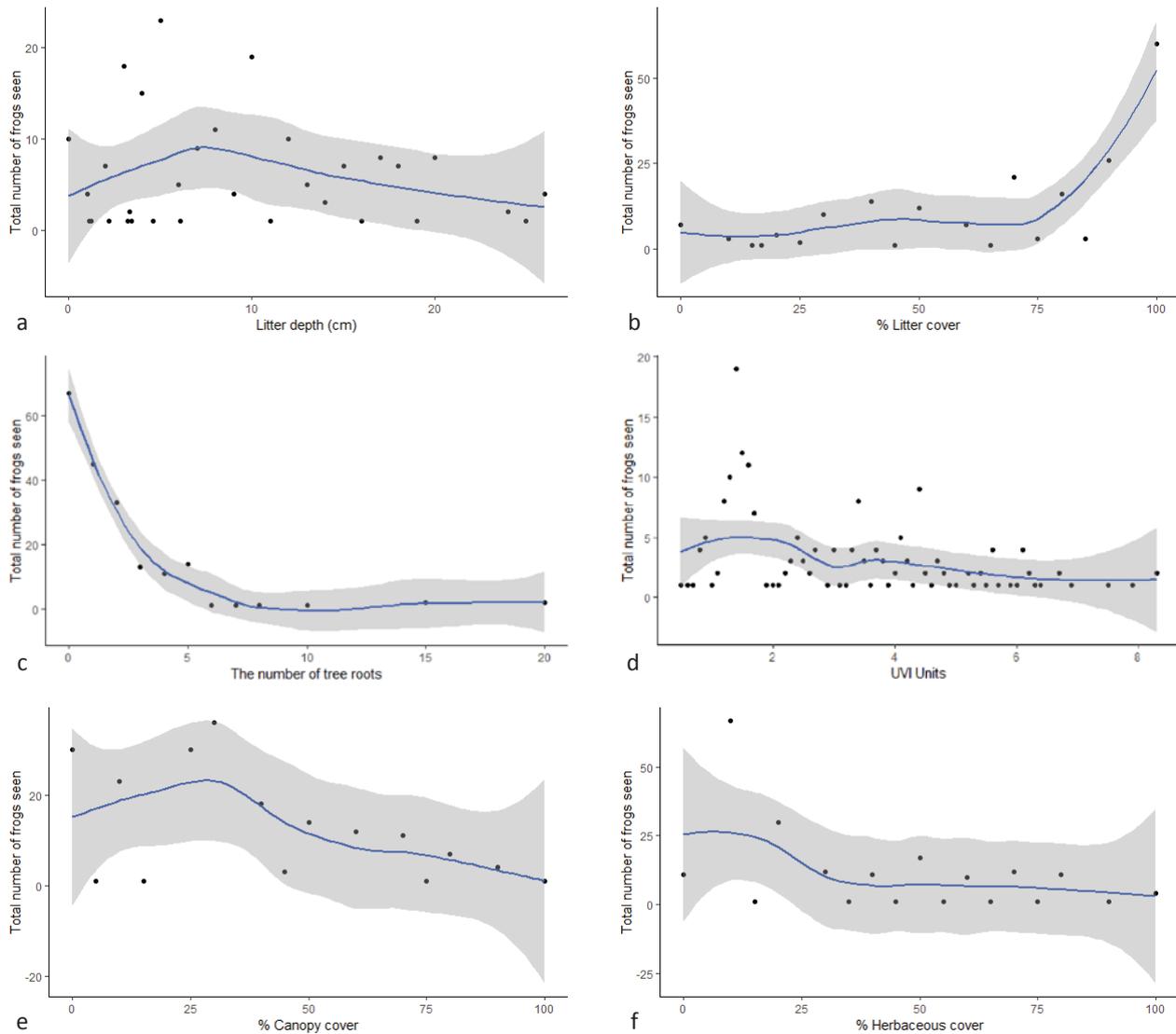


Figure 2. The total number of frogs observed combined for all ten sites versus **(a)** percentage litter cover, **(b)** litter depth in cm, **(c)** the number of tree roots, **(d)** UVB intensity (UVI units), **(e)** percentage canopy cover and **(f)** herbaceous cover. Each of the data points (black dots) represent the specific number of frogs recorded at each associated level of independent variable and are fitted with a LOESS smoother (blue line) to most closely model the relationship between independent variables and the total number of frogs seen. The shaded area represents a 95 % confidence interval.

dense canopy cover and tree roots. Golden mantellas are known to frequent sun-exposed areas within forest (Glaw & Vences, 2007) and the time of day or weather patterns may have an influence on mantella activity in these areas. Sunlight interception and irradiance at ground level depends to a certain extent on the height and positioning of the canopy (Dodd, 2010). The amount of cloud cover and orientation of the sun to the canopy gap can also be important in determining UVB and temperature levels at the forest floor (Pringle et al., 2003). Higher levels of UVB and herbaceous cover may be indicative of higher levels of disturbance or more extensive gaps in the canopy. Larger gaps in the canopy allow more solar radiation to penetrate further towards the forest floor which in turn increases soil and surface temperatures, lowers humidity, reduces leaf litter and food sources, these effects are amplified as canopy gap size increases (Carlson & Groot, 1997, Semlitsch et al., 2009).

It is plausible that as litter depth and the number of tree roots in a given quadrat increase, frog detectability becomes compromised. Greater coverage of herbaceous plants may also impede the ability of researchers to observe the frogs. According to an unpublished report by Rakotondrasoa et al. (2015), direct counts of golden mantella can be biased and challenging. An example is given where a count was carried out and around 400 mantellas were observed, yet further surveys were carried out and 2000 individuals were later captured in the same area. Indeed, it is generally acknowledged that at the population level count data for amphibians may be unreliable given imperfect detection, and where possible should be underpinned by capture mark recapture techniques, good quality habitat data and expert opinion (Schmidt, 2003; Sewell et al., 2010; Griffiths et al., 2015; Barata et al., 2017).

The rainy season begins in November in Madagascar, and this corresponds to the start of the breeding season

Table 3. Percentage of quadrats surveyed with or without golden mantellas at each of the ten forested sites. The range and mean of the predictor variables associated with mantella presence are also shown (% Litter cover, Litter depth, Number of tree roots, Surface temperature, UVI units, % Canopy cover and % Herbaceous cover). The percentage of quadrats not containing mantellas with associated ranges and means for predictor variables are also shown for each site. The bottom two rows show the differences between predictor variable means for quadrats with or without golden mantellas at all ten sites combined.

Site	Golden mantella	% of Quadrats	% Litter cover		Litter depth (cm)		Number of tree roots		Surface temp (°C)		UVB (UVI units)		% Canopy cover		% Herb cover	
			Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Sassarotra 25	with	51	0-100	65	0-24	10.4	0-8	1.6	18.1-27.7	22.3	3.0-8.3	5.0	0-100	30	0-100	32
	without	49	0-90	39	0-17	4.4	0-3	0.5	19.2-29.8	23.2	3.6-8.8	6.0	0-70	22	0-100	50
Sassarotra 17	with	47	30-100	74	2-26	13.0	0-20	4.7	19.5-27.8	22.4	1.2-3.6	1.6	0-60	29	0-80	42
	without	53	30-100	67	2-16	8.5	0-10	2.2	18.7-27.9	22.1	1.2-4.9	2.3	0-80	25	10-90	42
Antanimbarit-sara	with	36	0-100	75	0-18	6.0	0-3	1.1	16.9-22.9	20.0	0.9-1.7	1.4	0-90	36	0-100	25
	without	64	0-100	72	0-12	5.0	0-4	1.2	15.6-24.3	19.2	0.3-1.6	1.2	0-90	33	0-100	29
Andriamaro-hangotra	with	31	0-100	39	0-5	2.2	0-2	0.5	18.6-20.3	19.3	1.5-6.1	4.3	0-60	15	0-80	32
	without	69	0-90	51	0-10	3.2	0-4	0.4	19.2-20.4	19.7	1.6-6.4	3.7	0-70	21	0-100	37
Andravinala	with	27	40-100	79	10-25	16.5	0-5	1.4	18.6-21.1	20.1	2.7-5.6	3.9	0-60	27	0-50	14
	without	73	10-100	76	5-30	14.0	0-4	0.8	17.7-21.6	19.8	2.1-5.7	3.1	0-80	25	0-80	18
Andavaioaka 4	with	19	20-100	73	2-20	7.4	0-8	2.5	18.1-22.1	20.1	2.0-3.0	2.4	0-90	49	0-80	29
	without	81	10-100	65	1-30	9.1	0-7	1.9	15.4-25.8	20.2	1.6-3.2	2.3	0-100	27	0-100	30
Ambinanin'i	with	18	40-100	92	3-18	6.7	0-5	1.7	16.5-26.9	19.0	0.5-4.5	1.4	0-80	24	10-80	33
	without	82	0-100	61	0-18	6.9	0-5	1.1	15.1-43.2	24.3	0.3-7.8	1.9	0-80	18	0-90	40
Lemafy	with	14	0-85	58	1-7	3.2	0-1	0.1	16.8-22.9	19.1	3.3-7.5	4.6	0-80	38	10-75	39
	without	86	0-95	52	0-12	3.9	0-4	0.2	14.3-27.7	19.1	0.8-9.6	3.9	0-90	39	0-90	32
Bekomy	with	11	40-80	81	2-5	3.9	0-6	1.7	17.3-22.2	19.2	0.8-1.3	1.1	40-80	57	10-10	10
	without	89	10-100	73	0-22	6.1	0-4	1.2	17.0-36.7	21.4	0.9-5.8	1.7	0-80	40	0-90	16
Antoko	with	9	90-100	98	5-12	9.0	0-5	2.0	19.8-22.6	21.2	2.4-3.7	3.2	0-90	17	10-80	38
	without	91	10-100	84	1-18	5.5	0-15	1.6	18.2-40.4	22.0	1.3-6.3	2.8	0-100	40	10-90	33
Mean of Sites	with	26		73.4		7.8		1.73		20.2		2.9		32.2		29.4
	without	74		64.0		6.7		0.9		21.1		2.9		29.0		32.7

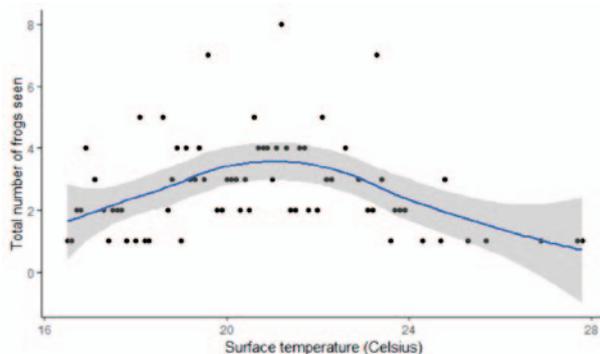


Figure 3. The total number of golden mantellas encountered combined for all ten sites and associated surface temperatures. Each of the data points (black dots) represent the specific number of frogs recorded at each temperature and are fitted with a LOESS smoother (blue line) to most closely model the relationship between surface temperature and the total number of frogs seen. The shaded area represents a 95 % confidence interval.

for golden mantellas. The Bejofo site was surveyed in March, towards the end of the breeding season when frogs may have migrated back up the hill away from ephemeral breeding ponds. Indeed, all golden mantellas encountered in Bejofo were recorded in the

hill-top quadrats. The other nine sites were surveyed in November and as such we would expect to observe more frogs in the valley bottom transects near to the breeding ponds. However, this was not the case, as more frogs were observed in the higher transects on the slope or crest of the hill. It may be that the frogs are migrating down to the breeding ponds and laying eggs in leaf litter, then migrating back up to warmer surface temperatures on the slope and crest. Lower average temperatures recorded in valley bottom transects may also mean fewer frogs are active outside of leaves and observed. The timing of the surveys was dictated by logistics and weather, but either way, there was no evidence that the difference in the timing of surveys between sites made any difference to observations of microhabitat use.

CONCLUSIONS

We recommend maintaining the integrity of current golden mantella forest habitat, increasing connectivity between breeding ponds and keeping disturbance of these areas to a minimum by increasing the levels of protection. Piludu et al. (2015) recommend an increase in effort or a new approach to safeguard breeding ponds, involving sampling and surveillance for detection of emerging pathogens, such as the chytrid

fungus *Batrachochytridium dendrobatidis* (e.g. Bletz et al., 2015). The monitoring of local climate and the study of predicted climate change effects and further development of species distribution and population viability models to determine future relevant sites should continue (Piludu et al., 2015). Like Rakotondrasoa et al. (2015), we recommend continuing the search for new ponds and the continued monitoring of existing ponds, as well as continuation of research and estimations of population sizes using capture-mark-recapture techniques. Understanding the relationship between rare species and subsequent avoidance by animals of certain microhabitats within their range is vital if we are to plan future management strategies in important forest habitat (Semlitsch et al., 2009; Irwin et al., 2010; Pike et al., 2010). Information on such factors as daytime surface temperatures, canopy cover and litter cover can be used to inform the identification, creation and restoration of suitable habitats in the wild, as well as the requirements of the species in captivity.

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Solving species quandary: why awareness programs are pivotal in snake conservation

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Snakes are feared but largely misunderstood by the public. In India, a country with ancient cultures and traditions, snakes were historically worshiped and their habitats were protected as sacred. However, over time these values have shifted to non-conservative approaches, even with people killing snakes on encountering them. This attitudinal change is an indication of knowledge erosion. A closed-ended survey was conducted for 300 randomly selected people in Kerala, India in the age range of 21-55 years to discern their skills in identifying common snake species, and evaluate their attitudes and knowledge about the importance of snakes in the environment. The study showed a lack of knowledge among the responders regarding snakes. Common snakes were not familiar, venomous and non-venomous snakes were mis-classified, and a lack of knowledge about post-bite treatment was observed. Therefore, there is an urgent need for planned conservation education and awareness programmes to build a snake-friendly society. A better understanding of snakes will supplement conservation in the future, and can minimise human-snake conflicts.

Keywords: snakes, conservation, identification, awareness

INTRODUCTION

Snakes are generally less liked compared to mammals and birds (Tisdell et al., 2015). They are often regarded as slimy, slithering creatures worthy of fear and disgust (Ohman & Mineka, 2003). Studies have shown snakes to be strongly and widely associated with fear in humans, independently of conscious cognition (Agras et al., 1969; Ohman & Mineka, 2001). A lack of knowledge and misguided perceptions of snakes have threatened their populations worldwide (Pandey et al., 2016). In India, with a long cultural history, traditional literature and mythological stories have depicted snakes as respected and fearful creatures (Mandlik, 1869; Menon, 1901; Sinha, 1979), leading to their worshipping as gods and killing them considered as sin (Allocco, 2009). This concept has led to the protection and conservation of snakes and their habitat in some parts of India, especially in Kerala as sacred groves ("Kaavu" in Malayalam), which are pristine patches of forest protected by a family or community for the conservation of biodiversity. Even though snakes are considered as symbols of power and worthy of worship worldwide (Miller, 1970; Hastings et al., 1922; Sasaki et al., 2010), when encountered in human-inhabited areas, they can be killed out of fear of a potentially life-threatening bite. The killing of snakes by humans has been identified as an important cause of their population decline (Dodd, 1987). India has one of the highest number of human deaths due to snakebites

(Kasturiratne et al., 2008), and people tend to kill snakes when encountered. This is contradictory to the culture and worshipping that was historically practiced. This attitudinal change has drawn the urgent need to educate the public regarding the importance and conservation of snake species.

Along with this change in attitude, human-induced impacts such as urbanisation and habitat loss have challenged the existence of snake species in both urban and rural areas. Rapid urbanisation has resulted in the alteration of most natural habitats, affecting local species (Czech et al., 2000), and is considered as one of the significant factors for current and future species extinctions (McDonald et al., 2008). Encroachment of natural habitat and poor waste management that appeals to rodents and other potential prey species has led to snakes occupying human-dominated and urban landscapes. This has resulted in increased human-snake conflict and casualties for both humans and snakes (Barve et al., 2013). In this context of rapid urbanisation, the study and conservation of urban wildlife is of great importance for minimising the extinction of urban wildlife. In addition to scientific research, public participation and support is mandatory for the conservation of urban wildlife. As the public is responsible for taking effective roles in solving environmental problems, they must be actively included in such actions to generate their interest in supporting long term conservation initiatives (Council of Environmental Quality, 1981).

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Public awareness programmes have shown positive effects on conservation (Zhang & Yin, 2014). Increasing awareness is often considered a precondition that can predict some conservation behaviours (Maibach, 1993; Beedell & Rehman, 2000). Formal education has often been found to be a predictor of an awareness towards nature (Rauwald & Moore, 2002). Peoples' attitudes towards conservation requires a clear understanding of various social, economic and cultural factors (Holmes, 2003). Whilst studies on attitudes and perceptions of charismatic megafauna are given priority (Sekhar, 2003; Shrivastava & Heinen, 2007; Heinen & Shrivastava, 2009), snakes were always given less attention. The aim of the present study is to understand the attitude and knowledge of the public regarding snakes, identification skills and post-bite treatments, from which we could develop an action plan for better education and awareness for promoting the conservation of snakes.

METHODS

A closed-ended questionnaire was distributed to 300 respondents (285 males and 15 females) during a snake awareness workshop conducted during October 2017 as part of a wildlife week celebration in Kannur District of Kerala, India. The interviewees are employees of the Kerala Police Department and the Kerala Armed Force, with ages ranging from 21 to 55 years old. To evaluate the knowledge and snake identification skills of the participants, pictures were shown to them using a slideshow presentation. They were then asked to identify snake species, and to identify correct snake bite treatment protocols. The data were then compiled and analysed to understand the perspectives and knowledge among the audience about snakes.

RESULTS

Among the participants, 80.3 % were afraid of snakes and 19.7 % were not afraid of snakes. When asked about willingness to co-exist with snakes, 68.0 % showed non-willingness while 32.0 % showed a willingness to co-exist with snakes (Fig. 1). The majority of individuals were curious to learn more about snakes (89.0 %), appreciate the ecological benefits of urban snakes (86.7 %), and to support the conservation of snakes (94.7 %). However, the majority (81.0 %) had a false conception that urban snakes should be translocated to nearby forest areas.

Even though 65.7 % of individuals were confident in identifying common snakes, actual results varied when

the responders were asked to identify the species shown in the pictures. When showed pictures of common snakes, the participants were able to identify and name the rat snake (*Ptyas mucosa*, 93.0 %) and the Indian rock python (*Python molurus*, 79.7 %) correctly, whereas the checkered keelback (*Xenochrophis piscator*) and common trinket snake (*Coelognathus helena*) were identified correctly by less responders, 13.0 % and 1.7 % respectively (Fig. 2).

In another question, we asked the responders to identify and name only the venomous snakes species among a total of eight species shown in a picture. The common krait (*Bungarus caeruleus*) and Russell's viper (*Daboia russelii*) were correctly identified as venomous by 74.3 % and 61.6 %, respectively, of the responders. However, the common wolf snake (*Lycodon capucinus*, 68.0 %), common sand boa (*Eryx conicus*, 57.0 %), travancore wolf snake (*Lycodon travancoricus*, 42.0 %), striped keelback (*Amphiesma stolatum*, 25.3 %), checkered keelback (24.6 %) and Indian rock python (8.3 %), which are the other common species in the study area, were all misidentified as venomous by responders (Fig. 3).

Knowledge on post-bite treatment was found to be weak among the responders (Table 1). Correct practices such as washing the bitten area with soap and taking the victim to hospital after immobilising the bitten area were suggested only by 58.0 % and 40.0 % responders, respectively. Incorrect practices such as tight tourniquets (45.3 %), spending time identifying the snake (49.0 %), cutting open the wound to suck out the venom (25.6 %), and applying ice on the bitten area (9.6 %) were also suggested by the responders.

DISCUSSION

Snakes are one of the most misunderstood and feared groups of animals around the world. Predatory snakes have played a central role in shaping the evolution of fear in mammalian and primate brains (Isbell, 2006). They trigger unique stimuli that are deeply grounded in evolutionarily shaped behaviour systems, causing alertness, rapid fear and escape tendencies (Ohman, 2009). It is therefore natural for humans to demonstrate hatred and fear towards snakes.

Translocation has been used as a mitigation measure against problematic animals for decades without any scientific evidence to support it (Athreya, 2006). This happens to be the case for most of the snakes rescued from urban areas, which are then translocated to forest areas. For example, Russell's viper, which is found in

Table 1. Knowledge on post snake bite practices

Post snake bite practices		Percentage of responders
Correct	Wash out the bitten area with soap	58.0
	Take the victim to the hospital at the earliest	40.0
Incorrect	Wrap bandage tightly across the bitten area	45.3
	Identify the bitten snake	49.0
	Cut open the wound and suck out the venom	25.6
	Apply ice	9.6

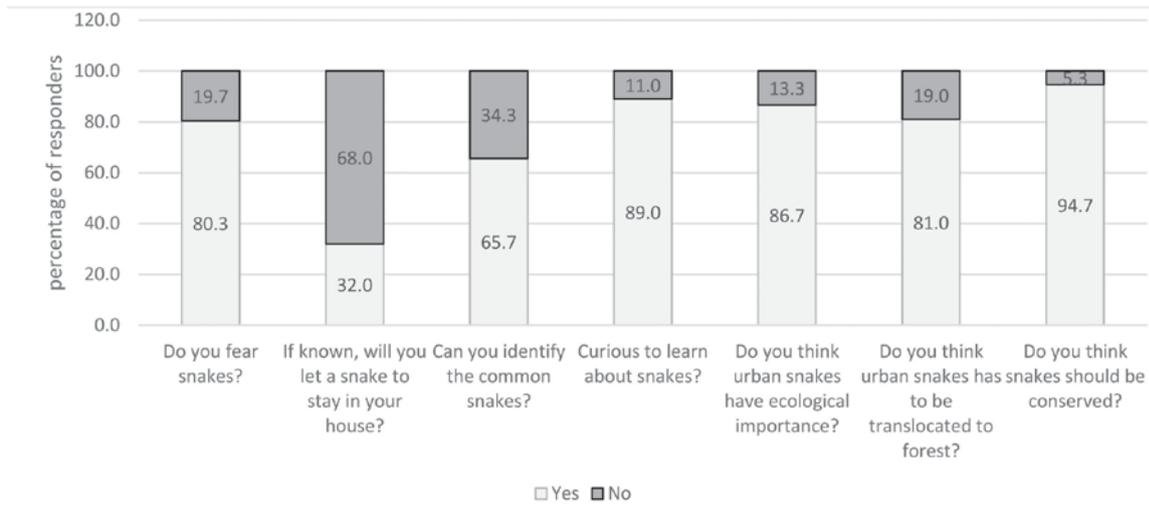


Figure 1. Attitude of the responders towards snakes

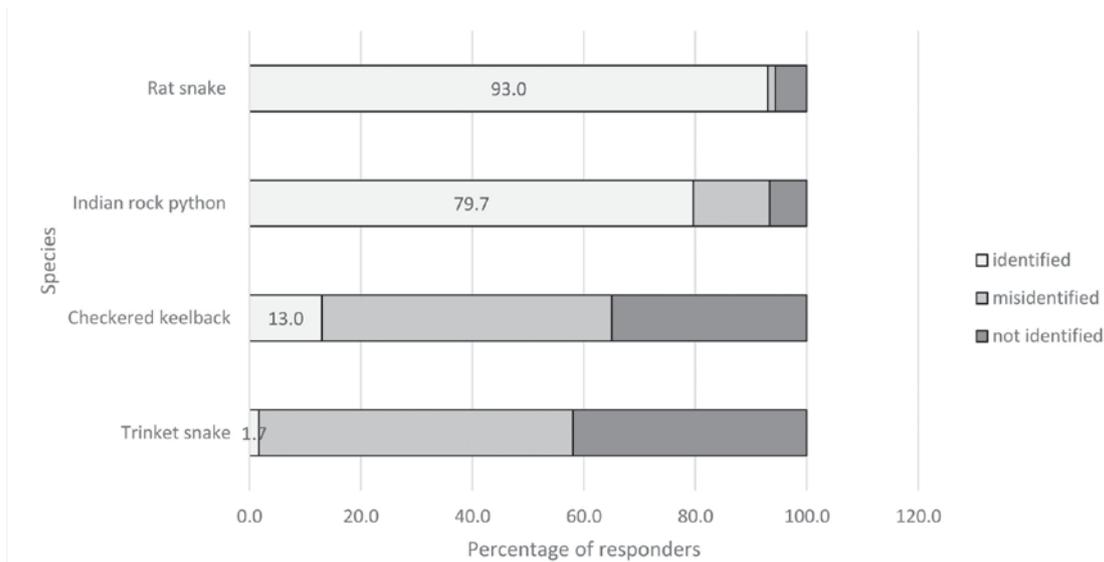


Figure 2. Percentage composition of responders in identifying common snakes

suburban habitats and laterite terrains, are released in the forest when caught. Translocated snakes have shown changes in behaviour such as ceasing reproductive activity and feeding, with deaths occurring due to the unavailability of their familiar prey (Barve et al., 2013). Cases of translocated snakes invading new territories, increasing home ranges, and travelling further distances were also recorded (Nowark et al., 1999; Butler et al., 2005). It was noted that majority of responders believed in the translocation of snakes to forest areas, suggesting the public believes that “snakes live in the forest”. This attitude shows lack of awareness about the ecological role of snakes as a main predator in urban environments, and their economical role in rodent control.

Responders were unable to identify common species such as checkered keelback, trinket snake, common wolf snake, and travancore wolf snake, and the suggestion that species like the checkered keelback and Indian rock python are venomous indicates a lack of understanding amongst the responders about common snakes in Kerala.

This lack of knowledge has implications for conservation, as many non-venomous snakes in human inhabited areas are killed by people, mostly due to their fear of snakes and snake bite deaths (Balakrishnan, 2010). Thus, along with these merciless killings, factors such as habitat loss and urbanisation have led to the decline in distribution and abundance of herpetofauna (Gibbons et al., 2000; Collins & Storfer, 2003; Beebee & Griffiths, 2005)

Good knowledge about potentially venomous snakes in our habitats and post snake bite practices are important lifesaving skills during snake bite emergencies. However, our results also showed lack of awareness in this context. Even though practices like tight tourniquets, spending time identifying the bitten snake, and sucking out venom are not advised (Annon, 2007, Ghosh et al., 2016), responders still follow such practices. A lack of knowledge by the general public on how to react when a bite occurs, and opting for discredited treatments, are major factors in the high rate of snake bite induced mortality in humans (Sivakumar & Jadeja, 2012).

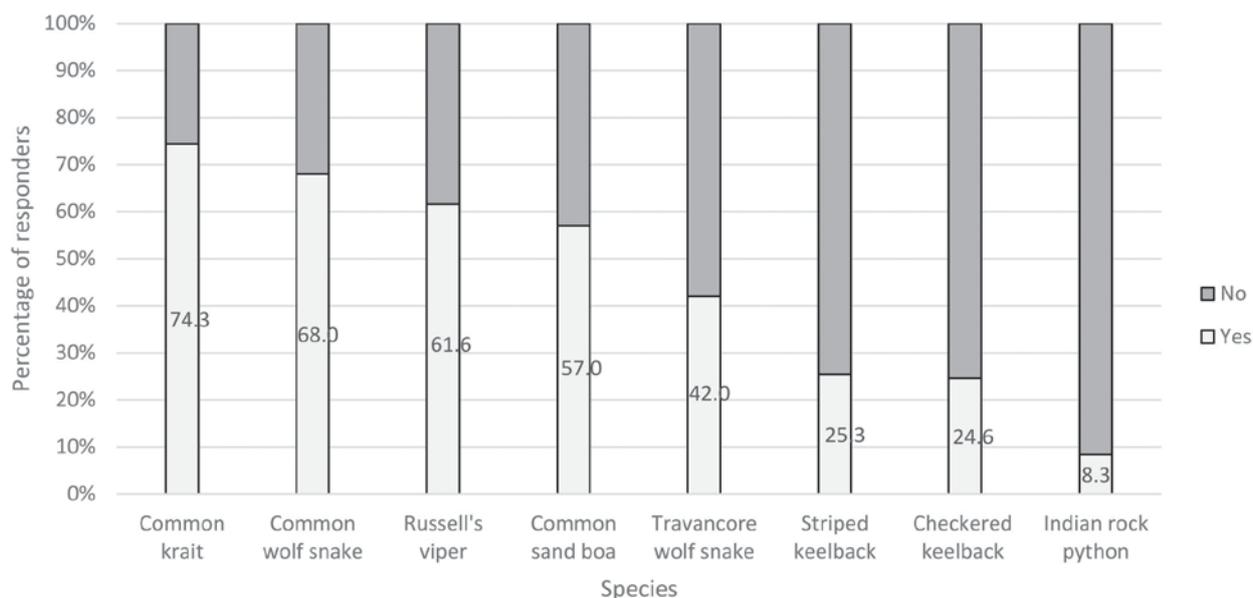


Figure 3. Responder's opinion on snakes; whether venomous or non-venomous

Increasing awareness of urban ecology through information dissemination is one of the best methods to conserve urban environments, leading to effective conservation (Kendle & Forbes, 1997). This was demonstrated during an educational programme on the conservation of non-venomous snakes in Nilambur, Kerala, which led to positive changes in the attitudes of the local public (Balakrishnan, 2010). With the completion of the programme, a considerable decrease in the killing of non-venomous snakes in that area was recorded (Balakrishnan, 2010).

Misconceptions regarding snakes and snakebites have drawn much attention and curiosity by people across a range of cultures and civilizations (Silva, 2013), which was also reflected in our study as many of the responders were curious to learn about snakes. The acceptance of their importance and readiness to co-exist shown by some responders could be a good sign for future conservation programmes. Therefore, more awareness programmes are required, targeting the public to educate them and change their attitudes towards snakes to promote conservation.

Media coverage on environmental issues have also shown potential positive change in the general public (Mikami et al., 1995). Popularising snakes and their importance using visual and print media, as well as developing snake parks and museums, are believed to be helpful in developing snake-friendly attitudes (Pandey et al., 2016). Thus, structured conservation educational programmes have to be prioritised to bring a positive attitude towards the conservation of urban wildlife. Promoting knowledge and awareness among public regarding snakes and snakebites will bring about an attitudinal change in the public, and can minimise casualties and fatalities in human-snake conflicts leading to a snake-friendly society.

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Review of chelid and emydid turtle distributions in southern South America with emphasis on extralimital populations and new records for Argentina

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There are many cases of animals reported in localities away from natural areas of distribution for the species. With respect to native freshwater turtles of Argentina and bordering countries, several populations (and single specimens) were reported for areas that cast doubts about their origin, due to not only the long distance from other known localities but also the geographical barriers that are in between. The present work provides a review of localities of the native turtle species *Hydromedusa tectifera*, *Phrynops hilarii*, *Acanthochelys pallidipectoris*, *A. spixii* and *Trachemys dorbigni* in Argentina, Bolivia, Brasil, Paraguay and Uruguay, and adds new records for some of these species for Argentina. We also employ an analysis to recognise core and extralimital populations, and a methodology based on five criteria that must be taken into account to elucidate if one extralimital turtle record is the result of anthropogenic action or a case of natural occurrence. Finally, we discuss about the origin of extralimital reports of turtles of the mentioned species.

Keywords: freshwater turtles, distribution, Argentina, bordering countries, translocation

INTRODUCTION

Translocation is defined as the intentional release of individuals of a species at a within-range location different from their capture site in order to 'establish, reestablish, or augment a population' (Griffith et al., 1989; Rittenhouse et al., 2007; 2008). This type of planned translocation is a useful conservation tool for population management, particularly when the habitat persists but the species to be translocated is locally extinct (Cook, 2004; Tuberville et al., 2005; Moore et al., 2013).

Officially planned turtle translocations of chelid and emydid turtles have never been performed in Argentina or in the bordering countries where they are distributed (Bolivia, Brasil, Paraguay and Uruguay). However, extralimital populations have been reported for certain freshwater turtle species. Some of these populations are arbitrarily believed to have been introduced by humans (Freiberg, 1938; Cei, 1993). They are (1) the South American snake-necked turtle *Hydromedusa tectifera* (Cope, 1869) recorded extraliminally in Atamisqui (Santiago del Estero province), and in mountain streams from western Córdoba province and southern Buenos Aires province (Cabrera et al., 1986; Cabrera, 1998; Di Pietro et al., 2012); (2) the spotted-bellied toad-headed turtle *Phrynops hilarii* (Duméril & Bibron, 1835), which

has extralimital records in three western provinces (San Juan, Mendoza and Tucumán) and the centre of Argentina (Santiago del Estero province and north-western Córdoba province; Laurent & Teran, 1981; Richard, 1987; Broin & de la Fuente, 1993; Cabrera, 1998; Prado et al., 2012); (3) the chaco side-necked turtle *Acanthochelys pallidipectoris* (Freiberg, 1945) and (4) the black spiny-necked swamp turtle *A. spixii* (Duméril & Bibron, 1835), both with extralimital records in Mendoza province (Richard, 1987; 1991a). Here, we review the distribution of these species and of the black-bellied slider turtle, *Trachemys dorbigni* (Duméril & Bibron, 1835), in South America with a focus on localities from Argentina. We also supply previously unknown core localities for this country and provide new extralimital localities for central and north-western Argentina: two for *H. tectifera*, one for *P. hilarii* and one for *T. dorbigni*. We also determine if the extralimital populations of the five species studied here are a result of anthropogenic action or a case of natural occurrence.

MATERIALS AND METHODS

The distributions of the chelids *H. tectifera*, *P. hilarii*, *A. pallidipectoris*, *A. spixii* and the emydid *T. dorbigni* in Argentina were constructed on the basis of specimens

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housed at the main herpetological collections of the country and from literature reports (Online Appendix). During the review of herpetological collections, we detected localities that were not previously reported in the literature. In addition, we provide other localities based on our field observations. The distribution of these species in other countries of South America were constructed on the basis of an exhaustive revision of literature reports. Localities with several reports were listed using only the first available one in order to avoid excessive references (Online Appendix). Records of water courses that lacked of precise location were mapped on the middle basin.

A Kernel Density Analysis (KDA) was conducted to determine the core of species distribution using ArcGIS software. This spatial analysis identifies one or more central points of distribution and calculates the mean and standard distances from them. This procedure generates concentric areas that correspond to different categories of significant point density. We worked with nine categories: four with the highest concentration of points (core of the species range), four with moderate to low concentration of records (peripheral distribution) and one with the lowest density (extralimital records, independently of their origin, natural or anthropogenic). Ringuelet (1956) listed four key factors to understand the current chorology of a given species. We used these combined with those established by Cabrera et al. (1986) to generate five criteria that a researcher must take into account to decide if a given extralimital turtle record is the result of anthropogenic action or a case of natural occurrence. It is important to note that just one of these criteria may be determinant to choose natural occurrence instead of anthropogenic mediated distribution. These points are (in order of importance according to our view): 1) paleochorology estimated from the fossil record: the

presence of the species (or related species) in the area in past geological times; 2) efficacy of the methods of dispersion associated with the presence of both current and ancient habitat corridors: it is important to know if it is possible that the turtle could have reached the area by its own through a water course; 3) ecological requirements and interactions with other species (e.g., competition among others); 4) current toponymy or place names that indicate the presence of certain fauna there (e.g., the turtles' stream); and 5) the certainty that official species introduction has not occurred in the past.

RESULTS

Hydromedusa tectifera

Our analysis showed that *Hydromedusa tectifera* has a distribution with two principal cores: one in Argentina and Uruguay, along the Uruguay-Río de la Plata river basin, and the other in southern Brazil, surrounding the Iguazú River (Fig. 1). We report 12 previously unknown localities corresponding to the first mentioned core from five departments of the north-east of Buenos Aires province (Río de la Plata and Salado river basins). These reports are based on our field observations, some of them supported with e-voucher specimens (Online Appendix). They are: (1) El Gato stream, (2) Rodríguez stream, (3) Carnaval stream, (4) Martín stream, (5) Pescado stream and (6) Cajaravilla stream (La Plata department); (7) del Petroleo channel (Ensenada department); (8) Tubichamini stream and the associated Cañada Arregui, (9) Zapata stream and (10) Juan Blanco stream (Magdalena department); (11) Juan Gerónimo ranch (Punta Indio department); and (12) Salada de Monasterio lagoon (Lezama department).

The extralimital populations of *H. tectifera* detected from the KDA are located in Argentina (north-center of Córdoba province, center of Santiago del Estero province

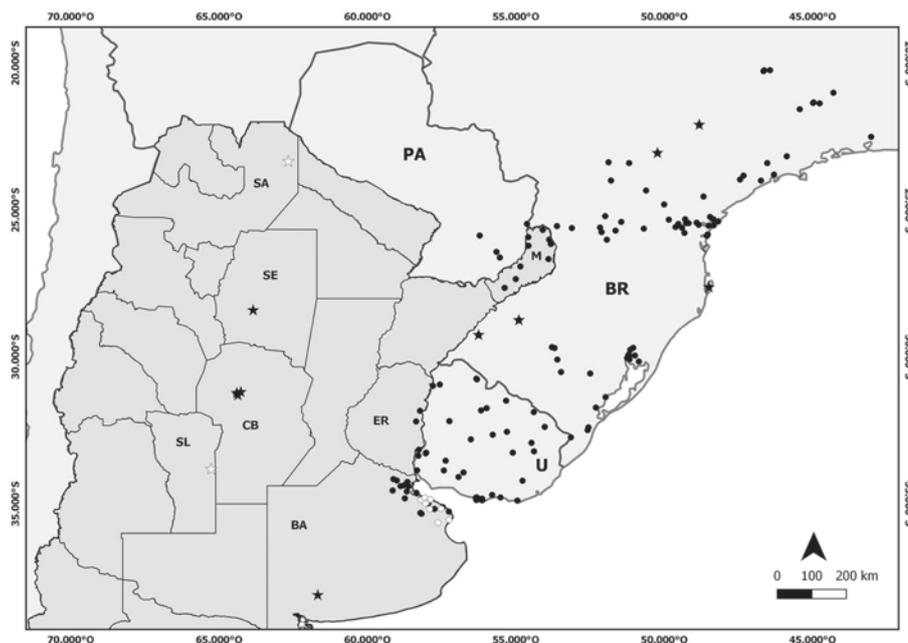


Figure 1. Localities recorded for *H. tectifera* in Argentina and in bordering countries. Spots indicate records corresponding to the core(s) of the species distribution; stars indicate extralimital records. Black colour represents previously known localities; white colour represents new records. Records with no precise locality were not mapped. BA, Buenos Aires; BR, Brazil; CB, Córdoba; ER, Entre Ríos; M, Misiones; PA, Paraguay; SA, Salta; SE, Santiago del Estero; SL, San Luis; U, Uruguay.

and south-west of Buenos Aires province), and in the south of Brazil (Tieté River, Andirá, Itaqui, Bossoroca, and Florianópolis; Online Appendix; Fig. 1).

Here we add two previously unknown and clearly extralimital localities for the species, which constitute the first records for two provinces. One of these is the Popopis River (also known as Quinto River), at Justo Daract, General Pedernera department, in eastern San Luis province (Fig. 1). We visited this river in October 2014 and found four females and two males of the species (the e-voucher of one of them is housed at the herpetological collection of the Universidad Nacional del Nordeste (Online Appendix; Fig. 2A-B). This locality is part of the Espinal Eco-region (Burkart et al., 1999) and the Popopis River flows from north-west of San Luis province to La Picaza lagoon system in the south of Córdoba province. The other extralimital locality we report here is Los Blancos, Rivadavia department, in north-eastern Salta province (Fig. 1). The voucher material is the carapace of an adult male housed at the herpetological collection of the Fundación Miguel Lillo (Online Appendix; Fig. 2C-D). Los Blancos is located in the Dry Chaco Eco-region (Burkart et al., 1999), about 50 km N Bermejo River; this river is a tributary of Paraguay River and belongs to the Paraná-Río de la Plata river basin.

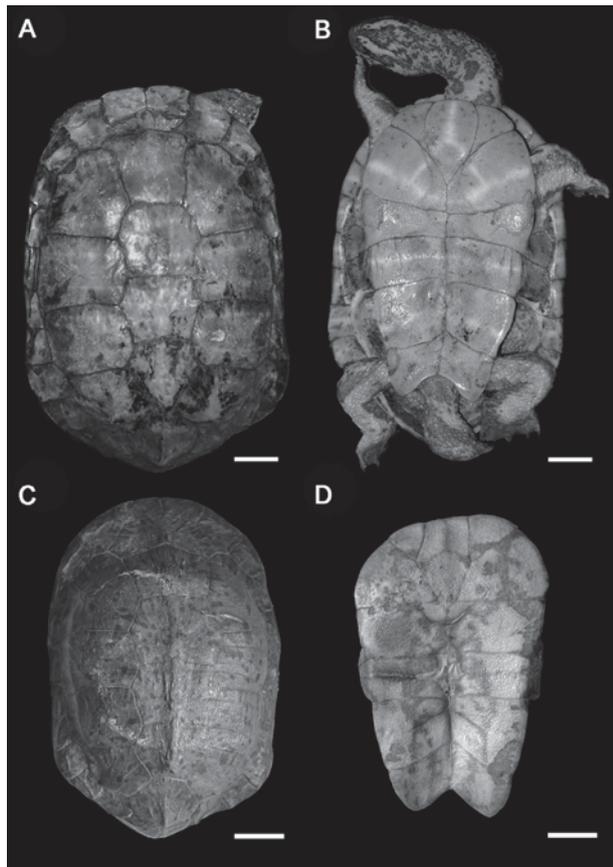


Figure 2. (A) Dorsal and (B) ventral views of a male specimen of *H. tectifera* from Popopis River, San Luis province (UNNEC-A 000010). (C) Dorsal and (D) ventral views of the carapace of a male specimen of *H. tectifera* from Los Blancos, Salta province (FML 13573). Scale bars = 3 cm.

Phrynops hilarii

According to the KDA, the distribution of *Phrynops hilarii* has two main cores: one is located in the Argentine Litoral region, mainly along the Paraná and Uruguay river basins and the Río de la Plata River, where these rivers flow into (Fig. 3). The other core occurs in southern Brazil and in northern Uruguay, mainly in the area drained by the Guaíba river basin and the system of dos Patos and Merin lagoons. We provide 19 previously unreported core localities of the species for five Argentine provinces (Buenos Aires, Santa Fe, Córdoba, Chaco and Corrientes). These records are mainly based on our field observations (some of them with e-voucher support; Online Appendix; Fig. 3) and communications from colleagues. Ten of the records correspond to Buenos Aires province: (1) El Gato stream, (2) Rodríguez stream, (3) Cajaravilla stream and (4) Pescado stream (La Plata department); (5) Aeroclub lagoon (Ensenada department); (6) Los Talas (Berisso department); (7) Juan Blanco stream and (8) Zapata stream (Magdalena department); (9) Cañada de los Peludos (Chacabuco department, M. V. Lazcoz, pers. comm.); and (10) "A" channel on Provincial Road 2, south of Dolores (Dolores department). The core records 11–13 are from Santa Fe province: two near Paraná River in north-eastern Santa Fe province: 11) Aguilar stream, General Obligado department, and 12) a small lagoon between Cayastá and Helvecia, in Garay department; record number 13 is near Salado River in the central-west of Santa Fe (San Cristobal department). The new records number 14 and 15 correspond to Corrientes province: a nameless stream near Paraná River, about 30 km NW Esquina (Esquina department) and Curupicay River at National Road 14 km 377 (Monte Caseros department). Records 16–17 are from Tercero River in south-eastern Córdoba province: Monte Buey (Marcos Juárez department) and Ballesteros Sud (Unión department). Finally, the new records 18 and 19 are from eastern Chaco province, near Paraguay River (El Cachapé farm and surroundings of Resistencia, on National Road 11).

The extralimital records of the species detected from the analysis in Argentina are: north-central Mendoza province, north-central Santiago del Estero province, central Tucumán province, southern San Juan province, northern Formosa province, north and central Córdoba province, and western Santa Fe (Online Appendix; Fig. 3). We incorporate two new extralimital reports (Fig. 3): one from the Cabra Corral dam in Salta province (first provincial record), and the other from a tributary of San Juan River, near San Agustín del Valle Fértil, in eastern San Juan province (E. Sanabria, pers. comm.). Both are supported by the finding of several specimens that were not collected nor photographed: the record from Cabra Corral consists of a group of about six adults detected basking on an emerged trunk on the reservoir margins, and the one from San Agustín del Valle Fértil is supported by nine specimens found during dredging works in streams in the area.

Regarding the bordering countries of Argentina, there are some records that are extralimital according to the analysis: three localities in Paraguay (two on the Paraguay River and one on the Paraná River), two in eastern Uruguay

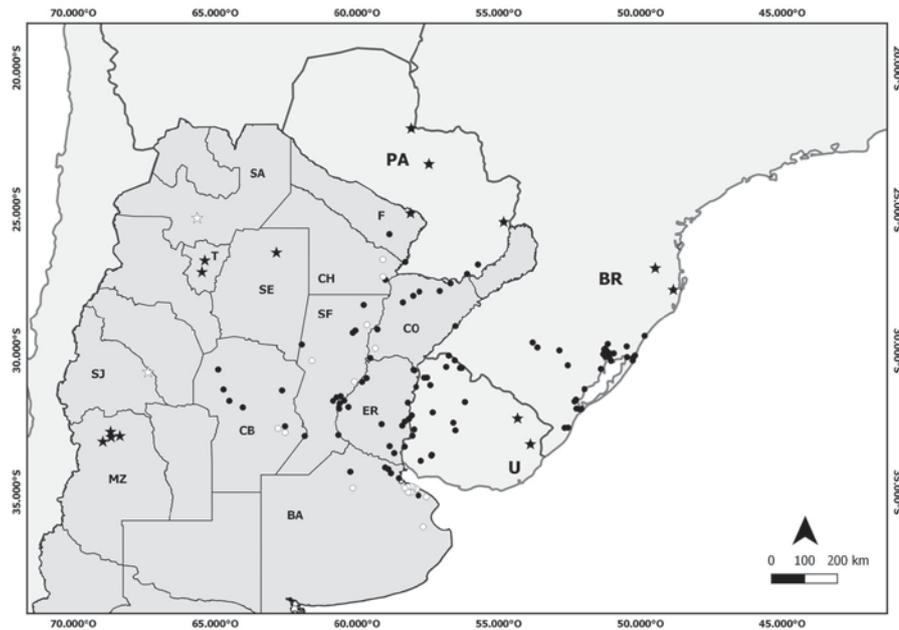


Figure 3. Localities recorded for *P. hilarii* in Argentina and in bordering countries. Spots indicate records corresponding to the core(s) of the species distribution; stars indicate relevant extralimital records (see Discussion). Black colour represents previously known localities; white colour represents new records. BA, Buenos Aires; BR, Brazil; CB, Córdoba; CH, Chaco; CO, Corrientes; ER, Entre Ríos; F, Formosa; MZ, Mendoza; PA, Paraguay; SA, Salta; SE, Santiago del Estero; SF, Santa Fe; SJ, San Juan; T, Tucumán; U, Uruguay.

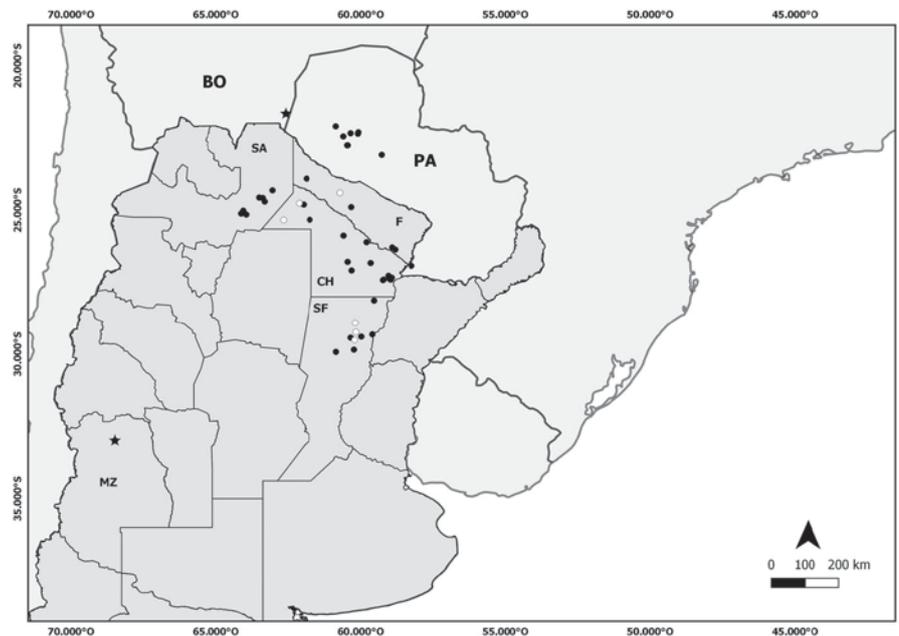


Figure 4. Localities recorded for *A. pallidipectoris* in Argentina and in bordering countries. Spots indicate records corresponding to the core(s) of the species distribution; black ones represent previously known localities; white ones are new records. Black stars indicate extralimital records. BO, Bolivia; CH, Chaco; F, Formosa; MZ, Mendoza; PA, Paraguay; SA, Salta; SF, Santa Fe.

(Cebolatti River and Tacuarí River) and the other two at the northern end of the species range, in Brazil (Ibirama and P.E. Serra do Tabuleiro; Online Appendix; Fig. 3).

Acanthochelys pallidipectoris

The analysis showed that the core of the species distribution is in north of Santa Fe province and Chaco province (Argentina), and continuous to north-west towards the south of Paraguay (Fig. 4). We report six new core localities for three of the four Argentine provinces

where *A. pallidipectoris* occurs: 1) Paraje La Guardia (Almirante Brown, new record for the department), and 2) Don Palomo (General Güemes department), both from Chaco province; 3) Yema Lagoon (Bermejo department, first record for the department), from Formosa province; 4) National Road 11 near Espin, 5) Ogilvie and 6) Campo Garabato Natural Reserve, from Vera department, Santa Fe province (Online Appendix; Fig. 4).

The KDA recognised two extralimital records for the species: one in Argentina and one in Bolivia. The former

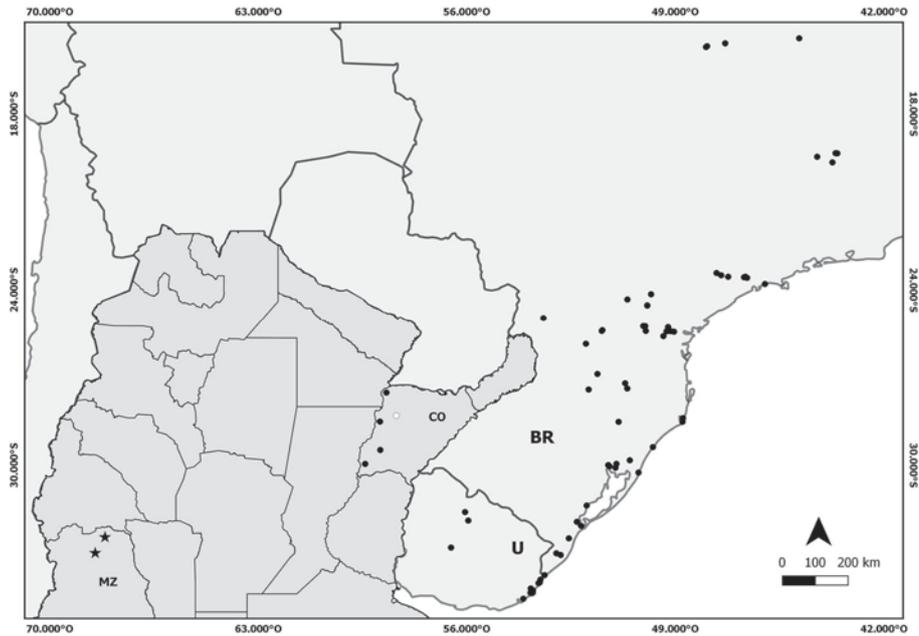


Figure 5. Localities recorded for *A. spixii* in Argentina and in bordering countries. Spots indicate records corresponding to the core(s) of the species distribution; black ones represent previously known localities, white one represents the new record. Black stars indicate extralimital records. BR, Brazil; CO, Corrientes; MZ, Mendoza; U, Uruguay.

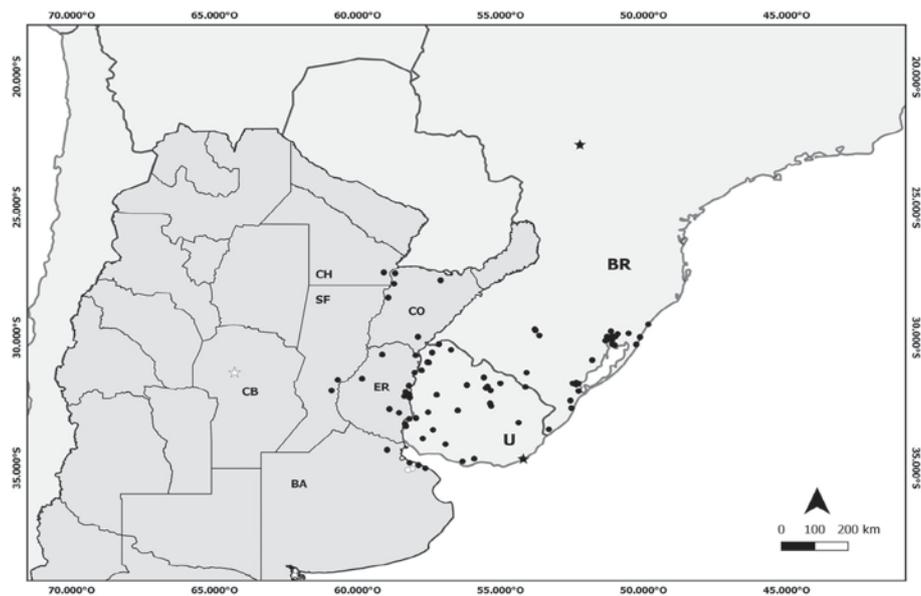


Figure 6. Localities recorded for *T. dorbigni* in Argentina and in bordering countries. Spots indicate records corresponding to the core(s) of the species distribution; stars indicate relevant extralimital records (see Discussion). Black colour represents previously known localities; white colour represents new records. BA, Buenos Aires; BR, Brazil; CB, Córdoba; CH, Chaco; CO, Corrientes; ER, Entre Ríos; SF, Santa Fe; U, Uruguay.

is in the north Mendoza province in Alto Verde lagoons (San Martín department; Online Appendix; Fig. 4). In October 2014 we visited this site, located in the farm of Figueroa family, at Las Violetas street, following the indications provided by Richard (1987). These “lagoons” are in fact artificial ponds and the area was completely modified for grape and olive cultivation. We interviewed local people and searched for turtles in the surroundings with no success. The new owners of the farm told us that the lagoons dried-up in 2000 with no signs of turtles there since (this is also valid for *A. spixii*).

Acanthochelys spixii

The species core is mainly along the Atlantic coastal area from northern Uruguay to southern Brazil into the Paraná State, with isolated populations corresponding to the peripheral distribution, in Corrientes province, Argentina, and the states of Minas Gerais and Distrito Federal, in Brazil (Online Appendix, Fig. 5). We provide one new locality for Corrientes province on National Road 12 near San Roque (Saladas department; Online Appendix; Fig. 5).

The analysis showed two extralimital reports in north of Mendoza province, Argentina: Alto Verde lagoons in San Martín department (as in the case of *A. pallidipectoris*) and

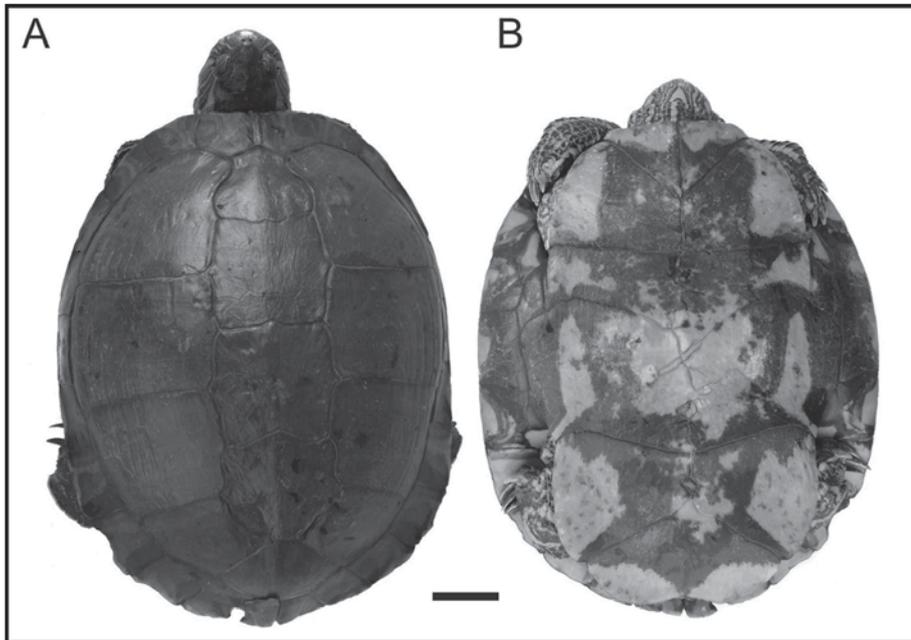


Figure 7. (A) Dorsal and (B) ventral views of a female specimen of *T. dorbigni* from Toro Muerto stream, Córdoba province (UNNEC-A 000009). Scale bar = 3 cm.

on Road 142 (Lavalle department, Online Appendix; Fig. 5).

Trachemys dorbigni

The core of *Trachemys dorbigni* distribution is from eastern Argentina, on the region drained by the Uruguay River and Río de la Plata River, almost Uruguay, to southern Brazil, into the area of influence of Guaíba River, Lagoa dos Patos and Grande River (Fig. 6). There are some isolated records associated to the Paraná River (Chaco, Corrientes, Santa Fe and Entre Ríos provinces), which suggests that the species has a peripheral distribution around this river basin. We add two new core localities for the species in Argentina: El Pescado stream (La Plata department, Buenos Aires province) and its tributary, Cajaravilla stream, both part of the Río de la Plata river basin (Online Appendix; Fig. 6).

Here we present the first extralimital report for central Argentina (Online Appendix). During field work in Córdoba province in March 2017, we found an adult female in the vegetated margins of Toro Muerto stream (Punilla department; Fig. 7A-B). This stream runs across a mountain range area where there is no important urbanisation but there are some houses near the water course.

With regard to bordering countries, our analysis identified two extralimital records for the species in Brazil: Morro do Diabo State Park and Santa Vitoria do Palmar; and two in Uruguay: one in the Atlantic coast and the other in a lagoon near Olimar River (Online Appendix; Fig. 6).

DISCUSSION

Hydromedusa tectifera

In Argentina, *H. tectifera* is mostly distributed in ponds, lagoons, streams and rivers that are part of the Río de la Plata and Uruguay river basins across three provinces (Misiones, Entre Ríos and Buenos Aires; Fig. 1). The species

was also generically mentioned for Chaco, Corrientes, Formosa and Santa Fe provinces, but with no specific localities and, except that of Corrientes, without voucher specimens (Freiberg, 1938; Ernst & Barbour, 1989; Richard & de la Fuente, 1992; Cei, 1993; Cabrera, 1998; Prado et al., 2012). The only precise locality mentioned for Corrientes province was published in a guide of the fauna and flora of Iberá Natural Reserve (Petraglia de Bolzón & Bolzón, 2003), but the referred specimen clearly corresponds to *Phrynops* (probably *P. williamsi*). In the third edition of this guide (Petraglia de Bolzón & Bolzón, 2009), the authors removed *H. tectifera* from the species list.

The extralimital and presumably isolated populations of the species are treated separately in the following paragraphs, since each case represents a different situation that deserves specific attention. *A priori*, the presence of *H. tectifera* in areas far away from the core of its range suggests anthropogenic origin, but a detailed examination of water courses of these regions does not necessarily favour this hypothesis. The records from Los Blancos (Salta province), Villa Atamisqui (Santiago del Estero province) and Popopis River (San Luis province) (Fig. 1) are clear examples of extralimital populations of *H. tectifera* connected with the core of the species distribution by rivers. Except for the significant absence of precise localities for Corrientes province, the species seems to be distributed along the large rivers that run from north to south in eastern Argentina. Los Blancos locality (Salta province) is about 50 km north from Bermejo River and about 500 km west from Paraguay River, a northern tributary of Paraná River. The species was also recorded in Asunción, Central department, Paraguay, east of Paraguay River (Bertoni, 1939; Cacciali et al., 2016). Therefore, the reported specimen of *H. tectifera* from Los Blancos should be considered within the natural range of the species distribution. In any case, since the record consists of a dried carapace collected in 1934, we consider that new

studies are needed to confirm the species there and its abundance in the area.

Regarding the populations from the western mountain chain of Córdoba province, first mentioned by Cabrera et al. (1986), we agree with the authors that they could be a relict population of a wider ancient distribution.

The record of Villa Atamisqui (Santiago del Estero province) is near Dulce River, which flows into Mar Chiquita Lake, on south-eastern Santiago del Estero and north-eastern Córdoba provinces. This lake also receives water from the Suquía River, which in turn is connected with the system of mountain streams and rivers where *H. tectifera* inhabits (Cabrera et al., 1986). Although currently isolated from the species distribution core, the populations of Córdoba and Santiago del Estero provinces are connected by the mentioned water courses.

The extralimital San Luis province record is from Popopis River. This river flows into south Córdoba province and forms an important wetland (La Amarga wetland). In the rainy season, this area overflows, connecting with Salado River in Buenos Aires province, which is part of the Río de la Plata river basin. Thus, based on 1) presence of certain Parano-Platense fish species (Bistoni et al., 1996; Garelis & Bistoni, 2010), 2) connection of water courses and 3) no official translocation; we consider the population of *H. tectifera* from Popopis River has a natural origin.

Finally, with respect to the presence of the species in the Sauce Grande River on south of Buenos Aires province, there are early-middle Pliocene fossils of *H. tectifera* (or a related species) from Quequén Salado River and Sauce Grande River (de la Fuente, 1992). Moreover, Ringuélet (1971; 1975) determined the south-west limit of the Parano-Platense biogeographic province in this area based on fish fauna, although the south of the province belongs to a different hydrogeological basin. Hence, we consider that the presence of *H. tectifera* in the Sauce Grande River might be part of the species ancestral distribution (Fig. 1). With regard to the Brazilian records, one corresponding to Florianópolis (Fig.1) occurs in an area that connects with the southern core of distribution through an Atlantic coast saline lagoon system. Hence, we think this record might be a case of natural occurrence because the presence of hydrological connection. The records of Tiete River and Andirá (Fig.1), although detected by the analysis as extralimital, are clearly part of the natural distribution of the species since both are situated in the Upper Paraná River basin. The other Brazilian records (Itaqui and Bossoroca) correspond to localities situated in the proximity of the Uruguay River, where the species is present. So, following the same criteria, they should be considered as natural occurrence.

In comparison with the maps published by Iverson (1992) and Rhodin et al. (2017), the most remarkable differences are: (1) we present three times more records than the Iverson's map and at least 50 more records than the map of Rhodin et al. (2017), mainly from the extreme south and the west of the distribution but also filling gaps in the south of Brazil; (2) there are currently several records for Paraguay (also reflected in the map of Rhodin et al., 2017), a country for which Iverson (1992) had

recognised the species as probably present; (3) contrary to previous maps we decided to exclude the species from two provinces (Corrientes and Santa Fe, Argentina) since no precise localities have been provided; and finally (4) the map of Rhodin et al. (2017) mentioned but had not mapped the species from Santiago del Estero province (Argentina). We included the species in this province based on the record from Atamisqui.

Phrynops hilarii

In Argentina, *P. hilarii* was mostly recorded for Corrientes, Entre Ríos, Santa Fe and Buenos Aires provinces. The species was generically mentioned for Misiones province by Freiberg (1938), without a precise locality. The single specific locality documented for this province was provided by Cabrera (1998). However, this report refers to San Juan Pirahui ranch, which in fact corresponds to Corrientes province. During the revision of material we found an unassigned record of *Phrynops* from Iguazú National Park, Misiones (FML 07764). It consists of a set of bones of a juvenile specimen regurgitated by a South American raccoon (*Procyon cancrivorus*). Thus, due to the state of the material, it is not possible to assign these remains to the species.

On the other hand, extralimital reports from Tucumán, San Juan (without a precise locality) and Mendoza provinces (Laurent & Teran, 1981; Richard, 1987; Broin & de la Fuente, 1993; Prado et al., 2012) are treated in the literature as translocation cases of the species, whereas the populations of Córdoba and Santiago del Estero provinces are considered isolated but part of the natural distribution of the species (Cabrera, 1998; Prado et al., 2012). The presence of *P. hilarii* in Tucumán province was first recorded by Laurent and Teran (1981), who determined it was introduced by humans without providing further explanations. The upwaters of Dulce River are in the north-west of the country. It then flows across Tucumán (where is called Salí River) and Santiago del Estero provinces, and ends into Mar Chiquita lagoon (south-eastern Santiago del Estero and north-eastern Córdoba province), where the species is present (Cabrera, 1998; Leynaud et al., 2006; Fig. 3). Although it is far from the species core of distribution, we think the extralimital records from Tucumán could be considered of the natural distribution of *P. hilarii* because there is a river system that connects with the Mar Chiquita Lake population. Moreover, there are fossil records of *Phrynops* sp. in Tiopunco, Tucumán province, dated to the Upper Tertiary (de la Fuente, 1992).

We present here the first report for Salta province in Cabra Corral water reservoir. This dams the Juramento River (also called Salado del Norte River), flows to the east across north of Santiago del Estero and north of Santa Fe provinces, and finally flows into Paraná River. The species is known in localities associated with Juramento River: Campo Gallo (Santiago del Estero), Villa Ana and San Cristóbal (Santa Fe). On the other hand, there is a fossil record from Angastaco, Salta province, presumably of *Phrynops* sp., dated to the Upper Tertiary (de la Fuente, 1992). This locality is about 75 km west from Cabra Corral reservoir. Thus, we consider that the Cabra Corral record

might be part of the natural distribution of the species, although extralimital. The reports from the east of the country (Mendoza and San Juan provinces) present a different situation: there are no water courses that currently connect this region with other populations of the species (Fig. 3). Therefore, and according to Richard (1999), the Mendoza population could have anthropogenic origin. Nevertheless, there are some reasons to be cautious about this: 1) certain fish fauna are mostly the Paraná-Río de la Plata Rivers species (Acosta et al., 2013; Fernandez et al., 2015); 2) an unassigned chelid was reported for the Upper Tertiary of Mendoza province (25 de Mayo, San Rafael department; de la Fuente, 1992; Broin & de la Fuente, 1993); and 3) although isolated and probably with a particular biology, there are recent records which confirm that the species is settled in the area (Valle Fértil, San Juan). Thus, with the available information, we cannot confirm if these populations have been introduced by humans or are a relict population of a wider ancient distribution. Further studies (e.g. genetic) are needed to elucidate this issue.

The rest of the records that the KDA considered as extralimital (two in Córdoba province and two in Santa Fe province) are not relevant, since these are too close to the peripheral distribution of the species.

Regarding the extralimital records of Paraguay River and Pilcomayo River (Formosa province) and Upper Paraná River in Paraguay (Fig. 3), we think they have a natural origin since these rivers flow directly towards the Argentine core of the species distribution. The extralimital localities of Uruguay (Cebolatti River and Tacuarí River, Fig. 3) are hydrographically connected to the Brazilian core of the species distribution (both rivers flow into the Merin lagoon), so we consider these to be of natural origin. The lack of records in between is surely related to a lack of sampling in the area, as also occurs for the records of Paraguay. Finally, the extralimital records of Brazil are situated on the north of the Brazilian core of distribution, near the Atlantic coast of Santa Catarina State (Fig. 3). Following the same criteria, although there is a significant distance in between, this area is connected by a system of rivers and coastal lagoons, suggesting these records are cases of natural occurrence.

Comparing our map and the one presented by Rhodin et al. (2017), these are mostly in agreement, but we enriched the distribution adding at least a quarter more records. The distribution of the species has greatly increased since Iverson (1992), with quadruple more records than in his map, particularly the localities from the Argentine Chaco and South of Brazil. The main differences with the map of Rhodin et al. (2017) are that we excluded the species from Misiones (since the single record for this province actually corresponds to a locality from Corrientes, Argentina) and the recognition of the species for Salta (Argentina).

Acanthochelys pallidipectoris and *A. spixii*

Acanthochelys pallidipectoris inhabits temporary fresh water ponds in the Chaco region: Chaco, Formosa, Salta and Santa Fe Argentine provinces, eastern Paraguay, and western Bolivia; and Monte (Mendoza province)

Eco-region (Burkart et al., 1999; Fig 4). The ability of *A. pallidipectoris* to perform terrestrial incursions is revealed from direct observations of specimens walking on land, in general during night, and also from the presence of ticks attached on the soft parts of the turtles (Vinke et al., 2011; MJC and LA personal observations). In fact, most freshwater turtles display to some degree the ability to do terrestrial displacements. In the case of *A. pallidipectoris*, we think such ability is restricted to movements between the temporary ponds the species inhabits, but it is weak to explain connections among isolated populations of the species along the Chaco region. The record of Bolivia, despite being isolated and distant from the area with the major concentration of localities for the species, belongs to the Chaco region, so it should not be unusual to find the species there. We believe that exhaustive searches in the area are necessary in order to complete the lack of data there.

The distribution pattern of *A. pallidipectoris* remains almost equal to that displayed in the map of Iverson (1992). There are many new records that fill gaps but that do not expand the distribution area (except by the presence of the species in Bolivia that had been suggested as probable by Iverson, 1992). Although supported by two times more localities, the distribution pattern of our map coincides in general with that published by Rhodin et al. (2017).

The other species, *A. spixii*, occurs more in permanent environments, such as lagoons and streams associated with big rivers. In Argentina, it is recorded for the tributaries of Paraná River in the province of Corrientes (Cabrera, 1998), and in temporary ponds in northern Mendoza province (Richard, 1999; Fig 5). The records from Mendoza (Alto Verde Lagoons, and Provincial Road 142 on Lavalle department) are clearly extralimital for both species. Richard (1999) established the anthropogenic origin of the Alto Verde populations of both species based on statements of local people who said that the owners of the farm (Figueroa family) had introduced them into the lagoons during the 1970s. As we related in the Results section, we visited the area and we can affirm that the populations of *A. pallidipectoris* and *A. spixii* from the Alto Verde Lagoons are extinct.

Regarding the record of the specimen of *A. spixii* found crossing the Provincial Road 142, the situation seems to be different from the case of Alto Verde lagoons, since it was found in a natural area with no human settlements nearby. Richard (1999) provided a series of arguments that support that the specimen may correspond to a natural population: (1) presence of fossil turtles in the area (de la Fuente, 1992; Broin & de la Fuente, 1993); (2) certain fish species are the same as those in the Paraná river basin. The presence of this fish fauna correlates with data from geological and palinological studies (see compilation in Ringuelet, 1956) that demonstrates the presence of a Late Pleistocen and Early Holocen extense waterbodies and laggons net, which has hydrologically connected the arid west with the humid east of the country; and (3) *A. spixii* usually has a distribution pattern based on isolated populations. Therefore, we agree with the author that it could be part of an extralimital natural population, but we

consider that exhaustive searches in the area are needed to find new specimens inhabiting there.

The main differences between our work and the maps of this species published by early authors are: (1) Rhodin et al. (2017) recognised the species for the Brazilian states of Bahia and Goiás (we are not sure of precise localities supporting that) and excluded the species (with no justification) from Corrientes province, Argentina; (2) Iverson's map showed a very similar pattern to the one known at present but based on very few localities (we present two times more records than in Iverson's map, filling many gaps for the extreme south of the distribution); and (3) the presence of the species for Paraguay was suggested by Iverson (1992) but, until now, there are no records supporting the presence of the species in this country.

Trachemys dorbigni

In Argentina, the species is distributed along the Paraná and Uruguay river basins, from Corrientes province to Río de la Plata River, as far as Buñirigo and Zapata streams in Buenos Aires province (Fig. 6). The record from Toro Muerto stream in Córdoba province is the first outside the species range. The documented *Trachemys* fossil from Mar Chiquita Lake (Cabrera & Luna, 2011), the accompanying fish fauna, mostly Parano-Platense, and the past water connections with the Paraná-Río de la Plata river basins, are reasons to believe that this locality is, although isolated, part of the natural distribution (as claimed for *H. tectifera*). However, the species has never been reported for the area, although several turtle studies were conducted there (Lescano et al., 2007; 2008; Bonino et al., 2009), the water is transparent, and it is a conspicuous, not secretive species. Thus, we consider that this record is a case of translocation, presumably due to the pet trade, given that *T. dorbigni* is the second most commercialised native reptile in the country (Prado et al., 2012).

The extralimital record for the species in Morro do Diabo State Park (Brazil) seems to be a case of natural occurrence since the turtle was found near the Paranapanema River, an affluent area of the Paraná River, where the species is present (Santos et al., 2009). Nonetheless *T. dorbigni* is a species usually sold as pets in this country, and is common that people release specimens in natural areas. With regard to the extralimital record of the Atlantic coast of Uruguay (surroundings of La Paloma), it is situated in a region that is hydrologically connected with the southern core of distribution, so it may well be part of the natural range of the species. Other extralimital records are not relevant since they are very close to the peripheral range.

Although our map of the species coincides with that presented by Rhodin et al. (2017), these authors excluded the species (with no justification) from two provinces (Chaco and Santa Fe) and presented very few localities for Corrientes. The main difference between our map and that of Iverson (1992) is a marked increase in the localities for Argentina and Brazil (we present four times more records than Iverson's map).

CONCLUSION

In the present work, we provide an updated revision of the geographic distribution of the South American turtles *Hydromedusa tectifera*, *Phrynops hilarii*, *Acanthochelys pallidipectoris*, *A. spixii* and *Trachemys dorbigni*, presenting the first provincial records of some of these species for Argentina, and formally excluding them from other provinces. Moreover, we exposed our position about the origin of all cases of extralimital populations of these species following a methodology that can be replicated in future work.

We would like to remark that contributions we make here tend to solve a relevant conservation issue about extralimital turtle populations. These types of population may be adapted to different habitats, or be genetically isolated and could represent distinct evolutionary units. In that sense, the possible origin about certain turtle populations should be the starting point for making conservation decisions by fauna agencies.

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The identity and probable origin of the *Hemidactylus* geckos of the Maldives

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The Maldives are coral reef islands of Holocene origin with no endemic terrestrial herpetofauna. The few species that have been reported from the region have affinities with Sri Lanka, south and south-east Asia, and are considered relatively recent introductions by humans. Recent collections of *Hemidactylus* geckos from the Maldives allowed us to identify and reconstruct the probable origin of this genus on the islands. We combine mitochondrial DNA (ND2) sequence data for the new collections with published sequences to reconstruct evolutionary relationships. The two species of *Hemidactylus* in the Maldives are *H. frenatus* and *H. parvimaaculatus*, with affinities to south-east Asia and Sri Lanka, respectively. Suggestive of multiple introductions, each species included multiple haplotypes, only one of which was previously recorded (in Sri Lanka); other haplotypes showed similarities to records from south-east Asia. Unravelling the colonisation patterns of house geckos in the Maldives requires a more complete knowledge of the natural variation across the range of the widely distributed source species.

Keywords: *Hemidactylus brookii*; *Hemidactylus frenatus*; *Hemidactylus parvimaaculatus*; human commensal; India; Sri Lanka

INTRODUCTION

With a maximum elevation of 2.4 m above sea level, the Maldives are the lowest-lying nation in the world, and its 26 atolls occupy a land area of 298 km². In addition to the threat of sea level rise with climate change, the islands are strongly influenced by human settlement and, in the last 45 years, by the expansion of tourism infrastructure. Given the lack of topographical relief and its uniform monsoonal climate, the vegetation is dominated by strand vegetation and human-associated plants (Fosberg, 1957). The Maldives are coral reef islands of Holocene origin and it is unclear how long each island has been land-positive (Kench et al., 2005). In combination with the small land area and low habitat diversity of the country, it is expected that the terrestrial herpetofauna is depauperate. There are no endemics, and all recorded reptiles show affinities with south Asia, and especially Sri Lanka (Moutou, 1985); it has been presumed that most, if not all of the species present arrived via either recent over water dispersal or by anthropogenic means. Indeed, Fosberg (1957) stated that there was no Maldivian (Dhivehi) word for lizard, suggesting that they are recent arrivals. The names *hoanu* (gecko) and *bundu* (lizard) are currently used in the Maldives, although it is uncertain if these words are autochthonous or imported from another language.

To date, no comprehensive survey has been conducted

on the herpetofauna of the Maldives (Hameed, 2002), with several authors having reported on the constituent species. Laidlaw (1903) listed eight terrestrial reptile species based on an extended expedition: *Calotes versicolor*, *Hemidactylus frenatus*, *H. gleadowi*, *Lygosoma albopunctata*, *Aspidura trachyprocta*, *Lycodon aulicus*, *Indotyphlops braminus*, and *Melanochelys trijuga thermalis*. Deraniyagala (1956), who recorded no snakes, added *Eutropis carinata* to this list and treated *H. gleadowi* as *H. brookii*. Subsequent authors have added no further species to the Maldivian reptile fauna. Indeed, in the most recent summaries (Das, 1994, 1996) only eight terrestrial species are listed (*Aspidura trachyprocta* being excluded). Although Moutou (1985) followed Laidlaw (1903) in listing *L. aulicus* for the Maldives, Philips (1958) cited *Lycodon aulicus capucinus*. This subspecies was recognised as distinct, and is now regarded as occurring in the Maldives (Das, 1994, 1996; David & Vogel, 1996). Its position in a recently described *L. aulicus* + *L. capucinus* clade, that lacked sampling from India or Java (the type localities of the nominate forms) (Siler et al., 2013) is, however, unknown.

Another uncertainty in the Maldivian reptile fauna regards the geckos. Moutou (1985) followed Philips (1958) in recognising *Hemidactylus frenatus* and *H. brookii*, whereas the geckos were listed simply as *Hemidactylus* (no species given) by Klausewitz (1958), “house geckos” by Hourston (1972), and “Palmgeckos”

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Figure 1. Colour and pattern variation of *H. frenatus* (A-C) and *H. parvimaculatus* (D-F) from the Maldives



Figure 2. Habitats where both species of *Hemidactylus* were observed on Huraa Island: **A** – palm forest; **B** – local houses

and “Hausgeckos” by Reichholf (1985). *Hemidactylus brookii* was also listed by Das (1994, 1996, 2002), recently replaced by *H. parvimaculatus* (Das & Das, 2017).

The instability in the identity of one of the two *Hemidactylus* is a reflection of the confusing taxonomic history of the *H. brookii* group. Bauer et al. (2010a, b), based on molecular phylogenetic evidence as well as morphological differences, elevated the Sri Lankan subspecies *H. b. parvimaculatus* Deraniyagala, 1953 to specific status, and showed its presence in Mauritius and part of peninsular India. Vences et al. (2004) and Rocha et al. (2005) had earlier identified low mtDNA differences between these populations and those from the Comoros, Réunion and Rodrigues. Deso et al. (2013) argued that putative *H. brookii* from Desroches in the Amirantes Group in the Seychelles were also referable to *H. parvimaculatus*. Thus, the majority of the island groups in the Indian Ocean that had previously been believed to harbor *H. brookii* have been demonstrated to support populations of *H. parvimaculatus*. Both Bauer et al. (2010a) and Deso et al. (2013) speculated that this species would also be present on the Maldives, but this has yet to be confirmed. The only explicit statements of the occurrence of *H. parvimaculatus* from the Maldives are in Rösler and Glaw (2010, citing Bauer et al., 2010a) and Das & Das (2017).

Despite a range of recent studies (Rösler & Glaw, 2010; Mahony, 2011; Kathriner et al., 2014; Lajmi et al., 2016), the identity of *H. brookii* as well as the

Table 1. Accession numbers, voucher references and localities for samples used in phylogenetic analyses. Accession numbers in bold are sequences generated in this study.

Species	Voucher	GenBank accession number	Locality
<i>Hemidactylus parvimaclulatus</i>	ADS 36	GQ458053	Sri Lanka, Kartivu
<i>H. parvimaclulatus</i>	AMB 7466	GQ458056	Sri Lanka, Mampuri
<i>H. parvimaclulatus</i>	AMB 7475	GQ458055	Sri Lanka, Kandy
<i>H. parvimaclulatus</i>	CES/14/174	MH454766	India, Visakhapatnam District, Araku
<i>H. parvimaclulatus</i>	DJ3428	MK559032	Maldives, Huraa Island
<i>H. parvimaclulatus</i>	DJ3429	MK559033	Maldives, Huraa Island
<i>H. parvimaclulatus</i>	DJ3430	MK559034	Maldives, Huraa Island
<i>H. parvimaclulatus</i>	DJ3431	MK559035	Maldives, Huraa Island
<i>H. frenatus</i>	AMB 7411	EU268357	Sri Lanka, Pidipitiya
<i>H. frenatus</i>	AMB 7420	EU268359	Sri Lanka, Anuradhapura District, Ritigala
<i>H. frenatus</i>	CAS 229633	HM559629	Myanmar, Tanintharyi Div., Kaw Thuang Dist
<i>H. frenatus</i>	CES G088	MK559036	India, Tripura, Rowa
<i>H. frenatus</i>	CES G115	MK559037	India, Assam, Kohora
<i>H. frenatus</i>	DJ1138	MK559038	Costa Rica, Cahuita
<i>H. frenatus</i>	DJ1150	MK559039	Costa Rica, Cahuita
<i>H. frenatus</i>	DJ1220	MK559040	Vietnam, Cuc Phuong NP
<i>H. frenatus</i>	DJ1252	MK559041	Vietnam, Cuc Phuong NP
<i>H. frenatus</i>	DJ1259	MK559042	Vietnam, Son Trach
<i>H. frenatus</i>	DJ1264	MK559043	Vietnam, Son Trach
<i>H. frenatus</i>	DJ1279	MK559044	Vietnam, Bach Ma NP
<i>H. frenatus</i>	DJ3432	MK559045	Maldives, Viligilimathidhahuraa Island
<i>H. frenatus</i>	DJ3433	MK559046	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3434	MK559047	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3435	MK559048	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3436	MK559049	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3437	MK559050	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3438	MK559051	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3439	MK559052	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3440	MK559053	Maldives, Huraa Island
<i>H. frenatus</i>	DJ3445	MK559054	Maldives, Viligilimathidhahuraa Island
<i>H. frenatus</i>	DJ640	MK559055	Malaysia, Tanah Rata, Malaysia
<i>H. frenatus</i>	LSUHC 4871	GQ458049	Malaysia, Pahang, Bukit Bakong
<i>H. frenatus</i>	LSUHC 6745	EU268358	Malaysia, Pulau Pinang, Empangon Air Hitam
<i>H. frenatus</i>	TNHC 62814	HM559630	Phillippines, Luzon Id., Albay Prov., Mt. Malinao
<i>H. frenatus</i>	USNM 579075	KM975949	Timor-Leste, E of Baucau
<i>H. frenatus</i>	USNM 579429	KM975950	Timor-Leste, Com
<i>H. gleadowi</i>	CES/11/014	MH454761	India, Karnataka, Bagalkot
<i>H. malcolmsmithi</i>	CES/11/065	MH454765	India, Himachal Pradesh, Sujanpur
<i>H. cf. malcolmsmithi</i>	CAS 252886	KM975948	USA, Louisiana, New Orleans
<i>H. murrayi</i>	CAS 206638	GQ458054	Myanmar, Mandalay Division
<i>H. murrayi</i>	CAS 208159	GQ458052	Myanmar, Yangon
<i>H. murrayi</i>	CAS 229632	GQ458051	Myanmar, Tanintharyi Division
<i>H. murrayi</i>	LSUHC 6754	EU268365	Malaysia, Pulau Pinang, Empangon Air Hitam
<i>H. murrayi</i>	LSUHC 6755	EU268366	Malaysia, Pulau Pinang, Empangon Air Hitam
<i>H. murrayi</i>	USNM 579430	KM975941	Timor-Leste, Com
<i>H. murrayi</i>	USNM 579441	KM975942	Timor-Leste, Com
<i>H. murrayi</i>	USNM 579442	KM975943	Timor-Leste, Com
<i>H. murrayi</i>	USNM 579443	KM975944	Timor-Leste, Com
<i>H. murrayi</i>	USNM 579728	KM975945	Timor-Leste, Dili
<i>H. murrayi</i>	USNM 579729	KM975946	Timor-Leste, Dili
<i>H. murrayi</i>	USNM 581298	KM975947	Timor-Leste, Dili
<i>H. murrayi</i>	ZRC 2.6167	GQ458050	Borneo, Sarawak, Loagan Bunut National Park
<i>H. treutleri</i>	CES/14/216	MH454770	India, Telangana, Hyderabad
<i>H. flaviviridis</i>	ID 7640	HM559628	India, Rajasthan, Jaisalmer
<i>H. giganteus</i>	JB03	HM559632	Captive

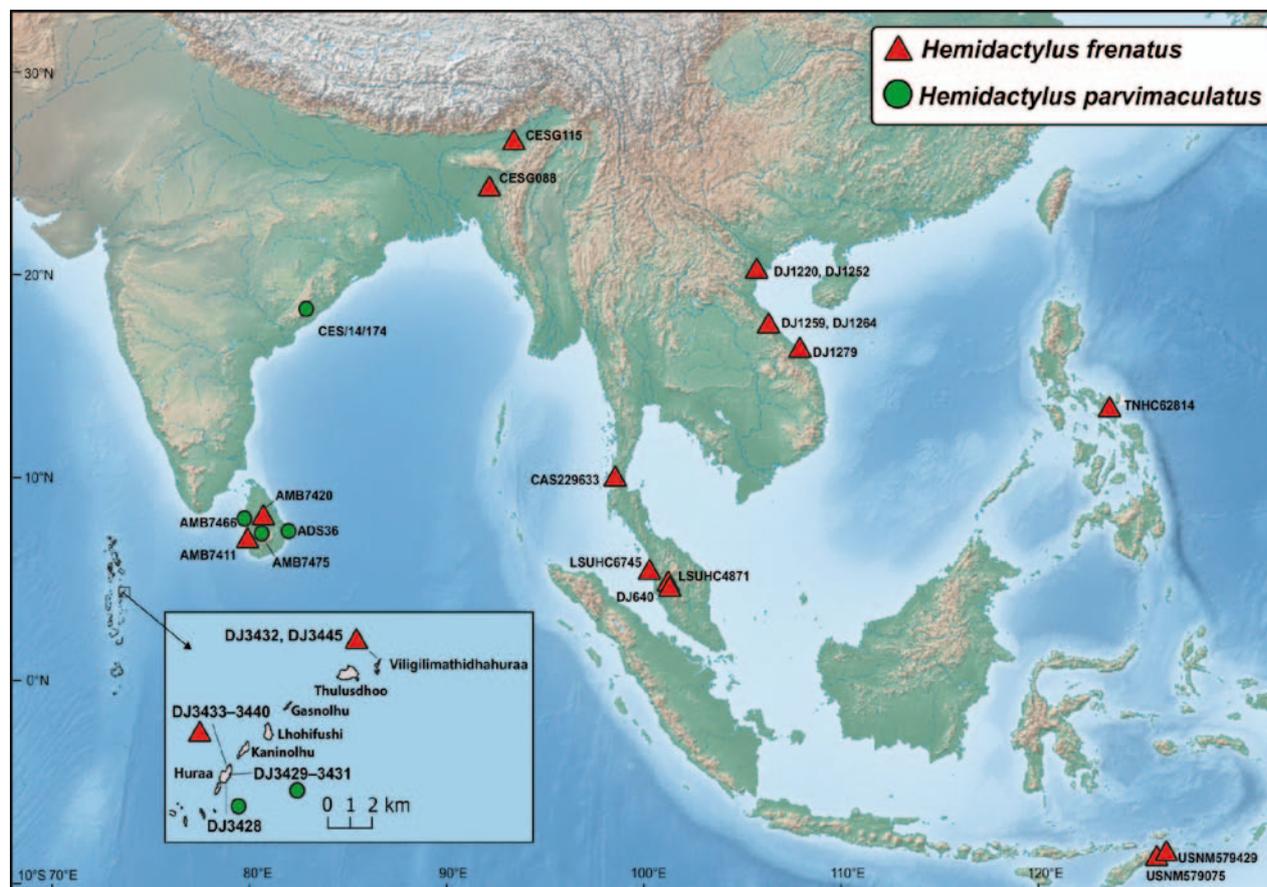


Figure 3. Map of south and south-east Asia showing sample locations for *H. frenatus* and *H. parvimaculatus*. Inset shows sample locations in the Maldives. Voucher number is given next to symbols, referenced in Table 1.

partitioning of the *H. brookii* group remain not yet fully resolved. With the elevation of several nominal taxa from synonymy, including *H. gleadowi* Murray, 1884 (Mahony, 2011) and *H. malcolmsmithi* (Constable, 1949) (Agarwal et al., 2018), and the description of several new species (Mahony, 2009; Mirza & Raju, 2017), the identity of the Maldives brookii-like geckos warrant an explicit clarification. A visit by one of us (DJ) to the Maldives in 2015 provided an opportunity to obtain further material of *Hemidactylus* geckos in order to ascertain the identity of the resident *H. brookii*. *Hemidactylus frenatus* were also collected in order to identify the potential source population(s) from which the other species of Maldivian house gecko was derived.

METHODS

We sampled 14 Maldivian *Hemidactylus* (Fig. 1) from natural vegetation (palm trees) and buildings (Fig. 2) from three localities on two islands including four from the *H. brookii* group (Fig. 1D-F) and 10 *H. frenatus* (Fig. 1A-C), besides eight *H. frenatus* from south-east Asia and two from Costa Rica (Table 1, Fig. 3). Blood or muscle samples were either preserved in 96 % ethanol or frozen after collection, and stored at -25 or -80 °C. We used the Qiagen DNeasy® Blood and Tissue Kit for DNA extraction and targeted up to 1041 nucleotides of the protein-coding mitochondrial gene ND2 using the primers L4437 and H5934 (Macey et al., 1997; flanking tRNAs excluded)

for PCR amplification (following Bauer et al., 2010a, b) and sequencing (performed by Macrogen Inc.; Seoul, South Korea and Amsterdam, Netherlands; <http://www.macrogen.com>). These sequences were combined with published ND2 sequences for *H. frenatus* and for the *H. brookii* group excluding the morphologically unique ground-dwelling clade (Mahony, 2011; Lajmi et al., 2016), with *H. flaviviridis* and *H. giganteus* as outgroups (Table 1). Sequences were aligned using ClustalW (Thompson et al., 1994) in Mega 5.2 (Tamura et al., 2011) and translated to check for stop codons. A maximum likelihood phylogeny was constructed using the codon partitioned dataset with the GTR+G model, 10 independent ML runs and 1000 rapid bootstraps through raxmlGUI 1.5 (Silvestro & Michalak, 2012) that implements RAXML HPC 8.1.2 (Stamatakis, 2014). Uncorrected genetic distances (p -distances) were calculated in MEGA 5.2 with the pairwise deletion option. Museum abbreviations are as follows: ADS, Ansem de Silva field series; AMB, Aaron M. Bauer field series (now deposited in the National Museum, Colombo, Sri Lanka); CAS, California Academy of Sciences, San Francisco; CES, Centre for Ecological Sciences, Bangalore, India; DJ, Daniel Jablonski field series; ID, Indraneil Das field series; JB, Jon Boone private collection; LSUHC, La Sierra University Herpetological Collection; TNHC, Texas Natural History Collection, Austin; USNM, United States National Museum, Washington D.C.; ZRC, Lee Kong Chian Natural History Museum (formerly Raffles Museum of Biodiversity Research), Singapore.

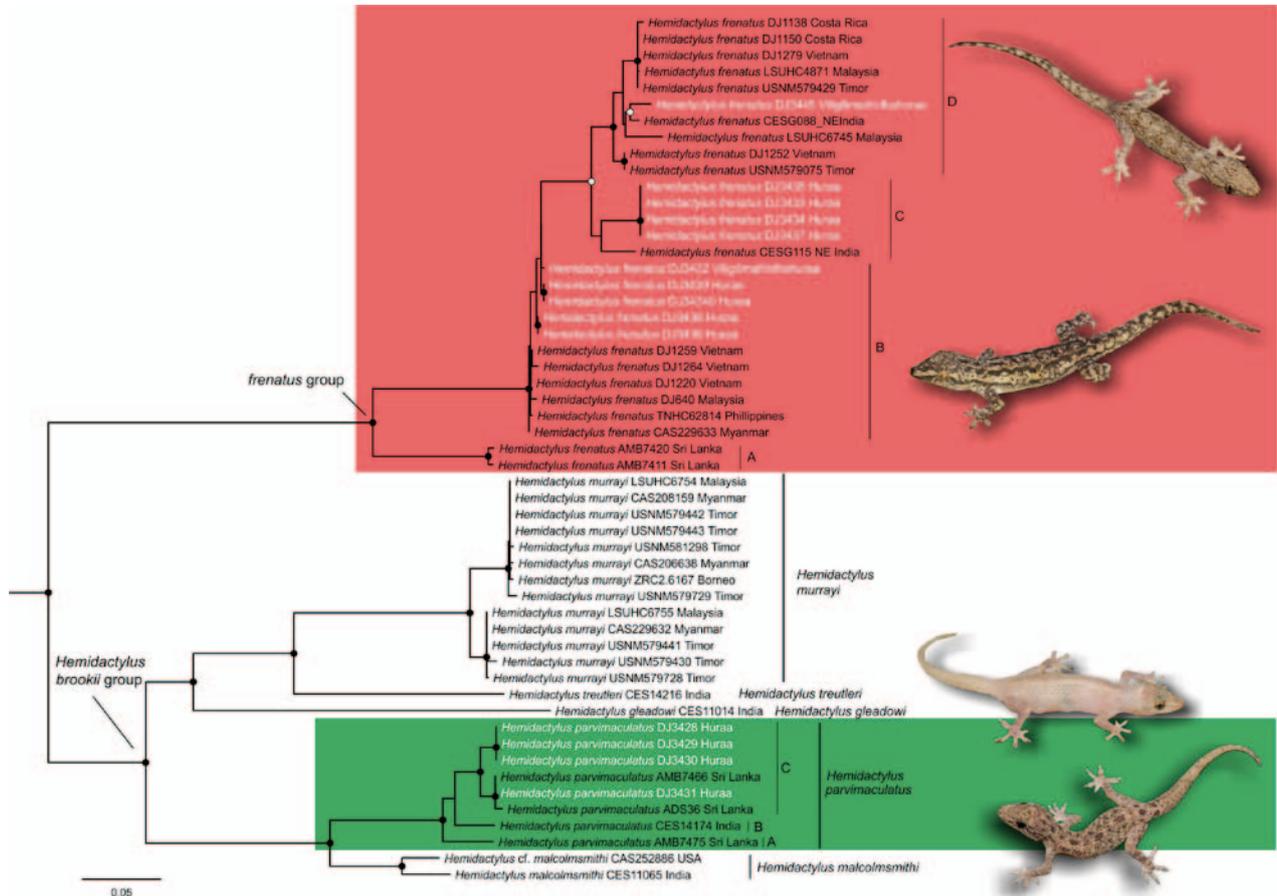


Figure 4. Maximum Likelihood phylogeny of *H. brookii* and *H. frenatus* groups with information on voucher number and country (NE India= north-east India). Samples from the Maldives are in white font and labelled by island; the *H. frenatus* group is labelled with red highlight and the *H. parvimaculatus* group by green highlight; solid black circles at nodes indicate bootstrap support >90 % and hollow circles indicate support from 80–90 %. Clades within *H. frenatus* and *H. parvimaculatus* are marked by capital letters. Representative photographs of sequenced Maldivian material.

RESULTS

The *H. brookii* and *H. frenatus* clades were each recovered with high bootstrap support (Fig. 4). The *H. brookii* complex includes *H. murrayi*, *H. treutleri*, and *H. gleadowi* in one subclade, sister to *H. parvimaculatus* + *H. malcolmsmithi*. A specimen previously identified as *H. parvimaculatus* (CAS 252886) is the divergent sister to *H. malcolmsmithi* (Fig. 4). *Hemidactylus parvimaculatus* was composed of three clades (A–C in Fig. 4). AMB 7475 from Kandy, Sri Lanka (A) is 5.5–5.7 % divergent from the rest of the clade, followed by CES14/174 from Araku, India (B) that is 4.5–4.9 % divergent from the remaining sampled individuals from the Maldives and Sri Lanka (C). The Maldivian *H. brookii* included two haplotypes in sister subclades within clade C, the first represented by a single individual (DJ3431) and identical to two individuals of *H. parvimaculatus* from Sri Lanka (AMB7466, ADS36), and the second represented by three individuals from the Maldives only (DJ 3428–3430), with a 1.8–2.3 % genetic divergence. The two Maldivian haplotypes of *H. parvimaculatus* were collected together, and the haplotype unique to the Maldives was also found at another locality 350 m away, all on Huraa Island.

Hemidactylus frenatus includes four divergent clades (A–D in Fig. 4). A Sri Lankan clade (A) was 13.1–18.2

% divergent from a SE Asian sister clade (B–D). The SE Asian clade included specimens from north-east India, Malaysia, Myanmar, the Philippines, Timor and Vietnam besides Costa Rica and the Maldives. The basal splits within this clade separate a shallow polytomy (B) which included three unique Maldivian haplotypes (DJ3432; DJ3436, DJ3438; DJ3439–3440) that are 0.5–0.8 % divergent from each other and 0.5–2.1 % divergent from sequences from Malaysia, Myanmar, Philippines and Vietnam. Clade C included four individuals from Huraa Island (DJ 3433–3435, DJ 3437) that are 3.7–4.3 % divergent from a sample from Assam, India (CES G115). Clade D separated a subclade including a sample each from Vietnam and Timor. The final two subclades include almost identical haplotypes from Costa Rica, Malaysia, Timor, and Vietnam as sister to a poorly supported clade, including another unique Maldivian haplotype of *H. frenatus* that is 2.1 % divergent from a sample from Tripura, India (CES G088), and a distinct individual from Malaysia (LSUHC 6745). Huraa harbours three different haplotypes, one with affinities to north-east India and the other two assigned to clade B. One haplotype from Viligilimathidhahuraa has affinities to north-east India and another haplotype is a member of clade B.

DISCUSSION

As is the prevailing view for the colonisation of the islands by humans and predominant trade routes (Litster, 2016), *Hemidactylus* geckos on the Maldives appear to have an Indian or Sri Lankan origin, besides affinities with South-east Asia. The Maldives was a major source of cowries when they were used as currency across many parts of Asia and Africa (Litster, 2016). The cowrie trade out of the Maldives was a major driver of shipping transport across Asia and Africa, besides the import of rice and wheat from India and Sri Lanka. Maldivian cowries that date back to as early as 500 CE have been found in India (Litster, 2016) and c. 950–1200 CE in Africa (Nixon, 2008, cited in Litster, 2016). Despite records of ships coming from as far west as Arabia and the east coast of Africa (Van Mehren 1866, cited in Litster, 2016), none of the sampled Maldivian geckos have African affinities.

We sampled only two islands and recovered patterns consistent with multiple colonisation events. *Hemidactylus parvimaaculatus* includes haplotypes identical to Sri Lankan samples as well as unique to the Maldives; the latter likely reflecting a lack of sampling in Sri Lanka and peninsular India (the presumed natural range of the species; Lajmi et al., 2016). Given the historical links and the fact that *H. parvimaaculatus* is widely distributed across other Indian Ocean islands (Bauer et al., 2010a; Deso et al., 2013), a source population of this widely distributed species in Sri Lanka was expected.

Hemidactylus frenatus includes five unique Maldivian haplotypes, two with affinities to north-east India and the other three to South-east Asia, suggesting that more sampling is needed across South-east Asia and India-Sri Lanka to ascertain source populations. Indicative of multiple introductions on the two sampled islands, each includes multiple haplotypes of both species (Tonione et al., 2011), in contrast to South American populations of *H. frenatus* that likely originated from a single source (Torres-Carvajal, 2015). Huraa is a small densely populated island less than one kilometer long and ~500 m at its widest. It is 8.5 km SW of Viligilimathidhahuraa, which is less than one km long and <200 m wide. Viligilimathidhahuraa is largely uninhabited but less than one kilometer away from the inhabited Thulusdhoo (Fig. 3). Huraa harbors two haplotypes of *H. parvimaaculatus* and three of *H. frenatus* in sympatry, whereas Viligilimathidhahuraa includes two *H. frenatus* haplotypes in sympatry.

This is the first study to use molecular data to ascertain the affinities of Maldivian terrestrial vertebrates. The revealed patterns are more complex than a simple Sri Lankan origin for all Maldives *Hemidactylus*, with one and five haplotypes unique to the Maldives for *H. parvimaaculatus* and *H. frenatus*, respectively. Unravelling patterns of colonisation of house geckos in the Maldives will require a more complete knowledge of the distribution of haplotypes across the range of these widely distributed species (Tonione et al., 2011), particularly across the regions from which the source populations were derived—India, Sri Lanka and South-east Asia. Only a few, largely anthropophilic, species in the genus *Hemidactylus* are invasive, but these taxa

(including *H. parvimaaculatus* and *H. frenatus*, and also e.g. *H. mabouia*, *H. turcicus*) are so readily transported by humans (Carrana & Arnold, 2006) that a complex mosaic of haplotypes from disparate source populations in locations such as the Maldives is not surprising.

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Morphological assessment raises the possibility of cryptic species within the Luristan newt, *Neurergus kaiseri* (Amphibia: Salamandridae)

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One of the main challenges in the conservation of biodiversity is to overcome inadequate knowledge about species and their intra-specific diversity. In the present study, we attempted to assess morphological distinction of the two previously identified genetic clades within the Luristan newt (*Neurergus kaiseri*, Schmidt 1952) endemic to Iran, which is essential for its conservation planning. Signals of the morphological variation in *N. kaiseri* were evaluated using landmark-based geometric morphometrics of body shape and characters of osteological structures. Morphological approaches revealed consistent groupings within the species, confirming the presence of two distinct lineages (previously named as the northern and southern clades). The morphological and genetic data provide evidence for the possible co-existence of two species in *N. kaiseri* and we recommend assigning the newly recognised forms to the species level.

Keywords: geometric morphometrics, Kaiser's spotted newt, intra-specific diversity, osteology, Zagros Mountains

INTRODUCTION

Morphological structures have been used, in the past 250 years, in various organisms to identify species, distinguish closely related taxa and demonstrate morphological variation among populations. Two types of morphometric techniques are commonly used, including traditional and geometric morphometrics. Geometric morphometric methods are widely used nowadays and are particularly useful in detecting morphological differences below the species level (Adams et al., 2004; Loy, 1996). Phenotypic differentiations at species and even subspecies level can be evaluated using landmark-based geometric morphometrics with a high discriminatory power (Bookstein, 1997; Nolte & Sheets, 2005; Rohlf & Marcus, 1993). Landmark-based geometric morphometric analysis allows visualising shape differences through thin-plate spline diagrams that can be used to portray the morphological evolution of a given lineage or group (Catalano et al., 2010).

Amphibians are in decline worldwide, yet there is a great amount of undiscovered diversity that calls for scientific explorations. The Luristan newt (*Neurergus kaiseri*, Schmidt, 1952) belongs to Salamandridae and is a vulnerable species, endemic to the southern Zagros Mountains of Iran (IUCN, 2018). It is highly dependent on ponds and streams and it is patchily distributed in mountainous areas. Developing conservation strategies

is of high priority, however, uncertainty in its taxonomic status and intra-specific diversity hamper effective management. Illegal collection of the newt for the pet trade is one of the major factors threatening the survival of its wild populations (IUCN, 2018). When the smuggled newts are retrieved from illegal hunters, returning them to their habitat is challenging and may cause the loss of individuals due to different climatic conditions throughout its range. If the released individual survives, there is a concern about the loss of genetic diversity due to genetic erosion (Frankham et al., 2004).

Limited knowledge is currently available on *N. kaiseri* including some aspects of its ecology such as its distribution (Mobaraki et al., 2014; Sharifi et al., 2013), and demography such as age structure, longevity and growth patterns (Farasat & Sharifi, 2015). Genetic studies based on the variation of mitochondrial control region (Farasat et al., 2016) and nuclear DNA (Rancilhac et al., 2019) revealed the presence of two genetically divergent clades (northern and southern clades) and a high geographical structuring within the species. Populations of the two clades have been isolated for about 1.5 million years and their habitats differ in temperature and precipitation (Farasat et al., 2016). Rancilhac et al. (2019) suggested that the two main clades of *N. kaiseri* could be considered distinct species, which is not currently reflected in the species taxonomy. This study did not lead to satisfactory conclusions about taxonomic

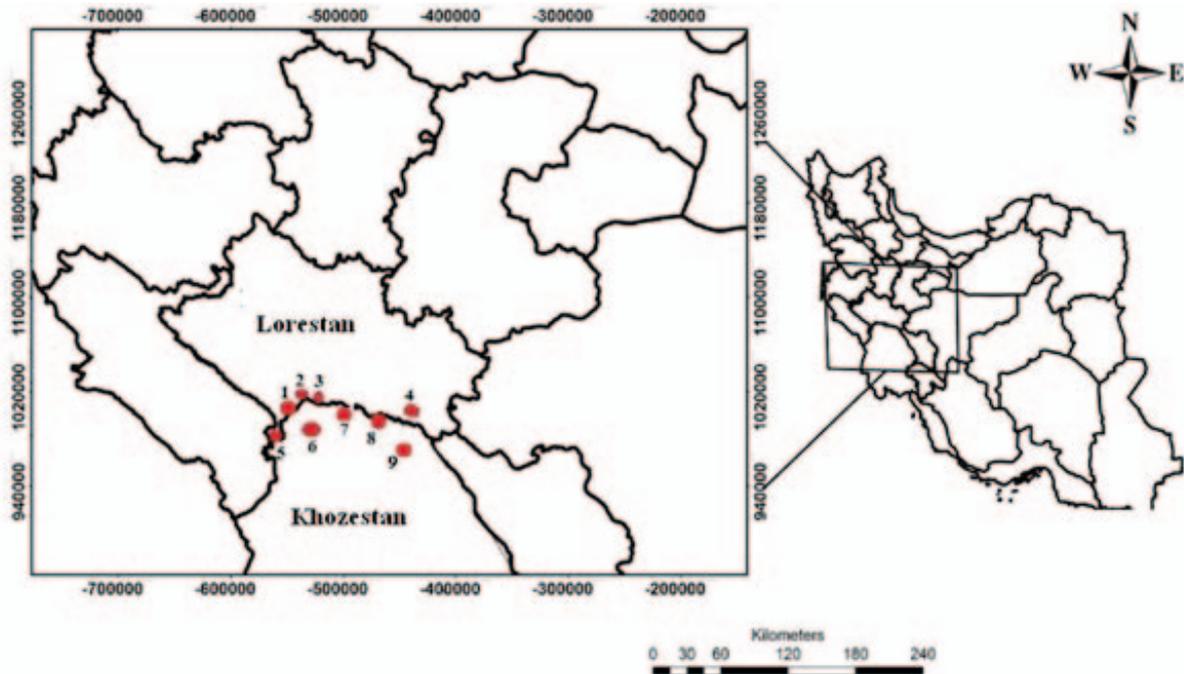


Figure 1. Sampling localities of *N. kaiseri* populations in the south-western Iran. Site names are given in Table 1.

status of the two clades because simulation studies have shown that the multiple species coalescent models do not necessarily infer true species boundaries, but might rather reflect population structure (Sukumaran & Knowles, 2017). The external morphological characters of specimens and skeleton structure of the species are yet to be described, which can provide additional data to further support the possibility of the recognised clades (northern and southern clades) as two species. We tested the delimitation of these clades by using landmark-based geometric morphometrics of larvae and adults. We also used characters of osteological structures to compare morphological variations in the skeleton of *N. kaiseri*. The information generated from this investigation may help clarify the identity of the species, and be useful for developing conservation and management programmes.

MATERIAL AND METHODS

Study area and sampling

The distribution of *N. kaiseri* in southern Zagros Mountains of Iran (Fig. 1) encompasses a minimum convex polygon of 900 km² between Lorestan and Khozestan Provinces (Mobaraki et al., 2014). Visual surveys for larvae or adults of the Luristan newt were conducted in the region in spring and autumn 2015 (see below). Due to the drought during the past few years, many of the ponds and springs in the region were dried out, limiting access to some of the known breeding sites (Farasat et al., 2016). However, we were able to collect samples from nine sites, covering both northern (Kolchap, Kerseer, Daregoand, Vogenab) and southern (Sh.Ahmad, Tove, Bozorgab, Talezang and Mongare) populations (Table 1). Sampling sites are mainly located at high elevations and separated from each other by steep and rocky mountains.

For morphometric analyses, individuals were

captured using a dip net (ring diameter of 40 cm) and photographed alive without anaesthesia using a Panasonic digital camera (8 MP) (Mitteroecker & Gunz, 2009). Larvae resemble miniature adults, differing in size, coloration and the presence of external gills, and usually reach maturity after three to four years (Consideration of Proposals For Amendment of Appendices I and II, 2010). To minimise interference and possible harm to animals, adults were sampled after the breeding season in late spring 2015. Sampling of larvae was carried out during one week in November 2015, when larvae were well developed. Individuals at the same larval development stage were selected for analyses (Alarcon-Rios et al., 2017). We were able to photograph a total of 90 adults and 112 larvae (Table 1) and all individuals were then released at the place of sampling.

The permit for measuring live *N. kaiseri* was issued by the Iranian Department of Environment. However, the vulnerable (VU) status of the species entails sampling restrictions for capturing and killing live individuals. Therefore, only deceased carcasses of *N. kaiseri* could be taken and used for osteological analyses. As the Luristan newt habitats are mainly streams, waterfalls and springs, located at high elevations (800 to 1500 m a.s.l) with relatively high water flow which could wash away the possible carcasses, 2 mm fish nets were installed in down streams and regularly checked. Dead individuals were captured by the fish net (Table 1) and adults with the body length of ~130 mm were used for the analyses. We selected similar sized specimens to minimise differences in ossification due to age. It has been suggested that body length is linearly correlated with age in many salamandrid species until maximum length is reached (Lima et al., 2000; Üzümlü, 2009). Collected specimens were preserved in 96 % ethanol prior to processing in the lab.



Figure 2. The defined bilaterally symmetric landmarks for extracting the shape data of *N. kaiseri*. 1. snout tip; 2. the beginning of the eye socket; 3. dorsal edge of the head perpendicular to the center of eye; 4. The bottom of the eye socket; 5. upper edge of the jaw; 6. bottom of the head; 7. tip of hand frame; 8. bottom of the hand frame; 9. tip of leg frame; 10. bottom of leg frame; 11-19. same as 10-2 (left side).

Table 1. Sampling sites and the number of samples obtained for geometric morphometrics (adults and larvae) and osteological structure of *N. kaiseri* in south-western Iran.

Site name	ID	Altitude (m)	Geographic location	No. samples		
				Geometric morphometrics		Osteology
				Adults	Larvae	
Kolchap	1	851	32° 55'N, 48° 22'E	-	14	1
Daregol	2	1050	32° 55'N, 48° 10'E	15	14	-
Kerser	3	1100	32°06'N, 48° 01'E	13	15	1
Vogenab	4	950	33° 00'N, 48° 4'E	15	12	-
Sh. Ahmad	5	890	32° 58'N, 48° 22'E	-	14	-
Tove	6	940	32° 6'N, 48° 53'E	12	14	1
Bozorgab	7	1080	49° 40'N, 48° 00' E	16	-	-
Talezang	8	980	32° 45'N, 48° 50'E	-	15	1
Mongare	9	960	32° 00'N, 48° 02' E	19	14	1

Geometric morphometrics of body shape

Nineteen bilaterally symmetric landmarks on two-dimensional images were selected on both larvae and adults of *N. kaiseri* (Fig. 2) using TpsDig2.04 (Rohlf, 2005). These landmarks included: snout tip; the beginning of the eye socket; dorsal edge of the head perpendicular to the centre of eye; the bottom of the eye socket; upper edge of the jaw; bottom of the head; tip of hand frame; bottom of the hand frame; tip of leg frame; bottom of leg frame. We followed landmark-based geometric morphometric methods (Rohlf & Marcus, 1993) to define landmarks that are widely used and best represent the head and body morphology (e.g. Adams et al., 2007; Ivanović et al., 2013; Ivanovic & Arntzen, 2014; Ivanović et al., 2009).

In order to minimise observer-induced errors, one person digitised the landmarks of individuals. Landmarks were then analysed using generalised Procrustes analysis (GPA, Rohlf and Slice (1990), which normalises shape data (annotated by landmarks) at equal scale, allowing for an accurate comparison of shapes regardless of their size. In order to properly evaluate whether two clades differ in their morphology, we assessed size, shape and allometric differences among clades. The snout-vent

length (SVL) of all individuals were obtained and a t-test was performed for differences. The relationship between size (independent variable) and shape (dependent variable) in the two clades (northern and southern) was tested by performing a multivariate regression of shape on size (averaged by clade). Statistical significance of regressions was assessed through permutation tests with 10,000 iterations under the null hypothesis of independence between size and shape. A Procrustes ANOVA was performed, looking for a significant value in the interaction term of size and clade to identify different allometric patterns. Principal components analysis (PCA) and canonical variates analysis (CVA) were applied on shape data. All analyses were performed using PAST (Hammer et al., 2001) and MorphoJ (Klingenberg, 2011).

Skeletal structure

The deceased carcasses of *N. kaiseri* (Table 1) were used for osteological analyses. To clean and stain bones, the following procedure was followed (Taylor & Van Dyke, 1985; Torres & Ramos, 2016). First, carcasses were fixed in 500 ml of 10 % neutral formalin for 4 days and washed thoroughly under running tap water for at least one hour to remove excess formalin. Second, specimens

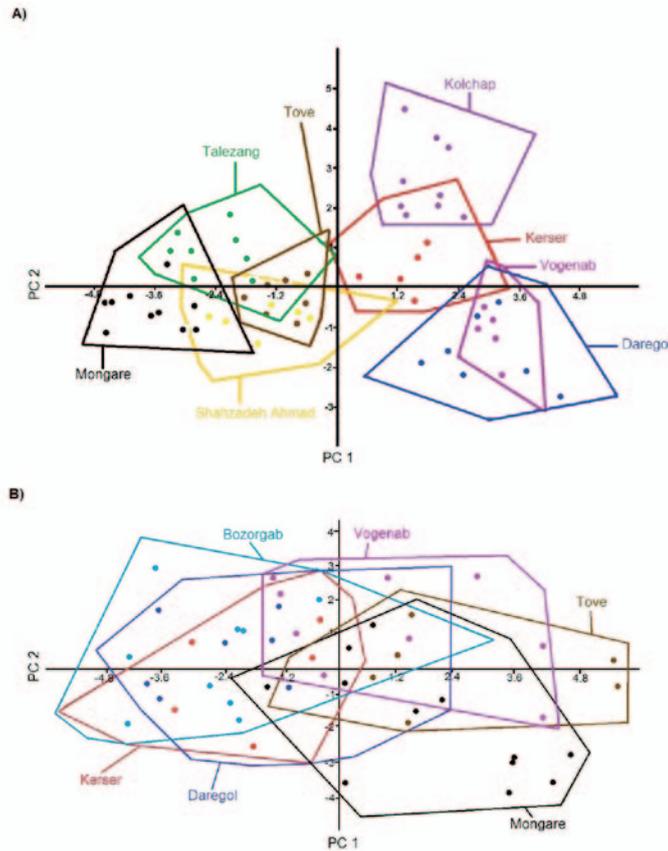


Figure 3. Scatter plots of the first two principal components with convex polygons of all larvae **(A)** and adults **(B)** of *N. kaiseri* populations in the south western Zagros Range, Iran.

Table 2. Osteological characters measured on skulls of *N. kaiseri*. Bone name, abbreviation, mean and standard deviation (SD) of each character are given for the northern and southern clades.

Bone name	Abbreviation	Northern clade	Southern clade
		Mean ± SD (mm)	Mean ± SD (mm)
Atlas	At	2.82 ± 0.02	3.21 ± 0.01
Maxilla	Max	9.92 ± 0.03	10.03 ± 0.02
Premaxilla	Pm	3.01 ± 0.01	3.12 ± 0.01
Nasal	Nas	2.95 ± 0.02	2.99 ± 0.01
Prefrontal	Prfl	2.39 ± 0.03	2.53 ± 0.01
Frontal	Fr	4.95 ± 0.03	5.22 ± 0.01
Parietal	Par	4.88 ± 0.01	5.00 ± 0.01
Squamosal	Sq	2.79 ± 0.01	2.85 ± 0.01
Prootic	Pro	4.12 ± 0.02	4.03 ± 0.02
Otic	Otic	3.02 ± 0.03	3.11 ± 0.01
Cavum internasale	Ca.in	2.20 ± 0.01	1.92 ± 0.01
Exoccipital	Exoc	1.07 ± 0.03	1.02 ± 0.01
Lacrimal	L	2.06 ± 0.02	2.10 ± 0.02
Processus pterygoid	Pt	3.22 ± 0.03	3.08 ± 0.01
Parasphenoid	Prsph	8.98 ± 0.01	9.12 ± 0.01
Orbitosphenoid	Osph	8.96 ± 0.02	9.15 ± 0.02

were placed in 15 ml of 1 % alizarin red solution, holding the specimens for three days. Third, the specimens were transferred into alkaline blue stain solution for two days. Fourth, specimens were transferred into trypsin solution

at ~37 °C, the enzyme solution was changed and the specimens were washed in distilled water every three days to avoid bacterial digestion. The stained specimens were examined using a stereomicroscope (HP SNP 120), and different skeletal elements were dissected and scanned in lateral, dorsal and ventral view, using a scanner (HP Scanjet G4050). For each specimen or view, a series of images were taken to produce a single image with maximum depth of field. The final image was drawn using CorelDrawX7 software and digitised using the TpsDig2.04 (Rohlf, 2005). The terminology of the skeletal elements follows Vassilieva et al. (2015). For comparisons, the mean of each variable was recorded for the northern and southern clades (Table 2), however, the low number of specimens precluded further quantitative comparative osteological analysis on size and allometric trends.

RESULTS

Geometric morphometrics of body shape

The results of t-tests evaluating differences in SVL showed a significant difference between the two clades in both adults and larvae. In adults, the SVL of the northern populations averaged 72.5 ± 1.34 mm and in the southern clade these averaged 61.19 ± 0.55 mm ($P = 0.001$). In larvae, the SVL of the northern populations averaged 61.5 ± 0.54 mm and in the southern clade this averaged 52.2 ± 0.45 mm ($P = 0.004$).

A PCA conducted on both adults and larvae showed that the sampled populations are separated into two

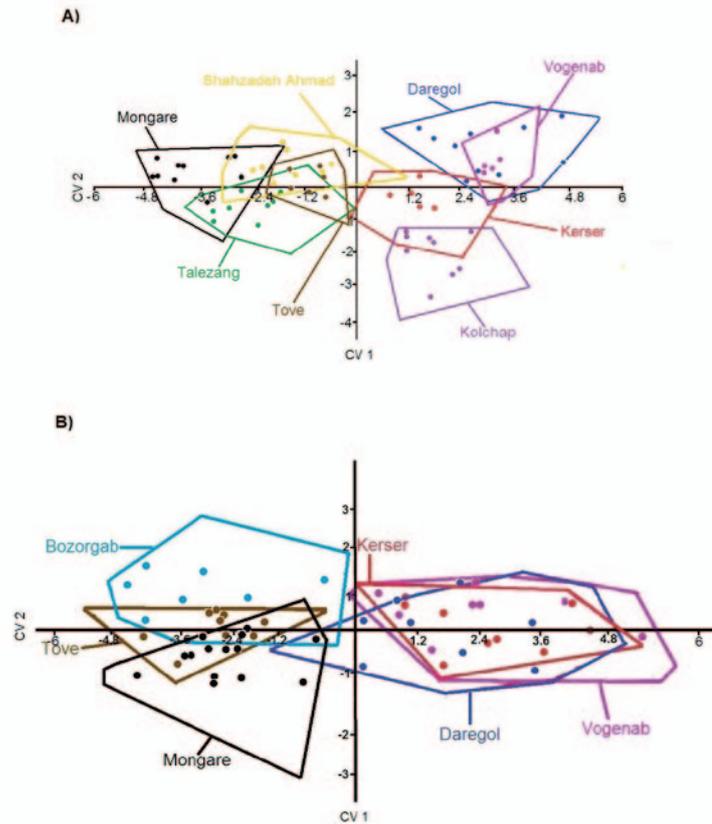


Figure 4. Scatterplots of the two first canonical axis resulted from canonical variate analysis (CVA), showing the distribution of all larvae **(A)** and adults **(B)** of *N. kaiseri* populations in the south western Zagros Range, Iran.

groups, corresponding to the two genetic clades (Fig. 3A-B). In the PCA of adults, the first two principal components (PC1 and PC2) accounted for 78 % of the total variance. In larvae, however, the first four PC accounted for a total of about 97 % of the variance. The scatter plot for the

first two principal components with convex polygons for larvae revealed two distinct groups including the northern and southern populations of *N. kaiseri* (Fig. 3A), though small overlaps were observed in the convex polygons of adults (Fig. 3B). The CVA further confirmed the distinction of the northern and southern clades with significant differences ($P < 0.01$, Fig. 4A-B). The Procrustes ANOVA analyses revealed significant differences in adults ($F = 8.75$, $P = 0.0001$) and larvae ($F = 6.81$, $P = 0.0009$) between the two clades, suggesting that they have different allometric patterns. Multivariate regressions also returned significant values for both adults ($r = 0.97$, $P < 0.000$) and larvae ($R = 0.94$, $P < 0.0001$) after 10,000 permutations (Fig. 5A-B).

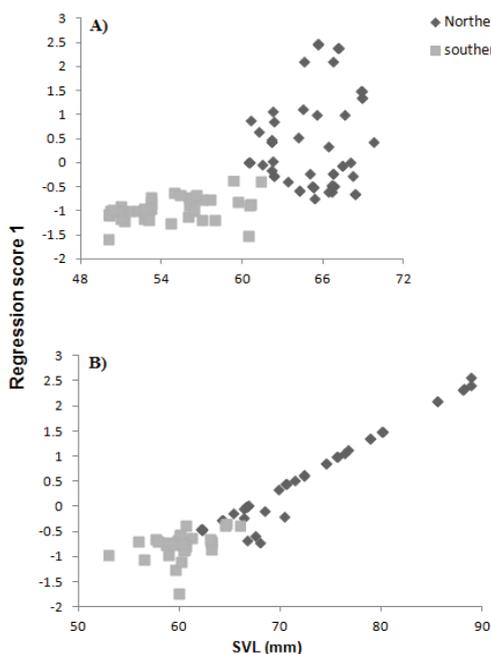


Figure 5. Multivariate regression scores of body shape variation by snout-vent length (SVL) for larvae **(A)** and adults **(B)** of *N. kaiseri* populations in the south western Zagros Range, Iran.

Skeletal structure

Overall, the skull of *N. kaiseri* showed a dense structure with severely ossified elements. Low amounts of cartilaginous elements were seen in mobile facets. Similarities were observed in the axial skeleton of both specimens. The vertebral number of each examined specimen was 50 and no variation was observed, however the number of specimens examined here was limited.

Arntzen et al. (2015) also found that in some groups such as *Neurergus* and *Lyciasalamandra*, the number of trunk vertebrae is stable, while in many groups it is subject to change. The vertebral column comprises two cervical, 16 abdominal and 32 caudal parts. Other similar components were hands and feet, which consisted of four fingers, having three (33 %, two specimens) or four phalanxes (67 %, four specimens). Seven bones

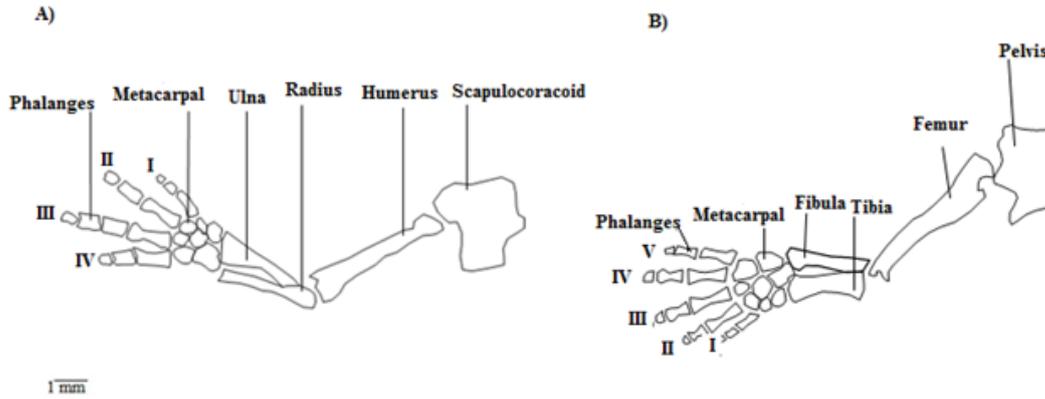


Figure 6. Hand (A) and leg (B) of *N. kaiseri*. I-IV: digits I-IV. Scale bar: 1 mm.

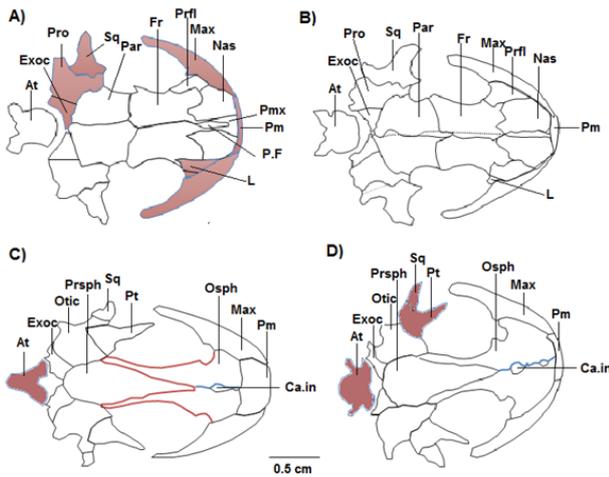


Figure 7. Osteological structures of *N. kaiseri*. Dorsal view of the skull, the northern (A) and southern (B) clade. Ventral view of the skull of the northern (C) and southern (D) clade of *N. kaiseri*. Abbreviations: At: Atlas; Ca.in: Cavum internasale; Fr: Frontal; L: Lacrimal; Max: Maxilla; Nas: Nasal; P.F: Premaxilla Foramen; Pm: Premaxilla; Par: Parietal; Prfl: Prefrontal; Pro: Prootic; Sq: Squamosal; Exoc: Exoccipital; Otic: Otic; Osph: Orbitosphenoid; Prsph: Parasphenoid; Pt: Processus Pterygoid.

are observed in the metacarpal and the number of metatarsus bones is eight (Fig. 6A-B).

Comparing the skull elements revealed some clear size (Table 2) and shape (Fig. 7A-D) differences between the northern and southern clades. For example, in dorsal views, shorter maxilla bones are seen in the northern clade compared to those in the southern (Table 2). Squamosal bones were completely different in size and shape in both specimens. Exoccipital bones are seen in the southern clade as separate bones, connected to the pterygoids bones and then to the occipitals. In the ventral view, the squamosal bones of the southern clade have longer parts compared to the northern (Table 2). The Atlas vertebra has two transverse processes in the southern clade. The premaxillary bone is bigger in the southern clade (3.12 ± 0.01) compared to the northern (3.01 ± 0.01). Prefrontal, frontal, parietal, otic, parasphenoid and orbitosphenoid were other larger skull elements in the southern clade.

The nasal bone is wide and elongated in the length of the skull roof in both clades. The vomer bone makes a pit between the nasal near the premaxilla, which is wider in the northern clade than the southern. The internasale cavity is larger in the northern clade (2.20 ± 0.01 mm) compared to the southern specimen (1.92 ± 0.01 mm) (Table 2). In addition, prootic, exoccipital and processus pterygoid were larger in the northern clade compared to the southern one (Table 2).

DISCUSSION

Based on the data presented here, there is significant morphological diversity within *N. kaiseri*. Our approaches (landmark-based geometric morphometrics of body shape and characters of osteological structures) revealed consistent groupings within the species, confirming the distinction of the two previously identified genetic clades (the northern and southern clades, Farasat et al., 2016; Rancilhac et al., 2019). Ecological differences also exist in the habitats of the two clades. The northern populations are located at higher elevations in a more humid climate than the southern clade. Habitat suitability models showed that northern populations occupy habitats that are cooler with lower winter temperatures and higher summer rainfall (Farasat et al., 2016). In contrast, southern populations are characterised by warmer habitats with higher winter temperatures and wetter winters (Farasat et al., 2016). The average elevation and average annual precipitation received by the northern and southern clades are 1050 m a. s. l. and 670 mm, and 870 m a. s. l. and 450 mm, respectively. In addition, based on the field surveys, habitats of the southern clade are mainly streams with fast-flowing water, while habitats are mostly ponds and springs with standing water in the northern clade. The morphological patterns of the southern clade may be an adaptation to lentic or lotic habitats to increase their ability to swim against the water flow. Differences in body morphology associated with an increase in maximising swimming speed is suggested for the European newts of the genus *Triturus* (Gvozdik & Van Damme, 2006). Research has shown that morphological differences may be attributed to environmental factors or genetic differences during the developmental processes (Van Buskirk, 2002, 2009), indicative of features that enable newts to live in different

aquatic ecosystems (Hopkins, 2007). Specific features of these newts, such as possible low dispersal abilities, may have also contributed to the intra-specific diversity of *N. kaiseri*. The results of this study provide adequate evidence for the potential co-existence of two species in the distribution range of *N. kaiseri* in south western Iran. Therefore, a taxonomic revision of *N. kaiseri* is recommended.

Due to the vulnerability of the species and sampling restrictions, we were only allowed to collect deceased individuals to perform the osteological analysis. The skull of *N. kaiseri* has a dense structure and is severely ossified. In salamanders that live in mountainous habitats (usually at altitudes above 900 m), skulls tend to be harder and stronger compared to those that inhabit the flatter areas (Buckley et al., 2010). The Luristan newt habitats are mainly streams and springs located at high elevations (800 to 1500 m a.s.l), separated from each other by steep and rocky mountains. Considering the habitats of *N. kaiseri* with high altitudes, a high degree of ossification may be expected.

Despite the small sample size, differences in the skull skeleton were observed between the two clades. Some of the skull elements were clearly larger or wider in the northern clade, such as internasale cavity. The size of the internasale cavities may be an adaptation to living in higher altitudes with lower oxygen pressures. Osteological features of *N. kaiseri* in both specimens may reflect changes in its composing structures with adaptations to the habitat and can be described as evolutionary process that gradually accumulated as modified anatomical structures. Amphibians living in high elevations tend to have lower metabolic rates, larger body sizes and greater longevity than their low altitude relatives (Morrison & Hero, 2003). Such variations have been attributed to low environmental temperatures and the short period of annual activity (Wells, 2007).

Our findings have important conservation implications. The Luristan newt is listed on the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2010), however, infrequent illegal collection of the newt for the pet trade threatens the survival of its wild populations. Releasing retrieved newts from illegal hunters into habitats should be conducted with adequate caution due to significant genetic (Farasat et al., 2016; Rancilhac et al., 2019), morphological (this study), and ecological differences (Farasat et al., 2016; Sharifi et al., 2013; Steinfartz et al., 2002) between the northern and southern clades. Released individuals may not survive in the wild and if they do, there is a concern about the loss of diversity due to genetic erosion or out-breeding depression (Frankham et al., 2004). Our findings can assist regional environmental managers to take informed decisions, preventing introgressions between the two clades.

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this manuscript and there has been no significant financial support for this research.

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Movements and habitat choice of resident and translocated adult female Grass Snakes (*Natrix natrix*) during the egg-laying period

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We used externally applied transmitters to study movements of female grass snakes (*Natrix natrix*) during the egg-laying period in a near-urban landscape in Sweden. Half of the studied snakes were residents while the other half were translocated individuals with no previous experience of the area. As predicted, resident females moved more goal-oriented and shorter distances than did translocated individuals. Habitat use did not differ between resident and translocated snakes; they were typically found in bushes, reeds, and tall vegetation. Habitat preference (use in relation to availability) showed that bushy habitats, tall grassy vegetation and reedbeds were over-used in proportion to availability, whereas forest and open grass lawns were used less than expected based on availability. Our study highlights the importance of preserving and restoring linear habitat components providing shelter and connectivity in conservation of grass snakes. We suggest that externally applied transmitters are a better option than surgically implanted ones in movement studies of grass snakes, and that translocation as a conservation method for snakes has drawbacks.

Keywords: colubridae; external transmitters; oviposition; radio tracking; telemetry; translocation

INTRODUCTION

Recent global biodiversity trends are of great concern, and arguably seeming especially gloomy for amphibians and reptiles (Gibbons et al., 2000; Wake & Vredenburg, 2008). Recent estimates show that 20 % of reptile species worldwide are threatened by extinction (Böhm et al., 2013). From a European perspective, the situation is hardly any better; several wide-ranging and previously common species are declining for reasons partly unknown (e.g. Reading et al., 2010).

The grass snake (*Natrix natrix*) is one of the most widespread snakes in the northern hemisphere. Several studies indicate that it is declining in parts of Europe, perhaps especially so in the north-western and northern parts of its range (Zuiderwijk et al., 1991; Hagman et al., 2012). There is an urgent need to understand the reasons behind this decline, so that conservation actions can be implemented to reverse the trend. Habitat loss and fragmentation have been suggested to be main drivers behind declines of amphibians and reptiles, not the least in semiaquatic species such as the grass snake (Gibbons et al., 2000). This species is geographically widespread, but at the same time stenotopic. Declines in such species

may be indicative of environmental changes potentially affecting also other species with similar habitat affinities, a fact that may offer valuable insights to conservation biology in general.

The grass snake is the world's northernmost oviparous (egg-laying) snake. In the northerly parts of its range, females are subjected to strong selective pressure to find the most suitable oviposition (nesting) sites. Specifically, finding a nesting site with suitable temperature is a matter of embryo survival, as well as ensuring a normal ontogenetic development versus developing abnormalities at suboptimal temperatures (Löwenborg et al., 2011; Löwenborg & Hagman, 2016; Idrisova & Khairutdinov, 2018). However, over a large swath of its geographical range, ambient heat is not sufficient for successful hatching of grass snake eggs, putting a premium on females finding oviposition sites with elevated temperatures. These sites can be of natural origin (reedbeds, decomposing plant material in littoral drift lines, and wood mould, i.e. loose material of dead wood in hollow trees), but a large proportion of grass snakes in cooler climes instead utilises anthropogenic heat sources such as manure heaps, composts, and sawdust piles for nesting (Nöllert et al.,

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1990; Zuiderwijk et al., 1991; Drews, 2006; Löwenborg et al., 2010; Löwenborg et al., 2012). This association with humans seems to have a long history, as indicated by the presence of grass snake bones and eggs in manure heaps at archaeological sites dating back at least 4000 years (van Wijngaarden-Bakker & Troostheide, 2003; Lenders & Janssen, 2014).

Like many other European reptiles, the grass snake experiences general area loss and fragmentation of its preferred landscapes. In addition, anthropogenic nesting sites have, and are still, declined rapidly due to changes in agricultural practices and environmental legislation pertaining to storage of manure (Hagman et al., 2012). As a consequence, successful conservation of grass snake populations rests on an understanding of preferred habitats, how snakes move in the landscape, and what role nesting sites have for these movements.

It is inherently hard to study movement patterns of reptiles living in temperate areas. They are secretive to start with; their annual period of activity is short, and they will typically stay hidden even during the active season when weather is unfavourable. All this is true for grass snakes, a species whose populations often comprise rather few individuals. The main objective of the present study was to investigate movement patterns and habitat use in female grass snakes during the egg-laying period in an area close to its northern range limit. A second aim was to evaluate if translocated (non-resident) females have a different movement behaviour compared to resident snakes. The rationale behind the translocation treatment is that natural selection will strongly favour individuals capable of finding high-quality nesting sites, which should be easier for local snakes than for translocated ones released in an area previously unknown to them. Moreover, translocations of snakes are already carried out, either intentionally as part of conservation programs, or unintentionally as long-distance stowaways (Fritts, 1987), or when people move unwanted snakes away from their premises (e.g. Reinert, 1991; Wolfe et al., 2018). Based on previous studies we predicted that: 1) translocated females would be less goal-oriented and move longer distance in search for nesting sites, 2) resident females would move goal-oriented towards nesting sites and then stay in their vicinity.

METHODS

Twelve female grass snakes were caught in April of 2014 (N=6) and 2015 (N=6), and were kept in captivity with one or two males in plastic boxes (matings were observed) with water bowls, heating mats, and offered fresh fish occasionally. All females were assumed to be potentially gravid, ranging in weight from 65 to 396 g (Table 1), which corresponds to a total length of >70 cm (unpublished data), e.g. sexually mature (Madsen, 1983). Six snakes (three per year) were caught within the study area (Stockholm: WGS84 coordinates: 59.365569, 18.077703), and six (three per year) were obtained from two populations located far from the study area (five at Uppsala; >50 km from the study area, WGS84:

59.77769, 17.581558 and one at Huddinge; >20 km from the study area, WGS84 59.229388, 18.009853). After spending time with males, all females were released in the beginning of June the same year they were caught. The study area comprised open lawn fields with groves, surrounded by urban areas, and forests with Norway spruce (*Picea abies*), scots pine (*Pinus sylvestris*), birch (*Betula* sp.) and aspen (*Populus tremula*) (Supplementary material, Fig. S1).

All snakes were equipped with an external radio transmitter (model PIP 3, Biotrack Ltd, Dorset, Wareham, UK), including a thermistor measuring body temperature. The transmitters were attached onto the snakes with cyanoacrylate glue (Tozetti & Martins, 2007; Madrid-Sotelo & García-Aguayo, 2008), thin thread, duct tape and surgical tape, laterally mounted behind the cloaca in order not to obstruct egg-laying and locomotion (Fig. 1). The weight of the transmitter package was approximately 4 g, which is less than 5 % of the body weight of the snakes (Blouin-Demers & Weatherhead, 2001; Tozetti et al., 2009; Conelli et al., 2011). The package's circumference did not exceed the thickest part of the snake.



Figure 1. Position of the attached transmitter, laterally behind the cloaca in order not to obstruct locomotion and egg-laying. Photo by Gustav Pettersson.

After being equipped with a transmitter, snakes were tracked during the egg-laying period from 17th June to 15th July in 2014, and from 16th June to 18th July in 2015 (Table 1). Each tracked position was recorded by a handheld GPS and the type of habitat was noted to validate the satellite data subsequently used for the models (see 'habitat choice model' below). The number of positions differed among individuals for several reasons, for example field work effort, sudden longer movements, detectability (above versus below ground), and technical issues with transmitters (Table 1). Snakes were usually tracked three times per day in 2014 and once a day in 2015, generally between 1000 and 1500 hrs. To be able to pool data from the two years, distance

Table 1. Twelve adult grass snake females ('Ind') were caught and fitted with a transmitter in either 2014 or 2015 ('year'). 'Origin' of individuals is either the study site ('resident') or from a population far away ('translocated'). 'Weight' is body mass in grams, 'Days' is the actual number of days each individual was tracked and 'Positions' is the number of recorded positions.

Ind	Year	Origin	Weight	Start	End	Days	Position
1	2014	resident	396	17 June 2014	11 July 2014	24	24
2	2014	resident	126	17 June 2014	11 July 2014	24	24
3	2014	translocated	150	17 June 2014	11 July 2014	24	24
4	2014	translocated	121	17 June 2014	15 July 2014	28	23
5	2014	resident	152	17 June 2014	6 July 2014	19	8
6	2014	translocated	390	17 June 2014	3 July 2014	16	16
7	2015	translocated	160	17 June 2015	6 July 2015	19	15
8	2015	translocated	75	16 June 2015	9 July 2015	23	18
9	2015	resident	200	16 June 2015	9 July 2015	23	16
10	2015	resident	65	16 June 2015	9 July 2015	23	16
11	2015	translocated	165	21 June 2015	18 July 2015	27	14
12	2015	resident	230	19 June 2015	18 July 2015	29	17

sums of multiple daily positions in 2014 were used in the movement distance analyses, resulting in 215 positions used in the movement model. However, in the habitat model all positions were used ($N = 258$). There was no difference in mean body mass, neither between years (t-test: $t = 1.21$, $p = 0.264$, $N = 12$) nor between resident and translocated individuals (t-test: $t = 9.98$, $p = 0.786$; $N = 12$).

Statistical Analyses

For analyses of movement and habitat choice (see below), mixed models were used to control for non-independence of data points, with individual as a random effect to obtain the correct level of replication for the fixed effects. Linear mixed models (LMM) were used for movement analyses and generalised mixed models (GLMM) were used for habitat analyses. In addition, for movement analyses we added body mass nested with individual to increase the fit, a recommended procedure for e.g. repeated measures (Schielzeth & Forstmeier, 2008), and year was included as a fixed factor in all models to control for inter-annual variations. To separate movement variations between years we included an interaction between year and origin (translocated versus resident snakes). Prior to movement analyses, all continuous variables were standardised to a mean of zero. All environmental variables in the habitat model were extracted from satellite data (raster; 2×2 m pixel size), and processed in ArcMap 10.4 (ArcGIS, ESRI, Redlands, CA, USA).

Movement Model

Data concerning moved distance (mid-June to mid-July) and turning angle (the relative movement angle measured by the change of direction between two subsequent relocations), were processed and analysed with the package *adehabitatLT* (Calenge, 2011) in R (R Core Team, 2018). A greater turning angle was assumed to indicate a less goal-oriented movement. Body temperature, body mass, air pressure, sampling day (day of the year) and origin (resident versus translocated) were included as explanatory variables in the models. The response

variable movement distance was set to $\log + 1$ to obtain normal distributions of the models. All twelve snakes were used for each sampling date with missing data (NA) included if no signal was recorded, resulting in regular trajectories. Data handling and statistical analyses were conducted in packages *adehabitatLT* (Calenge, 2011) and *lme4* (Bates, 2010) in R.

Habitat Choice Model

The study area was delineated by a minimum convex polygon (overall home range) enclosing the relocations of all individuals with a 100 m buffer zone added. This area was subsequently divided into 58,702 pixels (2×2 m resolution) corresponding to ca. 63 hectares. Habitats were extracted from rasterized land cover data (Swedish environmental protection agency; www.naturvardsverket.se). The habitat variables initially used in the study were bushes, forest, open areas (grass lawn), tall grass, reedbed, and water, which together covered all pixels. However, water was later excluded, as few individuals were found or located in this habitat. Binary variables were used in this model, i.e. presence or absence of snakes (response variable) and habitat presence (explanatory variables). In addition, individual was included as a random factor and year as a fixed factor to control for these variations.

RESULTS

Field Data

Cumulative movement distances of female in grass snakes varied substantially among individuals. Resident females moved between 174 m and 1578 m, whereas translocated moved 233 to 1985 m (Fig. 2). One resident female (#12) travelled 650 m (Euclidian distance) from the release point to a manure heap, probably to lay her eggs, and one translocated female (#4) travelled 540 m (Euclidian distance) from the release point, reaching the waterfront of the inner archipelago of the Baltic Sea (Fig. S1). Average daily movements of resident females were 26 m, in comparison to translocated that moved 33 m per day. However, most of the translocated grass snakes

Table 2. Output from a linear mixed model showing variables associated with distance moved (dependent variable) by female grass snakes. Individual was set as a random effect and body mass was also nested within the random factor. Sampling days is the number of days with relocation data. Bold *p*-values indicate significant results ($p < 0.05$) and italics denote marginal significance ($p < 0.10$).

Variables	Estimate	SE	t-value	<i>p</i> -value
Body temperatures	0.027	0.092	0.289	0.773
Body mass	0.446	0.246	1.814	<i>0.070</i>
Air pressure	0.125	0.097	1.285	0.199
Sampling days	-0.107	0.112	-0.951	0.342
Origin	-0.969	0.316	-3.069	0.002
Year	-0.652	0.299	-2.182	0.029
Origin*Year	1.031	0.477	2.163	0.031

Table 4. Output from a generalised linear mixed model of habitat utilisation (presence/absence) by female grass snakes and its association with different habitats. Individual was set as a random effect. Bold *p*-values indicate significant results ($p < 0.05$) and italics denote marginal significance ($p < 0.10$). Local and translocated females did not differ in habitat use ('origin' term non-significant in the model).

Variables	Estimate	SE	t-value	<i>p</i> -values
Bushes	2.379	0.386	6.164	<0.001
Forest	-0.530	0.341	-1.557	0.120
Grass lawns	-0.525	0.277	-1.895	<i>0.058</i>
Tall grass	1.530	0.237	6.447	<0.001
Reedbed	2.072	0.231	8.989	<0.001
Origin	0.270	0.485	0.557	0.578
Year	-0.690	0.489	-1.410	0.159

moved > 30 m per day on average (Fig. S2). Number of registered positions did not differ between resident (mean = 17.5) and translocated (mean = 18.3) individuals (t-test: $t = -0.278$, $p = 0.787$).

As is evident from the relocation positions, grass snakes were typically found in open habitats with some kind of cover, often hiding in bushes, reeds, and tall vegetation (Supplementary material, Fig. S1). Habitat preference (use in relation to availability) showed a somewhat different pattern; grass snakes used more bushes, tall grassy vegetation and reedbeds in proportion to availability (Fig. 3) whereas forest and open short-grass habitat were used less than expected based on availability.

Movement Model

In our modelling analysis, origin was the only variable significantly associated with distance moved and turning angle; in other words, translocated females moved longer distances than resident (Table 2) and were less goal orientated (Table 3). The difference in movement between snakes of different origin was most pronounced in the first study year (2014; see interaction term in Table 2). Neither body temperature nor air pressure was significantly associated with distance moved, but body mass came close to contributing significantly to the

Table 3. Model outputs (LMM) of variables associated with turning angle by female grass snakes (the relative movement angle, measured by the change of direction between two steps). Individual was set as a random factor and body mass was also nested within the random factor. Bold *p*-values indicate significant results ($p < 0.05$).

Variables	Estimate	SE	t-value	<i>p</i> -values
Body temperature	0.136	0.222	0.612	0.540
Body mass	0.175	0.286	0.613	0.540
Air pressure	0.194	0.237	0.821	0.412
Sampling days	-0.076	0.292	-0.259	0.796
Origin	-1.670	0.786	-2.125	0.034
Year	-0.329	0.823	-0.399	0.690
Origin*Year	1.424	1.069	1.332	0.183

model (Table 2). Also, sampling days was not significantly associated with distance moved, showing that there was not a bias from individuals tracked for a longer time.

Habitat Choice Model

The habitat model shows that bushes, tall grass and reedbeds were the most important variables significantly associated with presence of female grass snakes (Table 4 and Fig. 3). Open grass lawns were marginally negatively associated with habitat choice. Habitat choice was not associated with the individual snakes' origin (resident versus translocated) and did not differ between the years.

DISCUSSION

Movement patterns

Our initial predictions were supported by field data, that is, translocated snakes moved longer distances and were less goal-oriented (based on turning angle data) than resident snakes. It seems plausible that this is because translocated snakes were searching for familiar habitats, including new locations of oviposition sites, or exploring and becoming familiar with their new environment (Reinert & Rupert, 1999; Nowak et al., 2002). The fact that translocated snakes travelled greater distances concurs with studies of other snake species, including tiger snakes *Notechis scutatus* (Butler et al., 2005), rattlesnakes *Crotalus* spp. (Nowak et al., 1998; Brown et al., 2008; Reinert & Rupert, 1999), the hognose snake *Heterodon platirhino* (Plummer & Mills, 2000), and the dugite *Pseudonaja affinis* (Wolfie et al., 2018), for all of which resident individuals were more confined to home ranges compared to translocated individuals. The same pattern has been found in the common adder *Vipera berus* (largely sympatric with the grass snake), where translocated individuals undertook long-distance and more erratic movements, compared to resident individuals (Nash & Griffiths, 2018). Translocation of snakes is generally perceived as a humane conservation strategy (Reinert, 1991). Nonetheless, this study together with others suggest that such actions are negative, as longer and erratic movements of translocated snakes may result in higher mortality rates compared to

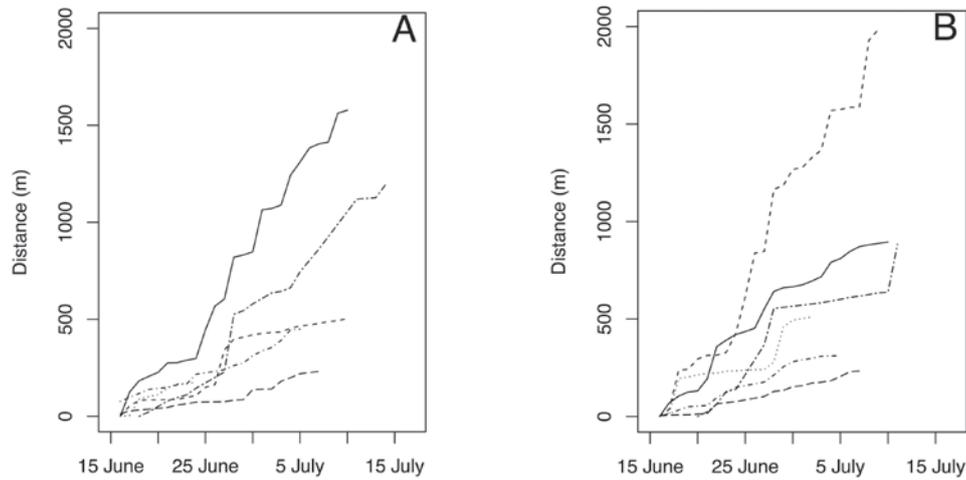


Figure 2. Cumulative moved distances of (A) resident and (B) translocated Grass Snakes (N=6 in both groups) during the egg-laying period (June and July). Different line types represent different individuals.

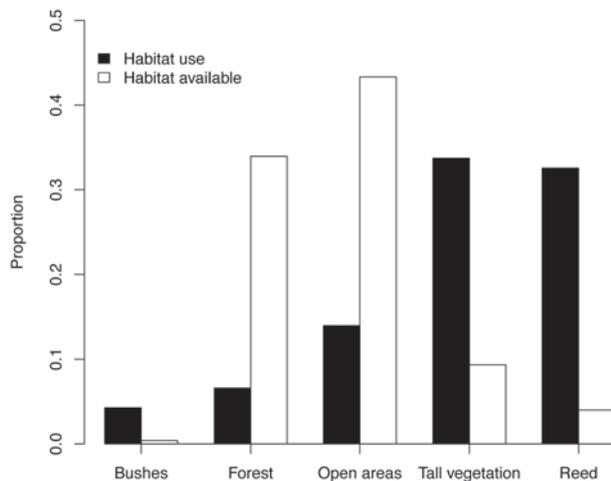


Figure 3. Proportion of used habitat (Habitat use) including 2 x 2-m pixels of bushes; 11 out of 258 pixels, forest; 17/258, open (lawn) areas; 36/258, tall vegetation; 87/258 and reed; 84/258 by all female grass snakes included in the study and proportion of the habitat available in the study area.

resident snakes (e.g. Reinert & Rupert, 1999; Plummer & Mills, 2000; Sullivan et al., 2015; see Implications). In our study, movement differences between resident and translocated females were more pronounced in the first study year, which may be due to greater fieldwork effort in this year. Sampling day had no significant effect on movement patterns among individuals, which may be related to the short study period.

There is little previous research on grass snake movements based on telemetry (but see Madsen, 1984; Nagy & Korsós, 1999; Wisler et al., 2008; Reading & Jofré, 2009). By and large, movements in gravid female grass snakes appear to be rather limited, except during the egg-laying period proper, when they have been reported to increase to 100 m/day on average (Madsen, 1984) and even 26-46 m per hour (Wisler et al., 2007). Although we do not know exactly where the resident snakes laid their eggs, there was at least one example of a female (#12; Supplementary material S1a) moving determinedly

to a manure heap where several other gravid females (without transmitters) most likely also came to lay their eggs. Other females in this study may potentially have found oviposition sites during days of missing tracks, or if they occasionally were out of the tracking range. The maximum Euclidian distance covered by female #12 (650 m) compares well to those documented for egg-laying grass snakes in Switzerland (e.g., ca 500 m from release site to oviposition site in Wisler et al., 2008). Daily movements of resident females in the present study are similar to those published by Madsen (1984), that is, 9-65 m versus 10-50 m.

Habitat choice

By and large, habitat use patterns in the present study fall well in line with previous research (Madsen, 1984; Nagy & Korsós, 1999; Wisler et al., 2007; Reading & Jofré 2009). Specifically, we found that bushes and reedbeds were important habitats for female grass snakes. Both offer thermal benefits and some protection against predators – avian as well as terrestrial – and both have a largely linear configuration in the landscape. A preference for linear habitats offering protection as seen in our study fits very well with results in Madsen (1984), Nagy & Korsós (1999), Wisler et al. (2007), and Reading & Jofré (2009), in which stone walls, embankments, bushes and ‘habitat interfaces’ were preferred.

The frequent use of reedbeds by females in our study may be explained by this habitat offering good opportunities for feeding as well as for thermoregulation. Reedbeds are typically a mix of dense stands and open patches. By moving between these microhabitats, it is easier for snakes to thermoregulate, which is important for gravid females (Madsen, 1987). Possibly for the same reason, cornfields and cereals can provide good habitat for grass snakes at this time of year (cf. Wisler et al., 2008). In comparison to previous studies, which generally tracked grass snakes over several months, the present study was conducted during a relatively short time period (a few weeks). Still, we found strong associations with similar habitat preferences, such as tall vegetation, reedbeds in riparian zones and bushes. This

indicates that grass snakes are strongly associated with these habitats during their active season, and that they may be particularly important for females during the egg-laying period (Wisler et al., 2007).

Implications

Human-mediated translocations of nuisance snakes from urban areas occur globally, including of non-venomous species (Shine & Koenig, 2001). The grass snake is frequently removed from private properties in Sweden and at the same rate as the common adder (T. Thunmark; Snake removal company, pers. comm). The present study identifies potential risks with translocations of female grass snakes, in the form of more erratic movements and longer moved distances post-release compared to resident snakes. Such behaviour may increase energy expenditure (Secor et al., 1992), the cost of reproduction (Devan-Song et al., 2016), and exposure to predators and motor vehicles (Shine & Koenig, 2001; Shine & Mason, 2004). Accordingly, translocated reptiles are commonly reported to have decreased survival rates (e.g. Nowak et al., 2002; Reinert & Rupert, 1999; Sullivan et al., 2015). Therefore, we suggest that long-distance translocations of female grass snakes should be avoided.

The present study also offers implications for habitat conservation. When preserving or restoring environments for grass snakes, it is important to secure linear structures and habitats that provide opportunities for shelter and thermoregulation (cf. Nagy & Korsós, 1999). Doing so will also facilitate dispersal and connectivity between other key habitats, such as foraging and oviposition sites. The crucial role of anthropogenic heat sources for egg-laying grass snakes has been highlighted in previous studies (Hagman et al., 2012; Löwenborg et al., 2010; Löwenborg et al., 2011; Löwenborg et al., 2012), a point further illustrated by a female in the present study that travelled more than one kilometre to a manure heap (cf. Wisler et al., 2007).

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The enigmatic palaeoecology and palaeobiogeography of the giant, horned, fossil turtles of Australasia: a review and reanalysis of the data

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The distribution pattern of the bizarre Australasian giant, horned, fossil turtles of the clade Meiolaniidae has puzzled biogeographers since their discovery late in the nineteenth century. While their distribution suggests a Gondwanan origin, the lack of fossil evidence from key times and places has inhibited a better understanding of their dispersal pathways to Australia and the south-west Pacific islands in which their fossils have been found. Much palaeoecological speculation related to their dispersal capabilities, ranging from purely terrestrial to freshwater, estuarine, and saltwater lifestyles, has been proposed to explain their enigmatic presence across a wide swath of Oceania. Various lines of fossil, anatomical and ecological evidence now strongly suggest a highly terrestrial lifestyle, and we believe these traits, reinforced by an abundance of marine predators and ever-widening saltwater gaps between land areas during the Late Mesozoic and Tertiary, minimise the importance of saltwater dispersal as an explanation for the observed meiolaniid distribution pattern. Here we propose that the fragmentation of Gondwana provided the main dispersal vehicle for the meiolaniids and that land connections were also used to access suitable habitats and expand their range. The recently recognised continent of Zealandia, along with Australia, South America, and probably Antarctica, transported all known meiolaniid turtles to their present locations. However, ice cover on Antarctica, and the nearly total submergence of Zealandia in essence preclude the current likelihood of fossil discovery in these critical locations. The islands of New Caledonia, Tige (in the Loyalty Islands), Walpole, and Lord Howe served as refugia for Zealandia meiolaniids as the continent submerged.

Keywords: Meiolaniidae, horned turtles, fossils, Australasia, Gondwana, Zealandia, palaeoecology, palaeobiogeography

INTRODUCTION

The giant, horned, fossil turtles (Fig. 1) are among the most bizarre and largest terrestrial chelonians in the world. The Australasian forms (Meiolaniidae) have been found in eastern Australia and on islands in the south-west Pacific Ocean east of Australia. Fossils date from Mid to Late Eocene into the Holocene. The closest taxonomic relatives are often considered to be the frilled turtles (Cretaceous and Paleocene) of Argentina.

The meiolaniids are quite large. In *Meiolania platyceps* the maximum length was probably greater than 3 metres (Gaffney, 1996). However, the carapace and plastron are quite thin except in the marginal areas such as the peripherals (Gaffney, 1996). The carapace is only moderately domed, unlike the higher domes of the terrestrial, testudinid, giant tortoises (Ritchie, 1978). The most unusual anatomical character is the presence of enlarged cow-like horns on the skull which project posteriorly or laterally. The distance between the horn tips for *Ninjemys* is almost 70 cm (Orenstein, 2012). However, in *Warkalania carinaminor*, horns are lacking, instead having a low horizontal ridge (Gaffney

et al., 1992). The skull is too massive to be withdrawn into the shell in all meiolaniids. The cervical vertebrae have long transverse processes (Boulenger, 1887) to attach or support muscles and ligaments to elevate, depress, and laterally move the huge head. Cervical ribs are absent in almost all other turtles (Gaffney, 1985). The tail is quite long with a club at the terminal end (Gaffney, 1985; Gaffney et al., 1992). A protective osseous sheath encases the caudal vertebrae. The tail is segmented and each ring bears a pair of stout spikes.

It is probable that the meiolaniids were potentially aggressive and very dangerous animals. Gaffney (1991:710) wrote "It is likely that the Pleistocene of Queensland had two, contemporary species of giant horned turtle wandering about, wreaking havoc". The horns and armoured tail may have been used for defence against predators (e.g. the large reptiles *Megalania* and *Quinkana*) or for pre-mating combat among males (as favoured by Jannel, 2015, based upon *M. platyceps* insularity and skull configuration), or both. The horns also may have been used to knock down vegetation for consumption.

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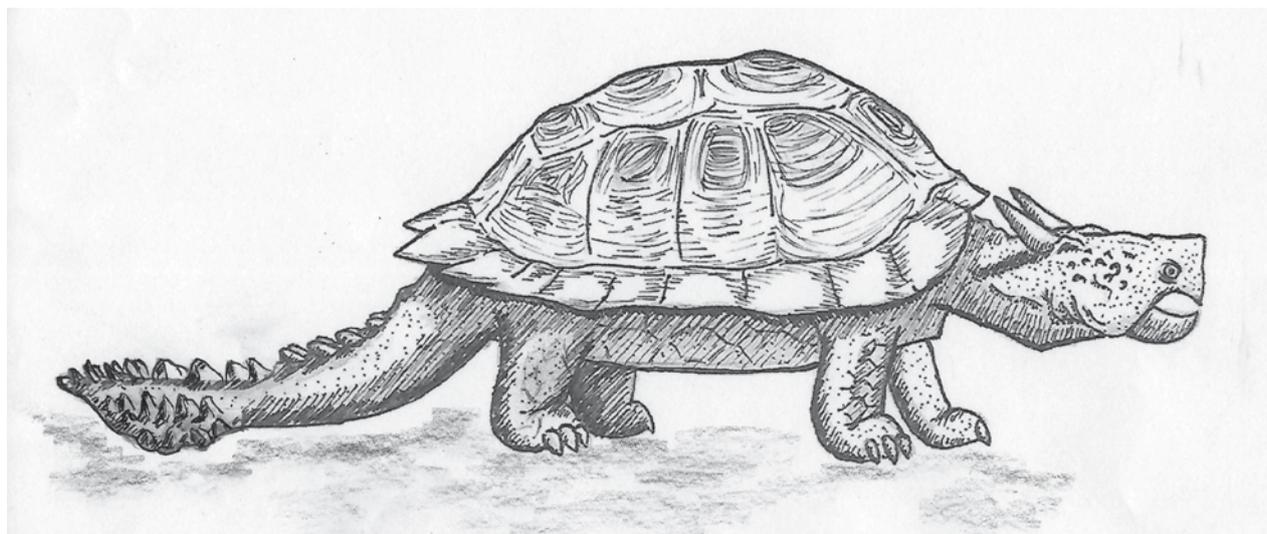


Figure 1. Reconstructed image of the giant, horned, fossil turtle (*Meiolania*). Drawing by Allyson Belcher, after Orenstein (2012) and by Janice Moll who portrayed the terminal tail club.

Many meiolaniid researchers have given their interpretation of the palaeoecology and palaeobiogeography of this group, particularly the mode of dispersal in relation to their geographical origin. However, there has been only one notable paper devoted to the palaeobiogeography of this fascinating group, in Mittermeier (1972) and in a slightly revised version in 1984. In the last 47 years, there has been considerable research on horned turtles. Herein, we review all research we found (to date) concerning their palaeoecology, dispersal, and biogeographical origin. Many possible explanations for the meiolaniid distribution patterns were provided with their merits and problems discussed.

The topics covered include taxonomy and distribution, convergent evolution, introduction by early humans, a brief history of perceptions concerning meiolaniid palaeoecology and dispersal modes, dispersal via water or land, requirements for successful colonisation, dispersal across land bridges, breakup of Gondwana, and the distributional significance of Zealandia for meiolaniid turtles.

METHODS

A comprehensive literature search was carried out following the methodologies of Brown et al. (2008); Moll & Brown (2017); and Brown et al. (2018). The search topics pertained to (1) the palaeontology of horned turtles, and (2) the geology of the breakup of Gondwana as well as the geological formation of islands in the south-west Pacific Ocean on which horned turtles have been found. We used a variety of search engines with numerous combinations of search words. Traditional searching for paper publications was carried out at Milner Library at Illinois State University. Some of these were on microfilm and many were from deep storage. In addition, the authors' extensive palaeontological and zoological libraries were searched. Interlibrary loan and I-Share were used frequently to obtain copies of publications

not available in the libraries heretofore mentioned. LEB translated from French to English the pages covering *Meiolania* in the Reptiles section of Dollo (1904). Everhart (2017) presented a translation of part of Dollo (1887).

RESULTS & DISCUSSION

Taxonomy and distribution

Gaffney (1996) and Sterli (2015) reviewed the species of meiolaniid turtles that have been described from Australasia. These include, with the known distributions, the following taxa:

- *Meiolania platyceps* Owen. Lord Howe Island, New South Wales, Australia (Gaffney, 1996).
- *Meiolania brevicollis* Megirian. Camfield Station, Northern Territory, Australia (Gaffney, 1996).
- *Meiolania mackayi* Anderson. New Caledonia, including Pindaï Caves on the main island, Tiga Island in the Loyalty Islands, and Walpole Island (Bauer & Sadlier, 2000).
- *Meiolania damelipi* White, et al. Teouma on Efate Island, Vao and Uripiv islands, Vanuatu; Naigani and Viti Levu islands, Fiji (White et al., 2010; Hawkins et al., 2016).
- *Ninjemys oweni* Woodward. Eastern Darling Downs, southern Queensland, and New South Wales, Australia (Gaffney, 1996).
- *Warkalania carinaminor* Gaffney, et al. Riversleigh Station, north-west Queensland, Australia (Gaffney, 1996).
- Gaffney (1996) reported localities of additional meiolaniid records of indeterminate identification from Queensland, New South Wales, and South Australia.
- Validity of the species names *mackayi* and *damelipi* was questioned by several workers (e.g. Gaffney, 1996; White et al., 2010; Sterli, 2015; Hawkins et al., 2016). Sterli (2015) indicated that *damelipi* was "not meiolaniid". Future research may further demonstrate these names to be erroneous.

In summary, there are three genera and 4-6 species of meiolaniids known from Australasia. The species have been reported from eastern Australia, Lord Howe Island, Tiga Island, Walpole Island, New Caledonia, Vanuatu, and Fiji.

Convergent evolution

Convergent evolution (or parallelism) has been frequently cited as an option for similarities of two biogeographically separated groups, but it was in most cases an explanation of last resort when nothing else seemed convincing. Simpson (1938) and Mittermeier (1972, 1984) were the only authors we found that suggested convergent evolution to explain similarities of the Australasian horned turtles and the frilled turtles of Argentina, but they presented no evidence. Basically, one needs to demonstrate the two groups do not have a common ancestor, which is very difficult because of lack of fossils. We found no evidence for or against convergent evolution in these turtles. The South American and Australasian forms have been treated as relatives within the clade Meiolaniformes (Sterli, 2015; Sterli et al., 2015).

Introduction by early humans

Bauer & Vindum (1990) suggested that meiolaniids may have been introduced into New Caledonia by humans, but we found no evidence that this occurred. However, this possibility should not be dismissed, even though there are several reasons described below why this activity seems unlikely. Presumably the reason for the human introduction would have been as a source of food. The capture and transport by boat of the 3 m long heavily armoured meiolaniids would have been particularly dangerous for humans. Young individuals of both sexes would have been the easiest and safest way to introduce the turtles, but due to typical turtle life history characteristics, including high juvenile mortality, slow growth, and slow generation times (~30 years in extant, testudinid, giant tortoises), it is unlikely that simple translocation and domestication would have been possible (Swingland & Coe, 1979; Cheke et al., 2017). In addition, larger reproductive adults probably could not have been harvested in turtle populations if the population was to remain viable (Congdon et al., 1993, 1994) and repeated introductions of new individuals from established populations would have been necessary to shore up population numbers. Another consideration is that translocated adult turtles may often quickly move out of the release area as observed by Sosa & Perry (2015) with ornate box turtles (*Terrapene ornata ornata*). They found that juveniles were more likely to remain closer to the release area than adults. Other studies have also shown similarly low release site fidelities for adult box turtles translocated into natural habitats (e.g. *T. ornata*: Doroff & Keith, 1990; and *T. carolina*: Cook, 2004; Hester et al., 2008).

However, a higher percentage of captive-bred juveniles ploughshare tortoises (*Astrochelys yniphora*) remained near their release site in studies conducted in Madagascar (Mandimbihasina & Woolaver, 2014). Although most

extant terrestrial turtles establish relatively small home ranges and usually return to them if displaced, some adult box turtles are considered transients and seem to wander over relatively long distances without ever establishing home ranges (see Dodd, 2001 for a review). We can only speculate upon the behaviour of meiolaniids which may have been transported long distances and translocated from a distant home to which they could never return. Perhaps we can gain some insight from “rewilding” projects in progress in some of the western Indian Ocean islands that involved the translocation of giant Aldabra tortoises (*Aldabrachelys gigantea*) and radiated tortoises (*Astrochelys radiata*) to other islands, hopefully to provide ecosystem characteristics once available to the now extinct native species (Cheke & Bour, 2014; Griffiths, 2014). The translocated species seem to be adapting successfully to their new homes in several of these locations (Griffiths, 2014), so it is possible that the meiolaniids adapted similarly to their translocation sites.

During the Holocene, the people of the Lapita culture became the first humans to colonise New Caledonia, Fiji, and Vanuatu (White et al., 2010). They were avid hunters of Meiolania and bones (some burnt) have been found in archaeological middens, possibly from turtles killed and transported from other locations (White et al., 2010; Orenstein, 2012; Hawkins et al., 2016). The Lapita seemed to prefer Meiolania over sea turtles (Orenstein, 2012). Hogs were introduced and raised for food by them (Orenstein, 2012), but we found no evidence of any attempt to keep meiolaniids in captivity or to domesticate them. Although people of archaic and extant cultures are known to keep freshwater turtles, sea turtles, and tortoises in captivity for food and other purposes (e.g. Parsons, 1962; Moll & Moll, 2004; Bell et al., 2005; Cheke & Bour, 2014) it is difficult to imagine the challenges that would have confronted the Lapita in domesticating or merely keeping in captivity adult, horned turtles as a source of food. The potential dangers of maintaining these formidable animals as well as the difficulties and time required to raise a “crop” to maturity due to the life history characteristics of most known turtles (see above) would have likely precluded domestication. Moreover, the questionable species identification of the turtles (*M. damelipi*) on Vanuatu and Fiji suggest the possibility that it could have been a more docile form (perhaps similar to extant, giant testudinids) that could have been more easily kept in captivity. Nevertheless, we found no evidence that the Lapita ever tried to keep in captivity or domesticate the turtles of questionable identity (*M. damelipi*) in Vanuatu and Fiji, although they hunted them avidly and travelled widely in search of better hunting grounds as more local stocks were depleted (White et al., 2010; Kinaston et al., 2014; Hawkins et al., 2016). It is reasonable to suggest that human hunting played an important role in the extinction of meiolaniids and other turtles elsewhere throughout the world during the Holocene.

History of perceptions concerning meiolaniid palaeoecology and dispersal modes

There has long been controversy as to whether this

group could have dispersed in water or if they were strictly terrestrial. Early researchers suggested they were terrestrial (Boulenger, 1887; Baur, 1889; Dollo, 1904) with Woodward (1901) indicating they were “truly” terrestrial or marsh dwelling. Anderson (1925) followed, suggesting they were probably strong swimmers, but also indicated terrestrialism. In 1926 Anderson again vacillated between marine, river, marsh, estuarine, shore-living, and land dwelling habitat, but by 1930 he indicated Meiolania was terrestrial.

Starting in 1925 and lasting nearly to the present there has been a series of papers all proposing that these turtles were capable of swimming, floating, walking, wading or drifting in water (e.g. McCulloch [in Anderson, 1925]; Anderson, 1925; Simpson, 1938, 1940; Paramonov, 1958; Fletcher, 1960; White et al., 2010; Orenstein, 2012; Sterli, 2015; Hawkins et al., 2016; Cheke et al., 2017; Lichtig & Lucas, 2018a, b). Many other publications suggested that the horned turtles were adapted for terrestrial life (e.g., Sutherland & Ritchie, 1974; Ritchie, 1978; Pritchard, 1979; Molnar, 1984; Gaffney, 1991; Molnar, 1991; White et al., 2010; Kinaston et al., 2014; Heinsvig, 2015; Jannel, 2015; Paulina-Carabajal et al., 2017).

Lichtig & Lucas (2018a, b) contradicted the most recent majority viewpoint supporting terrestrialism which is based upon evidence cited herein, and summarised in their own papers. They primarily employed an analysis based upon shell dimension ratios purported to identify whether extant and extinct chelonians were aquatic or terrestrial (Lichtig & Lucas, 2018a, b). They also examined the morphology of the forelimbs and femurs of *M. platyceps* for comparison with other chelonians. According to their analyses, *M. platyceps* was aquatic and was probably a bottom-walker similar to extant common snapping turtles (*Chelydra*) and alligator snapping turtles (*Macrochelys*).

We found no other information concerning aquatic habitat preferences for Australasian meiolaniids. We reject the conclusions of the Lichtig & Lucas (2018a, b) based upon the following considerations: 1) The use of ratios in statistical analyses is problematic in numerous respects that can lead to the misinterpretation of data and Allison et al. (1995), advocate that investigators should use regression-based approaches as alternatives; 2) The single meiolaniid specimen included in the Lichtig & Lucas (2018a, b) studies is itself a reconstructed composite specimen derived from skeletal elements obtained from multiple *M. platyceps* from Lord Howe Island (Burke et al., 1983). The main specimen used in the model had been found in an incomplete state (~60% complete, including only three fragments of carapace), was damaged in initial recovery efforts, and again in the reconstruction effort itself (Burke et al., 1983). Lichtig & Lucas (2018a, b) relied upon the reconstruction by Gaffney (1996) of the limb proportions described in Joyce & Gauthier (2004), and on images provided by Juliana Sterli (Lichtig & Lucas 2018a, b) to guide the development of their model. Lichtig & Lucas (2018a) admit that “working from reconstructions is not ideal, and an inherent result of this is the dependence of our results on the accuracy of the used reconstructions”. We

conclude that the use of a single composite specimen with shell damage and reconstruction from multiple sources, and with the inherent problems associated with the use of images and reconstructions is very problematic. The shell proportions used in the development of the critical ratios employed in habitat prediction are potentially unreliable, and therefore, suspect as a result. Even if the ratios obtained are considered valid only one (of two) meiolaniid shell ratios calculated by Lichtig & Lucas (2018a, b) suggests an aquatic lifestyle (i.e., the carapace width to plastron width ratio). The small sample size for *M. platyceps* greatly exaggerates the problems with their analysis (Matt Dugas, pers. comm.). In addition, the model specimen used for the analyses was relatively small, probably juvenile (Burke et al., 1983), and possibly with different shell proportions than adults, as is the case with many extant turtles and tortoises (Ernst & Lovich, 2009). Thus, its comparison with the adult chelonians used in the (Lichtig & Lucas, 2018a, b) analysis is not valid. An even more serious problem related to comparing shell proportion/ratios between meiolaniids and other chelonians is that the meiolaniids are evolutionarily and structurally archaic and unique among chelonians. They are arguably neither cryptodires nor pleurodires, and could represent a stem group more basal than any extant turtle suborder (we even question their inclusion in the Testudinata as currently defined); 3) The morphology of the forelimbs and femora of *Meiolania* are, of course, subject to individual and ontogenetic variation also, and a larger series than those that can be contributed by one composite specimen is necessary for valid, meaningful conclusions concerning their functional significance.

Under the circumstances, we reject the Lichtig & Lucas (2018a, b) reinterpretation of the habitat and lifestyle of *M. platyceps* as it does not rise to the level of the aphorism that “extraordinary claims require extraordinary evidence” (Deming, 2016). The evidence they provided is inadequate to support the radical changes in viewpoint they promote. These problems and uncertainties dictate caution in reinterpreting the habitat and lifestyle of *M. platyceps* in particular, and meiolaniids in general.

Very little information is available to precisely define preferred terrestrial habitats for Australasian meiolaniids either, although *M. platyceps* of Lord Howe Island has been proposed to be a beach dweller based on discovery of some fossils in aeolian calcarenite strata (i.e. indurated coral-sand rock common in coastal areas of the southwest Pacific and elsewhere initially deposited by aeolian processes [Etheridge, 1889a in Jannel, 2015]). Beaches are often difficult environments thermally and in terms of resource availability, however, and if they lived there they must have been well adapted for life in arid conditions as suggested by Paulina-Carabajal et al. (2017). The unusual elongation of the vestibulum of the nasal cavity in *M. platyceps* was also observed in iguanid lizards specialised for desert life as it limits the possibility of sand grains entering the nose (Parsons, 1959). A windy beach habitat would seem to be conducive to the evolution of such an adaptation. Alternatively, it is possible that meiolaniids used beaches for nesting, but lived elsewhere during

other stages of their life history. Meiolaniid egg clutches have also been found in calcarenite on Lord Howe Island, and many other extant reptiles are known to inhabit other, sometimes distant, habitats in non-reproductive periods before returning to beaches to nest (e.g. green iguanas, *Iguana iguana*, [Hirth, 1963]; painted terrapin, *Batagur borneoensis*, [Dunson & Moll, 1980]; all sea turtles, [Ernst & Barbour, 1989]; Central American slider turtle, *Trachemys venusta*, [Moll, 1994]).

Studies of neck vertebrae flexibility, the heavy, horn-bearing head, the suspected mass of soft tissue in the dorsal region of the neck, and the projecting anterior margin of the carapace of *M. platyceps* suggest that it was probably, primarily a terrestrial grazer, bending its neck downward to feed on fallen palm fruits, ferns and other herbaceous vegetation rather than raising it to browse (Heinsvig, 2015; Jannel, 2015). Jannel (2015) suggested it could have browsed occasionally however (perhaps on low hanging vegetation). Due to the stable, mild climate of Lord Howe Island during *M. platyceps*' tenure there an abundant and predictable food supply would have been present (Jannel, 2015). *Meiolania platyceps* probably wandered across the island searching for new food supplies which became seasonally available in different locations. Paulina-Carabajal et al. (2017) suggested the possibility that *M. platyceps* could have had acute olfactory capabilities, based upon analysis of its endocranial morphology. If so, this could have been important in identifying and locating new food sources as they appeared (Heinsvig, 2015; Jannel, 2015).

Isotopic evidence from Vanuatu (*M. damelipi*, which may not be meiolaniid; Sterli, 2015) supports the view that ?*M. damelipi* was terrestrial, herbivorous to omnivorous, may have utilised foods under a forest canopy, and possibly also used mangrove habitats as foraging sites (Kinaston et al., 2014).

Dispersal via water or land

Based upon the totality of the evidence presented above, and because all meiolaniid fossils have been found in terrestrial strata and locations, we strongly support the terrestrial niche concept for the Australasian meiolaniid turtles. In particular, we conclude that several specialised adaptations of meiolaniids could have made sea travel, and therefore continent and island colonisation by saltwater dispersal, nearly impossible for them. Most importantly, the skulls of meiolaniids are huge, well ossified, strongly ankylosed (Sterli, 2015), and adorned with horns or ridges, all of which would have made them extremely heavy and non-retractable into the shell (Heinsvig, 2015; Jannel, 2015). The neck of meiolaniids is quite rigid and is probably linked to the need to support the massive, heavy skull (Heinsvig, 2015; Jannel, 2015). It has somewhat greater flexibility in a downward plane than in an upward plane, probably related to its grazing, feeding style (Heinsvig, 2015; Jannel, 2015). All of these skull and neck characteristics would have driven the head end of the floating animal into and under the water as the neck muscles tired and extension of the neck upward to allow the turtle to see and breathe would have quickly become more difficult. The limbs and feet

were more similar in structure to those of the extant, terrestrial, testudinid tortoises, although the feet have been described as "ridiculously dainty" (Burke et al., 1983). Others have also noted the small size of the limbs (Sutherland & Ritchie, 1974; Sterli, 2015). They were not similar to the large, paddle-like forelimbs of extant sea turtles or the extant, estuarine pig-nosed turtle (*Carettochelys insculpta*). Therefore, efficient forward or upward propulsion would not have been possible for any extended period of time in water for meiolaniids. The extremities were probably covered with heavy osteoderms (Sterli, 2015). The shell is relatively thin and the carapace of meiolaniids is more oblong and lower domed than in the extant, testudinid tortoises (Ritchie, 1978). Therefore, the meiolaniid shell would have trapped less air and would not have increased buoyancy. Posteriorly, the long, osseous, sheathed tail with a heavy terminal club would have acted as a sea anchor. We conclude that these adaptations, undoubtedly useful in their terrestrial life, would have caused them to sink and drown after a short period of immersion in deep water. Lichtig & Lucas (2018a) considered *M. platyceps* to be negatively buoyant and would have sunk if immersed in water. The influence of turbulent seas, strong currents and winds, and thermal stress from sun exposure would have exacerbated the problems of flotation and movement in saltwater for these terrestrially adapted species. It seems unlikely that an overturned meiolaniid turtle could have righted itself, especially under the kinds of suboptimal conditions described above, and it would have surely drowned. A turtle fortunate enough to survive and stay upright long enough would soon have been afflicted by the debilitating physical and physiological impairments related to prolonged saltwater immersion (e.g. destruction of the nasal and alimentary canal epithelia, damage to the cornea of the eye). Furthermore, if the turtle drank the sea water it would have developed blood plasma ionic stress which requires a huge amount of energy to rectify, if even possible (Craig Gatto, pers. comm.).

Natural rafting, a saltwater dispersal mechanism suggested for meiolaniids by Mittermeier (1972, 1984), and by many other biogeographers for other species (see Darlington, 1963; Udvardy, 1969) would have theoretically allowed them to avoid many of the problems associated with immersion and movement through saltwater, but their great size and weight would have required a very large and unusually sturdy raft to transport them over long distances. Juvenile meiolaniids might have been more likely to successfully raft to suitable locations because of their smaller size, but their non-reproductive condition, vulnerability to predation, and the long generation times with delayed maturation typical of many turtles and tortoises (Congdon et al., 1993, 1994) would probably have reduced their chances for successful colonisation compared to adults.

Stepping stone dispersal was proposed by Anderson (1926) as another means of dispersal of meiolaniids. A variant of this, the escalator hopscotch model (McKenna, 1983), was also proposed for meiolaniids by Gaffney (1996). These would have required eliminating water

gaps between the mainland and islands by lowering sea levels and dispersal would have presumably been quite slow across the archipelago.

Ironically, extant, giant, testudinid tortoises are known to drift in saltwater and successfully cope with the harsh environmental conditions long enough to occasionally reach land and potentially colonise suitable habitats (e.g. Galapagos Islands, Mascarene Islands, and Seychelles Islands). Townsend (1936) reported two extant, giant tortoises (*Chelonoidis nigra* species complex) adrift 20 miles (~32.2 km) from land in the Gulf of Florida that had escaped from a captive colony. The presumption was that they were swept out to sea by a hurricane. Subsequently, Gerlach et al. (2006) reviewed a number of cases of Aldabra giant tortoises (*Aldabrachelys gigantea*) floating around Aldabra Atoll and in the open sea of the western Indian Ocean. Gerlach et al. (2006) also reported the first substantiated evidence of trans-oceanic dispersal by *A. gigantea*. In 2004 a female tortoise was found walking out of the Indian Ocean at Kimbiji, Tanzania. The animal was emaciated and had an excessive number of goose-necked barnacles, particularly on the lower limbs and carapace suggesting a long period of time spent at sea. Close examination of the tortoise indicated it was probably from Grande Terre, Aldabra Atoll. This tortoise had travelled a remarkable 740 km across the western Indian Ocean.

Testudinid tortoises and meiolaniids share a number of structural characteristics that are probably similar due to terrestrial lifestyles (e.g. walking and herbivory). However, there are several important differences between testudinid tortoises and meiolaniids that affect their seaworthiness. The testudinids have smaller, lighter retractable heads and tails than meiolaniids. Furthermore, testudinids have an elongated flexible neck which allows them to easily raise their head above water. The higher and thicker domed carapace of testudinids probably protected them from predators and contained more of an air pocket which enhanced their buoyancy. The shell shape in association with these other factors gives the testudinids a centre of gravity that enhances stability in water. Also, their ability to survive without food or freshwater for long time periods (Moll & Brown, 2017) adapts them for greater survivorship at sea than would otherwise be the case.

Mittermeier (1972, 1984) suggested that meiolaniid turtles might have had marine ancestors which have not yet been identified in the fossil record. If correct, they could have dispersed to their known locations via sea travel and subsequent terrestrial travel. This has also been proposed in other turtle groups (e.g., podocnemidid turtles). Until relevant, marine-adapted fossil forms should appear, it seems prudent to remain cautious concerning marine dispersal explanations for meiolaniid distribution patterns, and favour terrestrial dispersal pathways.

Immediately upon entering saltwater, meiolaniids would have also been exposed to the onslaught of a variety of turtle predators of the marine environment (see below). In order to move the head sideways during attack or defence, the feet of meiolaniids had to have

been firmly stabilised on the ground (Jannel, 2015). If swimming or drifting in water, such stability would have been non-existent. Moreover, movements of the non-retractable head and tail would have been slower because of the density of the water. These factors plus the thinness of the shell (e.g., Gaffney, 1996, *M. platyceps*) suggest that Meiolania would have been nearly defenceless if attacked by predators in water.

There were numerous carnivorous species of ancient oceanic animals that could have preyed on turtles, including horned turtles (if present). However, we found only one reference (Orenstein, 2012) that mentioned possible oceanic predation on meiolaniids (by Pacific, marine crocodylians). There are three groups, mosasaurs, sharks, and crocodylians that stand out as threats because of abundant evidence that they aggressively preyed on turtles (e.g. Dollo, 1887; Carr, 1940; Loveridge, 1946 [1974]; Neill, 1971; Cogger, 1975; Carpenter & Lindsey, 1980; McCoy, 1980; Erickson, 1984; Mazzotti & Dunson, 1989; Ellis, 2003; Karl & Tichy, 2004; Gandola et al., 2006; Fernández & Gasparini, 2008a, b; Ernst & Lovich, 2009; Schwimmer, 2010; Milàn et al., 2011; Erickson et al., 2012; Main et al., 2012; Amalfitano et al., 2017; Everhart, 2017; Scheyer et al., 2018).

The problem of successful colonisation

All successfully dispersing organisms must cope with the difficult and diverse problems posed by a newly entered habitat if successful colonisation is to be realised. Carlquist (1974) stated that “island elements are present in proportion to not only dissemination ability, but also to establishment ability”, and that the “difficulties of establishment seem much greater than those of transport”. MacArthur (1972) summarised many of the problems of island colonisation. These statements are scarcely different for the colonisation of continental habitats, except that dispersers arriving via terrestrial routes may often arrive in less weakened and emaciated condition than those drifters, swimmers, or rafters that have endured prolonged exposure to saltwater en route. Nevertheless, probably only a fraction of arrivals in either category manage to establish a successful colony in any case (Carlquist, 1974). Carlquist (1974) provided examples of some of the traits that influence success (or not). Australasian meiolaniids, present on both islands and the Australian continent, would seem to have had a colonisation advantage over many other dispersers since they could slowly adapt to changing climatic conditions and habitats, and forage as they rode their Gondwanan continental fragments to their final destinations without ever having to get their feet wet. The mechanisms that we presume allowed this to occur are explained in more detail below.

As a result of the factors described above we reject long distance dispersal via water as an explanation for Australasian meiolaniid distributions in all its forms that have been proposed. We support the view that the Australasian meiolaniid distribution pattern resulted solely from terrestrial movements of meiolaniids and/or their ancestors passively via the fragmentation of Gondwana and drifting of derivative continents, as well

as actively via the use of land connections when and where available.

Unlike other non-tortoise, giant, armoured species, such as ankylosaurid dinosaurs and the glyptodont mammals which were restricted to continents and perhaps a few continental islands (Olivero et al., 1991; Palmer, 1999; Arbour & Currie, 2016; Dixon, 2016), the meiolaniids were able to become established on a few remote Pacific islands. Their presence there has been one of the great enigmas of Pacific palaeobiogeography, and the need for an explanation of their presence has undoubtedly influenced the judgment of many meiolaniid researchers to envision aquatic capabilities for a group that is clearly highly specialised and ill-equipped for anything other than terrestrial life and dispersal. We propose a more likely explanation for meiolaniid presence on these islands below.

Dispersal across land bridges and breakup of Gondwana

During the 19th and 20th centuries, a plethora of biogeographers indiscriminately suggested the presence of land bridges in attempting to resolve the evolutionary history of organisms with puzzling geographical distributions. Many of these “bridges” were transoceanic, and the authors did not take into consideration the geology of the regions. Thus, they often should not be considered as viable hypotheses.

Over 100 years ago, (Moreno & Woodward, 1899) suggested that the discovery of *Miolania* (sic) on Lord Howe Island, Queensland, and Patagonia favoured the hypothesis of a former great Antarctic continent of which Australia and Patagonia were a part. Woodward (1901) was apparently the first to suggest an ancient land bridge connecting Australia, Antarctica, and South America across which *Meiolania* could have dispersed. Other researchers (e.g. Dollo, 1904; Anderson, 1926; Fletcher, 1960; Mittermeier, 1972, 1984; Molnar, 1991) agreed with this concept. Mittermeier (1972, 1984) stressed that the turtles could have passed in either direction. This 100+ year old concept became the precursor of the most recent research on the geographic origin of this turtle group.

There is a huge literature on the breakup of Gondwana that is beyond the scope of this paper to review. However, an excellent detailed description by Lomolino et al. (2017) is readily available. In this section, we summarise (after Lomolino et al., 2017) the breakup of this vast supercontinent and address problematic issues of palaeobiogeographic significance to the evolution of meiolaniids.

The landmass Gondwana separated from the much larger Pangea during the Jurassic (Lomolino et al., 2017). The original Gondwana consisted of the present day continents Africa, South America, Australia, Antarctica, Zealandia (including New Zealand), and India, as well as Madagascar. The breakup began with splitting off of South America and Africa from the rest of Gondwana. This was followed by the rifting of India and Madagascar from Gondwana. Lastly Australia and Zealandia rifted from Antarctica. The entire process probably lasted for a time span from ca. 160 mya to 80 mya years ago. The

speed of rifting varied. India’s northward migration was particularly fast (Lomolino et al., 2017). The direction of continental movement could have also been reversed. For example, Africa reunited with remnants of Gondwana and then rifted away a second time (Lawver et al., 1992).

The regions that were to become the future continents Australia, Antarctica, and South America were aligned in a row (with Antarctica in the middle) early in the history of Gondwana. Zealandia was connected to and adjacent to west Antarctica at this time (Mortimer et al., 2017).

Several researchers (e.g. Mittermeier, 1972, 1984; Gaffney, 1996; Sterli, 2015; Sterli et al., 2015) suggested that the breakup of Gondwana might have been involved in the distribution and evolution of horned and frilled turtles in Australasia and South America. We propose that Zealandia played a similar role for Australasian meiolaniids.

Mittermeier (1972, 1984) suggested the possibility that Antarctica (in the middle position) was the region of origin of meiolaniids. The dispersal-extinction-cladogenesis model (DEC) used by Sterli & de la Fuente (2013); Sterli (2015); and Sterli et al. (2015) also suggested Antarctica as an area of origin. However, they were reluctant to designate Antarctica as such because no fossil meiolaniids have been found there, and because of the scarcity of fossil turtles in the Cretaceous and Paleocene of Australia. Instead they considered South America as the more likely place of origin. Meiolaniid ancestors could have moved through and into the other, future continents from South America while all were connected, or in either direction if Antarctica turns out to be the centre of origin. Then they could ride with these land areas to their future locations as the continents continued to fragment and drift. The opportunities for vicariance in the populations inhabiting the separating fragments would be enhanced as they rifted further apart over time. The problems of determining the true place of origin might be resolved if the melting of considerable ice on Antarctica, resulting from extensive climate warming, allows more exploration for fossils there in the future.

Another problem concerns the dating of the rifting of South America from the remainder of Gondwana. Lawver et al. (1992) and Lomolino et al. (2017) indicated it occurred during the Mid to Late Jurassic (160 mya) which made it the first continent to break away from Gondwana. However, Sterli et al. (2015) followed Woodburne & Case (1996) and Lawver et al. (2011) indicating the separation occurred in the Early Eocene along the Eocene-Oligocene boundary (this was after the Cretaceous when the oldest fossil frilled turtles were found). This timing would have made the separation the final stage of the breakup of Gondwana. Other researchers (e.g., Royer & Sandwell, 1989; Blakey, 2008) indicated the separation occurred during the Mid Cretaceous. These extreme differences may reflect disparity in methodologies used. In effect, they represent the use of different proxies. Moll & Brown (2017) discussed the problem of inappropriate use of proxies. For instance, at a Quaternary site in southern Illinois, USA, no consensus was reached after nine methods were used over 25+ years to date the site (Curry et al., 2011; Blackwell et al., 2016).

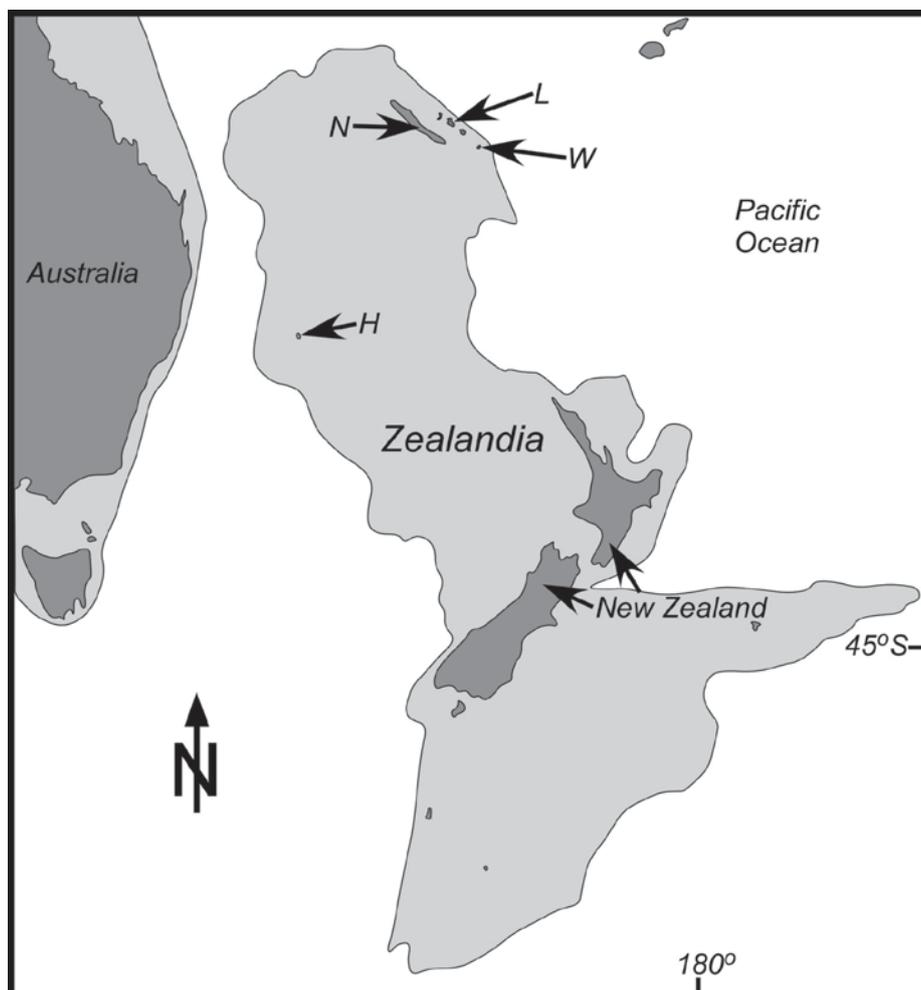


Figure 2. Map of the position of the mostly submerged continent of Zealandia showing emergent localities where fossil Meiolania were found. “N” designates New Caledonia; “H” designates Lord Howe Island; “L” designates the Loyalty Islands (arrow points to Tiga Island); “W” designates Walpole Island. Cartography by Dave Malone.

The timing of separation of continents has important implications for interpretation of the palaeobiogeography of horned turtles. Forty-seven years ago Mittermeier (1972) succinctly pointed out: “If common ancestors were not present in the continents in question before they split (and fossil evidence available at this time suggests that they were not) then it becomes most difficult to explain the distribution patterns of these turtles solely by continental drift”.

Distributional significance of Zealandia for meiolaniid turtles

Zealandia is a large mostly submerged continent (Fig. 2) east of Australia that has been recently recognised as such. Mortimer et al. (2017) presented a convincing argument that Zealandia is indeed a continent. Zealandia’s defining characteristics include elevation, geology, and crustal structure, as well as limits and area. Among the more important attributes are the presence of igneous, metamorphic, and sedimentary continental rocks, whereas oceanic crustal rocks are basalt and gabbro. In size, Zealandia is 4,500,000 km² of continental crust (Bache et al., 2014). At present it is 94 % submerged (Mortimer et al., 2017). It was part of Gondwana that was originally adjacent to present day West Antarctica.

The separation of Zealandia from Gondwana occurred between the Late Cretaceous to the Eocene (Bache et al., 2014; Mortimer et al., 2017). Isostatic balance and thermal relaxation led to Zealandia’s eventual submergence (Mortimer et al., 2017). New Caledonia, the Loyalty Islands, Walpole Island, and Lord Howe Island as well as New Zealand now occur as projections of Zealandia above the ocean surface (Fig. 2). The distribution of meiolaniids on these islands may reflect a past wider distribution on Zealandia, and the islands’ use as refugia when Zealandia submerged. There are many other archaic species resident in New Caledonia and Lord Howe Island (especially plants) that are also not recognised as good saltwater dispersers (Carlquist, 1965). Many probably were stranded there in similar fashion to the meiolaniid turtles. Fossil remains of a large, terrestrial turtle of early Miocene age were discovered on the South Island of New Zealand (a Zealandia remnant) also (Worthy et al., 2011), but its taxonomic designation remains uncertain (Sterli, 2015).

The islands of Vanuatu and Fiji occur north and north-east of Zealandia. The fossil turtles of these islands were thought to be ?*M. damelipi*, White et al. (2010). However, the identification of these fossils has been seriously questioned by White et al. (2010); Sterli (2015),

and Hawkins et al. (2016). It is very unlikely that they are even meiolaniids (Sterli, 2015). Thus, the locality records of this “species” of *Meiolania* from Vanuatu and Fiji may likewise be invalid.

CONCLUSIONS

Mittermeier (1972, 1984) argued that the lack of fossils at the right times and places casts doubt upon the importance of Gondwana fragmentation and drift as an explanation for the meiolaniid distribution pattern in Gondwana-derivative continents and islands. We submit that the absence of fossils from critical times and locations is frustrating, but it does not disqualify the fragmentation/drift-land connection scenario as the principal driving force resulting in the meiolaniiform distribution pattern as we see it today. A major problem is that it is often difficult to identify the critical times and places that relate to meiolaniid dispersal because there is still very imprecise understanding of the fine timing of fragmentation and rift (due to different methodologies and proxies employed [see above discussion under Dispersal across land bridges and Breakup of Gondwana]), and, also an inadequate knowledge of movement and interaction of rifting land masses and connections over shorter time frames. A better grasp of these issues would help to narrow down the best locations and time periods to search for critical meiolaniid fossils. We also stress that the critical locations from which relevant fossils have not been found, ice-covered Antarctica and submerged Zealandia, are among the most difficult places on earth in which fossiliferous strata could be investigated. Where critical locations and strata are accessible for exploration (e.g. South America and Australasia) meiolaniid fossils have been found.

Since sea travel is not a viable option to explain the meiolaniid distribution pattern then what explanation remains? The simplest explanation, with what we know now, is the Gondwana fragmentation/rift hypothesis, and terrestrial movement when and where possible. The recognition of the continent of Zealandia, its submergence, and its geographic location encompassing the south-west Pacific islands where meiolaniid fossils have been found provides a simple resolution to the formerly insoluble enigma of explaining their presence in these remote, insular locations.

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An assessment of funding and publication rates in Herpetology

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Currently, herpetofauna worldwide is facing enormous threats; the number of threatened species is increasing at an alarming rate and many species have gone extinct. Despite efforts of institutions and researchers to understand and address the causes of declines and raise awareness of herpetofauna conservation, there has been no systematic study to evaluate the allocation of funding for basic and applied research relevant to conservation, relative publication rates, and the relationship of these measures to a degree of threat among herpetological groups. This study addresses this gap and identifies strengths and weaknesses of herpetological research and conservation over the last 10 years (2008-2018). Frogs had the highest grant-publication index (1384), followed by lizards (695), turtles (678), snakes (461.5), salamanders (366.5), crocodiles (164), caecilians (25.5), worm lizards (23) and tuatara (10). Nonetheless, when the grant-publication index is divided by the number of threatened and data-deficient species within each group, it demonstrates that, proportionally and in ascending order, salamanders, snakes, lizards, worm lizards, frogs and caecilians are in most need of knowledge and on-going funding for their conservation and survival. I was able to document a continued shift in attention in herpetological research owing to the emergence of chytridiomycosis and the global decline of amphibians. Despite some caveats, these findings should represent a proxy for the allocation of research and conservation effort on herpetofauna worldwide. I suggest priorities for research and how to better direct efforts to herpetofauna conservation.

Keywords: amphibians, extinction, IUCN Red List, literature representation, natural history, reptiles

INTRODUCTION

Although amphibians and reptiles are not closely related in an evolutionary sense, they are often studied together, because as ectotherms they share many physiological, behavioural and ecological similarities (Vitt & Caldwell, 2014). Major extinction events in the past have reduced global diversity of amphibians and reptiles several times, only to be followed by relatively rapid diversification events within some of the surviving groups (Vitt & Caldwell, 2014). Currently, scientists recognise a contemporary extinction of species and populations of similar magnitude to those in the past, known as the sixth mass extinction (Wake & Vredenburg, 2008; Barnosky et al., 2011). Human activities, such as co-opting resources, fragmenting habitats, introducing non-native species, spreading pathogens, killing species directly and changing global climate are playing major roles in these extinctions (Barnosky et al., 2011). Amphibians and reptiles are affected by these globally threatening processes, and a global decline of herpetofauna is underway (Gibbons et al., 2000; Stuart et al., 2004; Hoffmann et al., 2010; Böhm et al., 2013).

An emerging amphibian chytrid fungal disease,

chytridiomycosis, has been responsible for massive die-offs of amphibians worldwide (Rovito et al., 2009; van Rooij et al., 2015). Combined with deforestation, chemical pollution, stochastic events and climate change, scores of amphibian species have gone extinct and 2421 species are currently listed as threatened (Gibbons et al., 2000; Stuart et al., 2004; IUCN, 2019). Reptiles are enduring declines similar to those experienced by amphibians in terms of taxonomic breadth, geographic scope, and severity (Gibbons et al., 2000; Sinervo et al., 2010), although on a global scale threat levels seems to be more severe in amphibians (Böhm et al., 2013). As with amphibians, causes of reptile declines are known with certainty in some cases, suspected in many, and unknown in others (Gibbons et al., 2000). Likely, overharvesting and habitat loss and fragmentation are the leading threats in the global decline of reptiles (Gibbons et al., 2000; Böhm et al., 2013).

Appreciation of the dire situation for herpetofauna has initiated efforts to investigate and conserve amphibians and reptiles around the globe, led by researchers, governments (Towns et al., 2001; McCarthy et al., 2012), zoos (Conde et al., 2011), and conservation organisations (see Appendix). Although awareness

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within the conservation community of herpetofauna biology, research and protection has increased recently (Gibbons et al., 2000; Urbina-Cardona, 2008; Ohmer & Bishop, 2011; Böhm et al., 2013; Roll et al., 2016), in addition to some studies that have dealt with publication rates (Gibbons, 1988; McCallum & McCallum, 2006; Urbina-Cardona, 2008; Christoffel & Lepczyk, 2012; Lovich & Ennen, 2013) and funding (Gibbons, 1988) in herpetology, there is still a lack of understanding on how current funding allocation and conservation publication outputs vary among herpetological taxonomic groups worldwide, and the relationship between funding levels and degree of threat. Such data are important to detect any bias arising from perceptions of need not related directly to actual levels of threat, and to realign priorities to better balance effort across need. To fill this gap, I gathered information on grants, publications and degree of threat among groups of amphibians (frogs, salamanders and caecilians) and reptiles (turtles, lizards, snakes, worm lizards, tuatara and crocodiles) as reported in conservation journals. I asked the following questions: (1) How is funding and generation of knowledge of strategic and tactical value distributed across taxonomic groups over the last 10 years (2008-2018)?, and (2) Does funding and the generation of knowledge reflect the level of threat faced by particular taxonomic groups? In answering those questions, I provide an improved understanding of the distribution of resources across herpetofauna to assist in setting priorities for conservation and research.

METHODS

Herpetological keywords

I searched for keywords that would cover amphibian and reptile classes, orders and families, including synonyms. For amphibians, I searched for “amphibian”, “amphibia”, “anura”, “frog”, “toad”, “caudata”, “urodela”, “salamander”, “newt”, “gymnophiona” and “caecilian”. For reptiles, I searched for “reptile”, “testudines”, “chelonian”, “chelonina”, “turtle”, “freshwater turtle”, “terrapin”, “cooter”, “marine turtle”, “sea turtle”, “tortoise”, “squamata”, “lizard”, “gecko”, “iguana”, “chameleon”, “monitor”, “snake”, “serpent”, “viper”, “rattlesnake”, “worm lizard”, “legless lizard”, “amphisbaenia”, “rhynchocephalia”, “tuatara”, “crocodylia”, “crocodilian”, “crocodile”, “alligator”, “caiman”, “gavial” and “gharial”. For general keywords such as “amphibian” or “reptile”, the records were subsequently examined to verify to which group they belonged. In some instances, some search engines were sensitive to “(s)”, and consequently the keywords were searched both in singular and plural. These keywords were used in searches in several funding agencies databases and the search engine Scopus for scientific publications, as detailed below.

Funding

I searched herpetological keywords for funding in herpetology across grants for basic and applied research, and grants specific for on-ground conservation, from 2008 to 2018, during which time all the funding agencies

were operating and results could be compared. For example, I searched databases from five funding agencies within North America, Australia, United Kingdom and the European Union, which cover basic and applied research in the field of herpetology. They were the National Science Foundation (NSF; available from <https://www.nsf.gov/awardsearch/> [accessed on 14th May 2019]); Australian Research Council (ARC; available from <http://www.arc.gov.au/grants-dataset-information> [accessed on 14th May 2019]); Research Councils UK (RCUK; available from <http://gtr.rcuk.ac.uk/> [accessed on 14th May 2019]); Natural Sciences and Engineering Research Council of Canada (NSERC; available from http://www.nserc-crsng.gc.ca/ase-oro/index_eng.asp [accessed on 31st May 2019]); and BiodiversERsA (available from <http://www.biodiversa.org/database/> [accessed on 31st May 2019]). The grants were subsequently filtered for herpetological keywords and the taxonomic group to which they belonged to, and to exclude keywords that had no link with biological research.

For on-ground conservation grants specifically, I searched the databases of six conservation agencies, five of which fund mainly conservation initiatives in the developing world: Conservation Leadership Program (CLP; available from <http://www.conservationleadershipprogramme.org/our-projects/supported-projects/> [accessed on 3rd June 2019]); Rufford Small Grant Foundation (RSG; available from <http://www.rufford.org/category> [accessed on 3rd June 2019]); Whitley Fund for Nature (WFN; available from <https://whitleyaward.org/winners/> [accessed on 5th June 2019]); The Mohamed bin Zayed Species Conservation Fund (MBZSCF; available from <https://www.speciesconservation.org/case-studies-projects/> [accessed on 9th May 2019]); and National Geographic Society (NGS; available from <https://www.nationalgeographic.org/funding-opportunities/grants/what-we-fund/our-focus/> [accessed on 9th May 2019]), searched on the wildlife focus area, which covered both research and conservation categories). I also included another agency that funds projects on species recovery and habitat protection in the United States of America and developing countries: National Fish and Wildlife Foundation (NFWF; available from <http://www.nfwf.org/whatwedo/grants/search/Pages/Grant-Search.aspx> [accessed on 17th June 2019]). Additionally, I searched for grants specific to freshwater turtles and tortoises awarded by the Turtle Conservation Fund (TCF; available from <http://www.turtleconservationfund.org/announcements/> [accessed on 9th May 2019]), to highlight this additional source of major funding to this taxonomic group.

Publications

I chose four leading conservation journals (*Conservation Biology*, *Biological Conservation*, *Biodiversity and Conservation*, and *Animal Conservation*), four high impact journals (*Nature*, *Science*, *Proceedings of the National Academy of Science of the United States of America*, and *Global Change Biology*), and six major herpetological journals (*Herpetologica*, *Amphibia-Reptilia*, *Journal of*

Herpetology, *Copeia*, *The Herpetological Journal*, and *African Journal of Herpetology*). The first two groups are broad in scope and not specific to any taxa, while the last one is specific to amphibians and reptiles. The goal was to cover the specialised literature in addition to conservation topics, cutting-edge research, and issues related to herpetology in these journals. The search engine Scopus was used to identify articles containing herpetological keywords in the title, abstract, and keywords from 2008 to 2018, which matched the period of funding searched for in this investigation. Scopus was chosen owing to the ability to organise the information and export it in several file formats, in addition to its accuracy in finding herpetological keywords.

A grant-publication index was calculated for each taxon by summing the number of grants (basic and applied research plus on-ground conservation) and the number of publications (conservation, high impact, and herpetological journals) and dividing by two. This index was created to summarise the efforts in both allocation of resources (grants) and biological and conservation knowledge (publications) for herpetofauna.

Threat category

The threat category for each class, order, family, genus and species was obtained from IUCN Red List of Threatened Species (IUCN, 2019) for threatened (critically endangered - CR, endangered - EN and vulnerable - VU) amphibians and reptiles. In addition, the number of data-deficient (DD) species was also recorded. The data were subsequently organised into total (absolute) number of threatened (CR, EN, VU) and data-deficient species (DD) per lower taxonomic group (i.e. frogs, salamanders, caecilians, turtles, lizards, snakes, worm lizards, tuatara and crocodiles). In addition, percentages were obtained by dividing the number of threatened species and the number of data-deficient species by the total number of species in each lower taxonomic group (number of species for amphibians, Frost, 2019; for reptiles, Uetz et al., 2018). To answer the question if funding and knowledge reflect the level of threat faced by herpetological taxonomic groups, giving the unequalness of size of taxonomic groups, the grant-publication index was divided by the absolute number of threatened and data-deficient species in each taxonomic group.

RESULTS

Considering an overall picture in terms of funding and publications, amphibians have received slightly more basic and applied research funding (US\$ 212,629,001.29) than reptiles (US\$ 200,813,308.73; Table 1, 2), but reptiles attracted more on-ground conservation grants and had a slightly greater publication record ($n = 831$ and $n = 2330$, respectively) compared to amphibians ($n = 542$ and $n = 2010$, respectively; Table 3, 4).

When considering lower taxonomic levels, frogs led with the highest grant-publication index (1384; Fig. 1). Lizards (695), turtles (678), and snakes (461.5) also had an above average grant-publication index (mean = $423 \pm$ [SD] 450.3 [range, 10–1384], $n = 9$). On the other hand, salamanders (366.5), crocodiles (164), caecilians (25.5), worm lizards (23) and tuatara (10) had below average values (Fig. 1, Table 1, 3, 4). With regard to on-ground conservation grants, turtles (37.8 %) and frogs (32.7 %) received the greatest number, followed by lizards (9.2 %), snakes (8.2 %), salamanders (6.1 %), and crocodiles (5.1 %) (Table 3). Caecilians (0.7 %), worm lizards (0.2 %) and tuatara (0 %) have received little or no on-ground conservation funding from the agencies analysed in this study (Table 3). Publications were highest in number for frogs (34.4 %), followed by lizards (20.2 %), snakes (14.6 %), turtles (13.8 %), and salamanders (11.3 %). The other taxonomic groups showed below average values (mean = $482.2 \pm$ [SD] 492 [range, 16–1494], $n = 9$; Table 4).

Proportionally, tuatara (100 %), turtles (46.2 %), crocodiles (45.8 %), salamanders (38.7 %) and frogs (30.1 %) are the most threatened groups of herpetofauna, while lizards (12.9 %), worm lizards (8.7 %), snakes (7.5 %), and caecilians (6.1 %) are the least (Table 5).

Nonetheless, when dividing the amount of funding and knowledge (grant-publication index) by the absolute number of threatened species, crocodiles, tuatara, and turtles are proportionally considered better funded and studied groups, while caecilians, snakes, worm lizards, salamanders, lizards, and frogs less so (Fig. 2a). If data-deficient species are also considered, crocodiles, tuatara, and turtles proportionally continue to be the most funded and studied groups of herpetofauna, whilst salamanders, snakes, lizards, worm lizards, frogs, and caecilians the least (Fig. 2b).

Table 1. Number of basic and applied science grants for herpetological research and conservation during 2008 to 2018.

Class	Order	Group	NSF	ARC	RCUK	NSERC	BiodivERsA	Total	Projects %
Amphibia	Anura	Frogs	284	52	96	266	127	825	43.4
Amphibia	Caudata	Salamanders	99	0	12	41	7	159	8.4
Amphibia	Gymnophiona	Caecilians	9	0	0	5	2	16	0.8
Reptilia	Testudines	Turtles	88	8	6	86	50	238	12.5
Reptilia	Squamata	Lizards	243	30	14	64	36	387	20.3
Reptilia	Squamata	Snakes	98	10	12	50	9	179	9.4
Reptilia	Squamata	Worm lizards	2	1	0	2	1	6	0.3
Reptilia	Rhynchocephalia	Tuatara	4	0	0	0	0	4	0.2
Reptilia	Crocodylia	Crocodiles	53	6	3	14	12	88	4.6

NSF - National Science Foundation; ARC - Australian Research Council; RCUK - Research Councils UK; NSERC - Natural Sciences and Engineering Research Council of Canada; Projects % - percentage of grants among herpetological groups

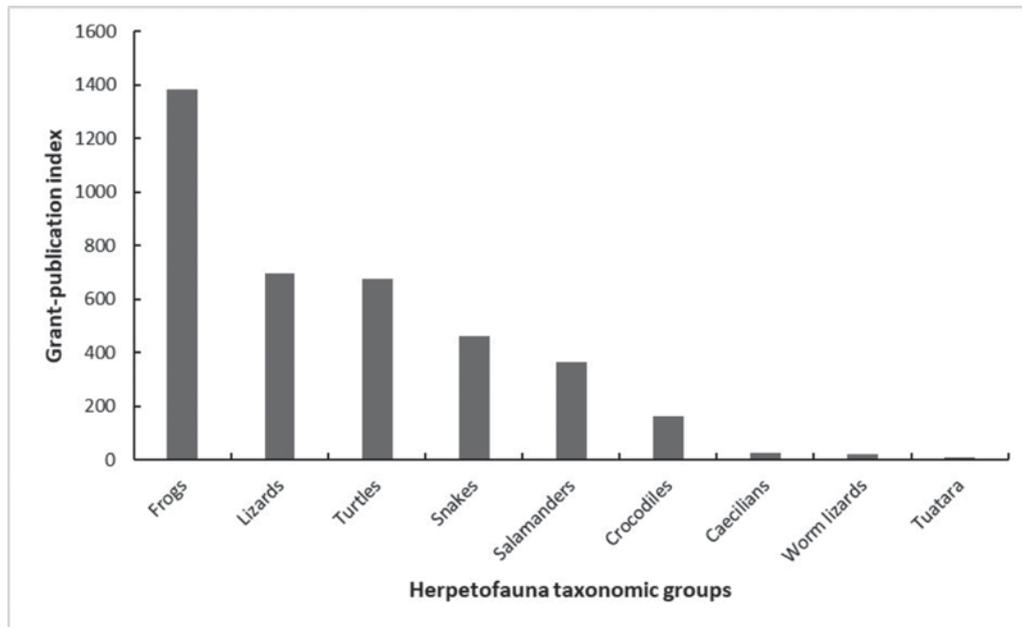


Figure 1. Grant-publication index (during 2008-2018, see Methods) across herpetological taxonomic groups.

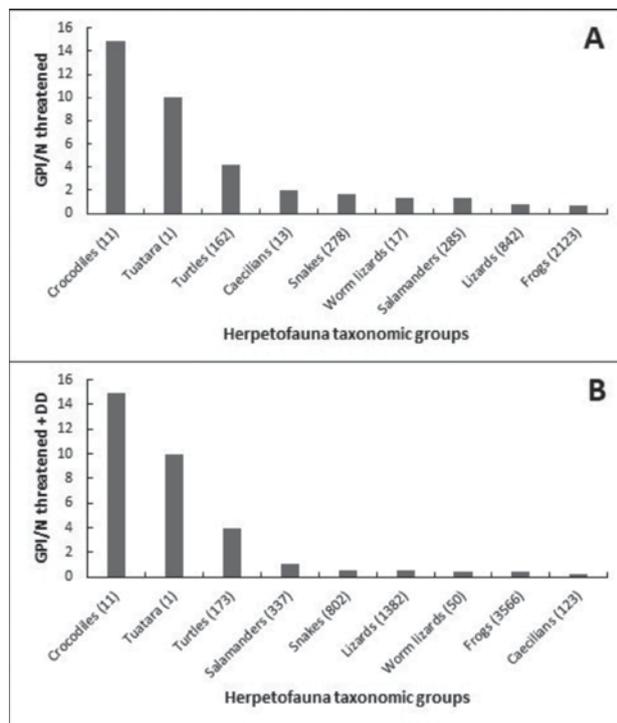


Figure 2. Grant-publication index (during 2008-2018, see Methods) divided by the total number of threatened species (critically endangered, endangered, vulnerable- IUCN), and threatened and data-deficient species (DD), in amphibians and reptiles. In parentheses, total number of threatened species in each group (A), and threatened and data-deficient species (B).

When a finer resolution was used for turtles, dividing up tortoises, marine and freshwater turtles, marine turtles had the highest percentage of threatened species (85.7 %) and attracted more on-ground conservation funding (52.5 %), but had a smaller publication rate (34.4 %) and less basic and applied research funding (31.9 %)

compared to freshwater turtles (40.1 %, 27.5 %, 43.7 %, 61%, respectively) and tortoises (70 %, 20 %, 21.9 %, 7.1 %, respectively). And when the grant-publication index is divided by the total number of threatened species within the turtle subgroups, marine turtles (47.5) are largely better funded and studied than tortoises (3.09) and freshwater turtles (2.51; Table 1, 3, 4, 5). Additionally, considering funds specific for freshwater turtles and tortoises through the Turtle Conservation Fund (see Methods), of a total of 192 proposals, 67.2 % were granted to freshwater turtles and 32.8 % to tortoises.

DISCUSSION

Although setting priorities to protect and fund the most threatened taxa should be the norm, current research on vertebrates show that conservation efforts and biological research are generally biased toward large-bodied and charismatic mammals (Sitas et al., 2009), and common bird species (Roberts et al., 2016), leaving most threatened taxa with little or no biological data to inform their conservation (Roberts et al., 2016). Here, I show that funding and publications are not uniformly distributed among herpetological groups, with frogs, lizards, turtles, and snakes at the forefront of grants awarded and conservation knowledge (i.e., grant-publication index), whilst salamanders and crocodiles are in an intermediate position, and caecilians, worm lizards and tuatara are at the bottom. Even though some of the most threatened groups of herpetofauna showed a small grant-publication index, when the absolute number of threatened and data-deficient species is considered, it demonstrates that, proportionally and in ascending order, salamanders, snakes, lizards, worm lizards, frogs and caecilians are in most need of continued knowledge and on-going funding for their conservation and survival. Potentially, the main driver of the findings in the present study is the description of the chytridiomycosis

Table 2. Amount of funding for basic and applied science grants for herpetological research and conservation during 2008 to 2018*.

Class	Order	Group	NSF	ARC	RCUK	NSERC	Total	Funding %
Amphibia	Anura	Frogs	\$90,632,022.00	\$16,890,638.80	\$34,782,850.17	\$6,505,562.76	\$148,811,073.73	36.0
Amphibia	Caudata	Salamanders	\$51,794,459.00	\$0.00	\$6,788,856.12	\$639,463.24	\$59,222,778.36	14.3
Amphibia	Gymnophiona	Caecilians	\$4,492,686.00	\$0.00	\$0.00	\$102,463.20	\$4,595,149.20	1.1
Reptilia	Testudines	Turtles	\$32,600,709.00	\$1,804,096.00	\$1,388,084.60	\$2,045,102.24	\$37,837,991.84	9.2
Reptilia	Squamata	Lizards	\$75,737,365.00	\$8,617,134.40	\$4,031,933.77	\$1,371,951.24	\$89,758,384.41	21.7
Reptilia	Squamata	Snakes	\$46,914,666.00	\$2,758,161.70	\$4,296,841.80	\$843,004.92	\$54,812,674.42	13.3
Reptilia	Squamata	Worm lizards	\$1,476,018.00	\$184,800.00	\$0.00	\$66,880.00	\$1,727,698.00	0.4
Reptilia	Rhynchocephalia	Tuatara	\$1,626,934.00	\$0.00	\$0.00	\$0.00	\$1,626,934.00	0.4
Reptilia	Crocodylia	Crocodyles	\$13,225,972.00	\$1,257,900.00	\$217,721.18	\$348,032.88	\$15,049,626.06	3.6

* BiodivERsA was not included as the database did not contain grant amounts. All the values were converted to US dollars for comparisons. NSF - National Science Foundation; ARC - Australian Research Council; RCUK - Research Councils UK; NSERC - Natural Sciences and Engineering Research Council of Canada; Funding % - percentage of funding among herpetological groups

Table 3. Number of on-ground conservation grants for herpetology during 2008 to 2018.

Class	Order	Group	CLP	RSG	WFN	NFWF	MBZSCF	NGS	Total	Projects %
Amphibia	Anura	Frogs	22	127	3	124	165	8	449	32.7
Amphibia	Caudata	Salamanders	3	13	1	37	30	0	84	6.1
Amphibia	Gymnophiona	Caecilians	0	4	0	0	5	0	9	0.7
Reptilia	Testudines	Turtles	15	169	9	208	115	3	519	37.8
Reptilia	Squamata	Lizards	1	40	1	9	72	4	127	9.2
Reptilia	Squamata	Snakes	1	31	1	42	36	1	112	8.2
Reptilia	Squamata	Worm lizards	0	1	0	0	2	0	3	0.2
Reptilia	Rhynchocephalia	Tuatara	0	0	0	0	0	0	0	0.0
Reptilia	Crocodylia	Crocodyles	5	36	1	7	21	0	70	5.1

CLP - Conservation Leadership Program; RSG - Rufford Small Grant Foundation; WFN - Whitley Fund for Nature; NFWF - National Fish and Wildlife Foundation; MBZSCF - The Mohamed bin Zayed Species Conservation Fund; NGS - National Geographic Society; Projects % - percentage of grants among herpetological groups

outbreak in late 1990s and the recognition of the global decline of amphibians (Stuart et al., 2004; Ohmer & Bishop, 2011). The breadth and scope of this fungal disease in amphibians is so extensive and fatal (Daszak et al., 2003; van Rooij et al., 2015), that it may have prompted a response by the scientific community to understand the mechanisms involved in the disease (Daszak et al., 2003), which could still reflect the last ten years (2008-2018) of research funding, by the number of publications and basic and applied grants to amphibians in this study, especially for frogs. It has been noted that new scientific discoveries, as in the case of chytridiomycosis, spark ideas and hypotheses which may draw a disproportionate amount of funding and both scientific and public attention (Ohmer & Bishop, 2011). A three-fold increase in scientific knowledge about amphibians was also observed by the number of manuscripts published in wildlife research journals from the 1990s to the 2000s (Christoffel & Lepczyk, 2012). This may be viewed, together with the findings in the present study, as a shift in attention in herpetological research, where previously reptiles (n = 29) had received almost six times more grants than amphibians (n = 5) during 1987-1988 by U.S. funding agencies, and reptiles (n = 42 and n = 6) were more commonly featured than amphibians (n = 27 and n = 1) in general ecology and wildlife ecology journals during 1983-1988, respectively

(Gibbons, 1988). Also, over a 30-year period (1980-2010), reptile publications (n = 202) were much more commonly featured than amphibians (n = 95) within six wildlife research journals (Christoffel & Lepczyk, 2012), as number of papers per taxonomic group followed the order: turtles (n = 84), squamata (lizards and snakes; n = 78), frogs (n = 57), crocodyles (n = 30), salamanders (n = 19), and worm lizards, tuatara and caecilians had no studies (Christoffel & Lepczyk, 2012). Interestingly, I showed that proportionally to the total number of frogs in risk of extinction, continued funding and conservation knowledge are needed to protect frogs worldwide (Table 3, Fig. 2), and help to halt the effects of chytridiomycosis. Although frogs had the highest grant-publication index, they did not secure the greatest number of on-ground conservation grants. Turtles, especially marine turtles, have received the majority of on-ground conservation funding by the agencies examined in this study. This could be due to the high degree of threat faced by marine turtles (85.7 % threatened; IUCN, 2019), their global distribution, in addition to their charismatic profile (McClenachan et al., 2012), helping to yield public attention and funding. Even though freshwater turtles and tortoises face an unprecedented threat worldwide (40.1 % and 70 %, respectively; IUCN, 2019), they do not garner the same levels of funding specific for conservation, and compared to the number of threatened species, they are

Table 4. Publication rates in herpetology in leading conservation journals (Conservation Biology, Biological Conservation, Biodiversity and Conservation, Animal Conservation), high impact journals (Nature, Science, Proceedings of the National Academy of Science of the United States of America, and Global Change Biology), and major herpetological journals (*Herpetologica*, *Amphibia-Reptilia*, *Journal of Herpetology*, *Copeia*, *The Herpetological Journal*, and *African Journal of Herpetology*) during 2008 to 2018.

Class	Order	Group	C. J. (n, % ¹)	H.I.J. (n, % ¹)	H.J. (n, % ¹)	Total (n, % ²)
Amphibia	Anura	Frogs	263 (39.8)	249 (35.1)	982 (33.1)	1494 (34.4)
Amphibia	Caudata	Salamanders	65 (9.8)	75 (10.6)	350 (11.8)	490 (11.3)
Amphibia	Gymnophiona	Caecilians	4 (0.6)	3 (0.4)	19 (0.6)	26 (0.6)
Reptilia	Testudines	Turtles	190 (28.7)	62 (8.7)	347 (11.7)	599 (13.8)
Reptilia	Squamata	Lizards	68 (10.3)	142 (20.0)	666 (22.4)	876 (20.2)
Reptilia	Squamata	Snakes	42 (6.4)	123 (17.3)	467 (15.7)	632 (14.6)
Reptilia	Squamata	Worm lizards	3 (0.5)	2 (0.3)	32 (1.1)	37 (0.9)
Reptilia	Rhynchocephalia	Tuatara	7 (1.1)	2 (0.3)	7 (0.2)	16 (0.4)
Reptilia	Crocodylia	Crocodiles	19 (2.9)	51 (7.2)	100 (3.4)	170 (3.9)

C. J. - Conservation Journals; H.I.J. - High Impact Journals; H.J. - Herpetological Journals; n = number of publications; %¹ = percentage within journal group; %² = total percentage

Table 5. Number of threatened and data-deficient amphibians and reptiles worldwide (CR – critically endangered; EN – endangered; VU – vulnerable; DD – data-deficient; IUCN 2019).

Class	Order	Group	CR	EN	VU	Total threatened*	DD	N.Species**	Threatened %	DD %	TDD %***
Amphibia	Anura	Frogs	567	924	632	2123	1443	7062	30.1	20.4	50.5
Amphibia	Caudata	Salamanders	79	111	95	285	52	736	38.7	7.1	45.8
Amphibia	Gymnophiona	Caecilians	1	8	4	13	110	212	6.1	51.9	58.0
Reptilia	Testudines	Turtles	50	45	67	162	11	351	46.2	3.1	49.3
Reptilia	Squamata	Lizards	175	346	321	842	540	6512	12.9	8.3	21.2
Reptilia	Squamata	Snakes	56	115	107	278	524	3709	7.5	14.1	21.6
Reptilia	Squamata	Worm lizards	5	8	4	17	33	196	8.7	16.8	25.5
Reptilia	Rhynchocephalia	Tuatara	0	0	1	1	0	1	100.0	0.0	100.0
Reptilia	Crocodylia	Crocodiles	7	0	4	11	0	24	45.8	0.0	45.8

* CR + EN + VU

** Number of species per group obtained for amphibians (Frost, 2019) and reptiles (Uetz et al., 2018) *** Threatened species (%) + DD species (%)

almost 19 and 15 times, respectively, less studied and funded than marine turtles. In terms of funding specific for tortoises and freshwater turtles only, tortoises are two times less funded than freshwater turtles. This suggests that even though turtles as a taxonomic group are well studied and funded (Fig. 2), proportionally, the majority of these resources are channelled to marine turtles. Contrary to examples of marine turtle recoveries (Balazs & Chaloupka, 2004), freshwater turtles and tortoises have shown no sign of resilience in the wild, and overharvesting and habitat degradation are currently the main threats (van Dijk, 2000; Turtle Taxonomy Working Group, 2014; Nijman & Shepherd, 2015).

It has been noted that there is a decrease in number of manuscripts published on natural history and field ecology studies in herpetological journals, which are essential to comprehend the crisis facing many herpetofauna (McCallum & McCallum, 2006). There is also evidence that changes in the priorities by funding agencies could play a role, owing to the rise of modern molecular and mathematical techniques, such as the case of the National Science Foundation establishing programmes to boost systematics training after a steep decline in this

research area (McCallum & McCallum, 2006). Perhaps such trends in the decrease of field studies and increase in cutting-edge research being funded by agencies could be reflected in the present findings, in terms of grant-publication index, where several groups have fallen behind frogs, lizards, turtles and snakes, especially the more cryptic groups, such as worm lizards and caecilians (Fig. 1). Additionally, it is interesting to note that there were several similarities in the order and proportion of publications in herpetological, conservation and high impact journals among groups in this study (Table 4).

One major finding was that researchers conducting studies on frogs, turtles, lizards and snakes publish at similar rates in herpetological and high impact journals, whilst in conservation journals, frogs, turtles and lizards tend to be the most featured groups (Table 4). In the case of the least published groups, tuatara had more studies featured in conservation journals, worm lizards were more featured in herpetological journals than high impact and conservation ones, while caecilians were similarly featured in all journal groups (Table 4). Considering that many species in the data-deficient category could be classified as threatened (Morais et

al., 2013; Howard & Bickford, 2014), it is alarming that worm lizards and caecilians are facing increasing threats (Gower & Wilkinson, 2005; Colli et al., 2016) but are not adequately funded or studied (Fig. 2b, Table 4), consequently they should be considered as high priority. Potential reasons for their inattention by funders and researchers may be that worm lizards and caecilians are mainly found in developing countries where there is less funding for research (Fazey et al., 2005), and their cryptic habits make them harder to sample (Gower & Wilkinson, 2005; Colli et al., 2016).

Besides common threats for amphibians (Gibbons et al., 2000), salamanders are not only exposed to the chytrid fungus *Batrachochytrium dendrobatidis*, which has caused mortalities in all amphibian orders (van Rooij et al., 2015), but also by another chytrid fungus which is specific to salamanders and newts (*B. salamandrivorans*; Martel et al., 2013; van Rooij et al., 2015). Both *B. dendrobatidis* and *B. salamandrivorans* have been linked to the decline and are recognised as a conservation threat to several salamander species (Cheng et al., 2011; Martel et al., 2014). In the present study, I show that salamanders are highly threatened (Table 5) and in need of more funding for research, on-ground conservation and recovery programmes (Fig. 1, 2).

Among Squamata, lizards have the highest grant-publication index and on-ground conservation funding. Lizards (12.9 %, IUCN, 2019) are slightly more threatened than worm lizards (8.7 %, IUCN, 2019) and snakes (7.5 %, IUCN, 2019), nonetheless when the number of threatened and data-deficient species is considered in relation to the grant-publication index, these three groups are still in need of more conservation funding and attention (Fig. 2b). Life-history traits of many lizards, such as high fecundity, short generation times, and high population densities can make them less susceptible to declines from anthropogenic factors as they may be able to rebound quickly (Todd et al., 2010). However, lizard species characterised by endemism, restricted geographic ranges, large body size, late maturity and long lives are more prone to population declines and endangerment (Todd et al., 2010). Despite snakes not presenting an overall high degree of threat, studies have indicated that several species not listed as threatened share ecological traits of threatened groups (Reed & Shine, 2002), or could have their status reviewed from data-deficient to threatened (Maritz et al., 2016). In addition, extinction risk may be underestimated owing to a lack of population information (Böhm et al., 2013).

Crocodiles and tuatara are considered the most studied and funded groups of herpetofauna, in relation to the number of threatened species they possess (Fig. 2). Even though they are relatively well researched and have some success stories in terms of recovery (Nelson et al., 2002; Gibbons et al., 2000; Todd et al., 2010), they are still highly threatened and have particularities that deserve monitoring. For example, tuatara is the sole remnant of the order Rhynchocephalia and is endemic to New Zealand (Daugherty et al., 1990; Hay et al., 2010). According to the present study, tuatara is the most threatened group of herpetofauna, has the lowest grant-

publication index, and did not procure any on-ground conservation grants included in this study. It is possible that the grant-publication index and conservation grants are underestimated for tuatara for two reasons. First, tuatara is a species that endemic to New Zealand, unlike other herpetological groups that have greater global distribution. Second, on-ground conservation agencies analysed for this study fund mainly projects in developing countries. In fact, most of tuatara funding and recovery plans are sponsored by the New Zealand government (Towns et al., 2001). On the other hand, tuatara risk of extinction is high as many populations live on small islands and are declining despite absolute protection (Daugherty et al., 1990). Despite intensive hunting pressure on crocodilians during the mid to late 20th century, protection measures and management programmes were established during the 1970s, and for most species for which habitat loss was not a significant threat factor, there were many cases of population recovery (Thorbjarnarson, 1999; Gibbons et al., 2000; Todd et al., 2010). Nonetheless, for some species, viable populations are no longer extant in the wild, such as the Siamese crocodile (Platt & Ngo, 2000), or have scattered and isolated populations, such as the Indian gharial (Gad, 2008) and Chinese alligator (Thorbjarnarson et al., 2002). Potentially, a limitation in the present study was that certain taxa lend themselves more to addressing fundamental questions in ecology and evolution (e.g. short-lived taxa compared with long lived taxa). Consequently, frogs and lizards (Hopkins, 2007; Losos, 2009) could attract more funding (e.g. basic and applied science grants) compared to turtles, crocodiles and tuatara, for example. However, on-ground conservation grants should be independent of this aspect. Perhaps another caveat was that the funding metrics were based on agencies in developed countries and six conservation grant providers focusing mainly on developing countries. This should be a concern for herpetological groups with restricted distribution, as previously discussed in the case of tuatara. Even though the bulk of herpetofaunal biodiversity is found in tropical regions in developing areas of the world (Stuart et al., 2004; Böhm et al., 2013), the majority of groups are distributed globally and the collaborative nature of research projects, nationally and internationally, among universities (Grueber & Studt, 2011; Suresh, 2012), and the range of projects supported by on-ground conservation agencies in several countries investigated in this study, should represent an appropriate coverage of herpetological research. For example, within the NSF grants for both amphibians and reptiles investigated in this study, 5.8 % were specifically targeted to the Office of International Science and Engineering (OISE), which fosters international collaboration. Still, many more grants from different NSF programmes researched here involved the study of herpetofauna in developing and tropical countries.

Another aspect to be mentioned is that countries such as the U.S., Canada, Australia, Costa Rica and South Africa have specific policies to protect critical habitat, establish recovery programmes and mandate work to prevent extinction in threatened species (Waples et al., 2013).

Though some of the countries where funding agencies investigated here operate do not have mechanisms such as the Endangered Species Act (Waples et al., 2013), the majority of countries do have national laws to protect threatened species (www.bagheera.com/endangered-species-laws/), hence national red lists and the IUCN Red listing are important mechanisms for researchers and wildlife managers to attract funding to study and protect threatened wildlife (Rodrigues et al., 2006). The bulk of the research output investigated here was not necessarily being directly funded by the grant agencies searched in this study. Investigators funded by the research funding agencies are expected to promptly have their results disseminated and published, while the on-ground conservation agencies only encourage the grantees to have their results published, and this difference can account for some of the differences in publication rates. Despite this fact, the intention was to have a systematic approach to understand funding levels and knowledge within herpetofauna groups, and by examining a diverse set of granting bodies and journals, this should generally represent a reliable overview of grants and knowledge for amphibians and reptiles worldwide.

CONCLUSIONS

This manuscript aimed to demonstrate strengths and weaknesses in herpetological research and conservation (as represented by fourteen research journals, five research funding agencies, and six on-ground conservation agencies) and shed some light on the groups needing action. I was able to substantiate a continued shift in attention in herpetological research owing to the emergence of the chytridiomycosis as other authors have (e.g. Christoffel & Lepczyk, 2012), the imbalance of funding and scientific information among groups and that degree of threat does not always translate into enough grants and publications.

The main message of this manuscript is that funding should increase as a whole for herpetofauna conservation and biology. This argument is based on the degree of threat faced by several groups of amphibians and reptiles, which is comparatively, more than birds (ca. 13 %), mammals (ca. 21–25 %), and cartilaginous and bony fishes (ca. 17–31 %; Turtle Taxonomy Working Group, 2014). Research, funding and management efforts of amphibians and reptiles have historically lagged behind those of other vertebrates which have a high economic value or are considered pests, such as game species of large mammals, birds and fishes (Gibbons, 1988; Christoffel & Lepczyk, 2012). Making studies more representative at the current insufficient level of funding will not have as substantial an impact as increasing funding generally. By securing more funding for herpetofauna from funding agencies, governments, universities, NGO's and citizen science programmes, it would be possible to continue research on high quality projects on more commonly studied taxa; protection of highly threatened groups of herpetofauna, such as tuatara, crocodiles and marine turtles; and more focus on groups that proportionally have fewer funds and

less knowledge in relation to the overall number of threatened and data-deficient species, such as caecilians, frogs, worm lizards, lizards, snakes, and salamanders (Fig. 2b), in addition to tortoises and freshwater turtles.

Another example to be followed by governments is of employing herpetologists as done by the U.S. Department of the Interior, where science can foster knowledge and protection of amphibians and reptiles (Lovich et al., 2012). Also, it is important to consider more research on the usefulness and effectiveness of species of reptiles and amphibians as “umbrella” and/or “flagship” species (Simberloff, 1998; Rondinini & Boitani, 2006; Kalinkat et al., 2017), considering their ecological similarities and shared habitats (Vitt & Caldwell, 2014). Finally, a follow-up study could investigate which categories of projects are being funded in herpetology. For example, a break down into categories (such as behaviour; ecology; distribution; disease; conservation evidence; evolution; physiology) could help to understand which study areas are currently more active and where more attention should be focused.

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APPENDIX

List of conservation organisations involved in herpetological conservation worldwide: Amphibian Survival Alliance, Partners in Amphibian and Reptile Conservation, Amphibian and Reptile Conservation Trust, Save the Frogs!, Turtle Survival Alliance, Turtle Conservancy, The Nature Conservancy, Conservation International, Wildlife Conservation Society, World Wildlife Fund, Durrell Wildlife Conservation Trust, and Disney Conservation Fund, among others.

Priorities for research and action are determined by a range of agencies, in particular the IUCN Species Survival Commission networks, such as Amphibian Specialist Group, Anole Lizard Specialist Group, Boa and Python Specialist Group, Chameleon Specialist Group, Crocodile Specialist Group, Iguana Specialist Group, Marine Turtle Specialist Group, Monitor Lizard Specialist Group, Sea Snake Specialist Group, Snake and Lizard Red List Authority, Tortoise and Freshwater Turtle Specialist Group, and the Viper Specialist Group.

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Spatial patterns of snake diversity in an urban area of north-east Brazil

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The distribution of animal populations within an assemblage includes a wide variety of patterns, which are fundamental to understanding population dynamics and aid in conservation actions. We examined a snake assemblage in an urban area to describe species distribution patterns and to identify which places are more likely to contain snakes, both venomous and non-venomous. The study was conducted in the urban area of the municipality of Rio Tinto in north-east Brazil. We used a geostatistical modelling technique called ordinary kriging to identify which places were more likely to contain snakes, and a statistical spatial method (average nearest neighbour distance) to detect distribution patterns of snake species within the study area. A total of 291 individuals distributed among 28 species were recorded. The snakes were found in streets, homes, churches, university campus, streams, and even in local supermarkets. Ordinary kriging showed that the area of distribution of individuals was concentrated at three distinct points located in the centre of the urban area. The significant results of the average nearest neighbour distance analysis showed a clustered distribution for two species and dispersed distributions for eight species. Information on urban sites where snakes are more likely to be found is important not only for conservation, but also to help local citizens better understand and live amongst snakes.

Keywords: geostatistical, kriging, urban herpetology, interpolation, urbanisation

INTRODUCTION

The anthropogenic transformation of natural ecosystems is a serious threat to worldwide biological diversity (Hamer & McDonnell, 2010), as it is a major cause of species extinction (McKinney, 2006). As the human population grows and urban areas expand, original habitats are lost, fragmented, or modified, reducing local biodiversity. However, few studies have been conducted on wildlife in urban environments, especially snakes (Mitchell et al., 2008). Furthermore, even when conducted in urban areas, most studies focus on protected areas instead of the surrounding urban matrix. Indeed, very few studies have been conducted in areas with intense anthropogenic influence, particularly in South and Central America (Mitchell & Jung Brown, 2008; Rojas-Morales, 2012).

Studies on snake distribution patterns in urban areas can help both snakes and people. This knowledge is essential to assist the local population, since the places where venomous species are found will be known; also, it is possible to prevent and support local medical staff in cases of snakebite accidents (Brites & Bauab, 1988). Additionally, these studies allow us to evaluate the degree of plasticity of species, pointing out which species

are restricted to preserved areas and which can deal with the alteration of the natural environment (Barbo, 2008). In Brazil, some studies have addressed the ecology of snakes inhabiting urban areas (e.g. Silva & Ferreira, 1988; Brites & Bauab, 1988; Marques et al., 2009; Barbo et al., 2011; França et al., 2012), while others only mentioned species found in these anthropic environments (e.g. São Pedro & Pires, 2009; Bernarde & Machado, 2002; Santos et al., 2005; Costa et al., 2010). Furthermore, most studies were restricted to south and south-east Brazil, and almost none were conducted in north-east Brazil (but see Sales et al., 2009; França et al., 2012).

The distribution of animal populations includes a wide variety of patterns, and although they are key to understanding a population's ecology, they are extremely difficult to describe precisely (Clark & Evans, 1954). Once distribution patterns are found and described, it is also important to discover which factors are responsible for each pattern and what mechanisms produce and maintain them (Levin, 1992).

Methods using modelling have become important tools to understand the spatial distribution of species (Jenkins et al., 2009). Several approaches to modelling have been used to determine variations in the spatial distribution of snakes, such as logical approaches (rule-

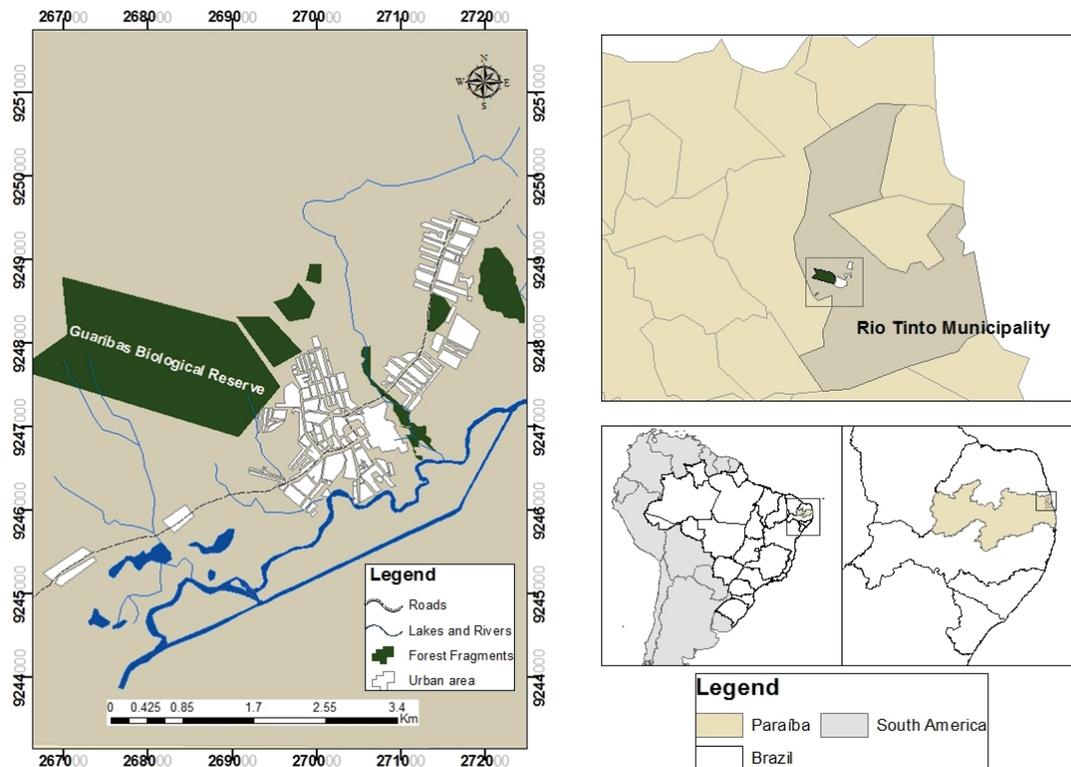


Figure 1. Study area showing the northern coast of Paraíba State, underscoring the urban area of Rio Tinto municipality and the Guaribas Biological Reserve.

based; e.g. Scott et al., 2002); statistical approaches (trapping/observation probability; e.g. Peterson et al., 2002), Mahalanobis distances (e.g. Browning et al., 2005), logistic regression (e.g. Cooper-Doering, 2005), Euclidean distance analysis (e.g. Bieser, 2008; Degregorio, 2008), and geostatistical approaches (kriging; e.g. Lee & Peterson, 2003). Among these, the geostatistical approach is different from the others because it does not require habitat information to determine the potential distribution of species (Jenkins et al., 2009). Furthermore, while most traditional statistical techniques assume data independence, the geostatistical approach is based on the principle that the difference in the values of a given variable at two points depends on the distance between them (Dal Prá et al., 2011). Although Lee & Peterson (2003) showed that geostatistical models are more suitable for determining the potential distribution of some snake species than models that use information on habitats, few studies have used this analysis for snakes (e.g. Brito et al., 2008; Martínez-Freiria et al., 2009; Lee & Peterson, 2003), and none of these were conducted with Brazilian snake populations.

The aim of this study was to examine the spatial distribution of the snake assemblage in the urban area of the municipality of Rio Tinto, north-east Brazil, using a geostatistical modelling technique and statistical spatial method to describe the distribution pattern of the species in the area. We tested the hypothesis that snakes should present a clustered distribution because they use only suitable spots in the urban matrix. Finally, we identified which places are more likely to contain snakes, both venomous and harmless to humans.

METHODS

Study area

The spatial distribution of snakes in the assemblage was studied between August 2009 and September 2012 in the urban area of the Rio Tinto municipality (06°48'10"S and 35°04'51" W), north-east Brazil (Fig. 1). The Rio Tinto municipality covers approximately 466 km² and has a humid tropical or non-arid climate (Aw in the Köppen classification), with a rainy season between February and October and average annual precipitation of 1.634 mm (Beltrão et al., 2005). The urban area of Rio Tinto is located in the centre of the municipality. At the north-west of the urban area, there is a 327.0 ha patch of protected area (Guaribas Biological Reserve) (Fig. 1) with two different types of forest formations: a forested habitat (Floresta Estacional semidecidual) and an arboreous open savanna (Tabuleiro) (Oliveira-Filho & Carvalho, 1993; Mesquita et al., 2018). It is considered a refuge for local biodiversity.

The Rio Tinto municipality is located in the Brazilian Atlantic Forest. It has approximately 24,000 human inhabitants and has suffered from increasing urbanisation since 1920s, when a Swedish family (Lundgren family) decided to install a textile factory in the city (the Companhia de Tecidos Rio Tinto) (Rosa, 2010). There are 28 species of snakes known for Rio Tinto, which also has the second highest rate of accidents caused by the genus *Bothrops* in the state of Paraíba (Albuquerque et al., 2005).

Sample collection

Snakes were collected in the urban area of the

municipality of Rio Tinto through active searches and occasional encounters with the human population. Active searches were conducted four times a month, two hours each, by two or three researchers who searched for snakes on roads, rivers, vacant lots, and forest areas along the Rio Tinto urban from August 2009 to September 2012. These were previously known sites to find snakes. Local collectors provided additional records of snakes during this period. We informed the general populace about our research through community radio (once a week). We did not encourage people to search for, catch, or kill snakes, but usually when local residents find these animals in the urban area, they do kill them (França et al., 2012). We simply reported our phone numbers to local residents and asked them to contact one of our researchers to collect the animal. In addition, we also visited local community leaders and presented our research, requesting them also to call if there were any snakes in the area.

GIS Analyses

Each individual found was identified to the species level, and its geographic location recorded with a GPS unit. The perimeter of the study area was delimited using the SUDENE SB 25-Y-A-V4 NE (1974) map and a map of the Rio Tinto municipality (2005). The area was georeferenced and divided into 100 x 100 m regular grids using ArcGIS 10.1 software developed by ESRI (2004), and all individuals present in each grid were counted.

Ordinary kriging

We used the spatial interpolation method called ordinary kriging, which uses mathematical functions to add more weight to the closest sample points and less weight to more distant sample points. From these data, new points are created based on these linear combinations. This procedure allows the generation of continuous surfaces from punctual sampling units. This analysis included all snake individuals and was not separated by species, as the purpose was to determine the points in the urban area where we could find more snakes. The data were run in the Geostatistical Analyst extension of ArcGIS 10.1 software (ESRI, 2004).

Before executing the kriging, we analysed the spatial variability of the data. This was achieved by examining the semivariogram, which is a graphical representation of the semivariances and the distances between sampled points. ArcGIS 10.1 uses a least squares method to fit the models, and as a criterion for model selection it uses the coefficient of determination and the sum of squares of residuals. From this procedure, the exponential model was adjusted to the interpolation of variables and acquisition of their parameters (nugget effect, partial sill, and major range). These parameters were used to create a map of snake density through ordinary kriging. The major range was also used to determine the appropriate distance between sampling points, as it represents the influence of the point on its neighbours. Thus, variables located at distances greater than the range have random spatial distribution and are considered independent between locations (Ge et al., 2005). As the maximum range found

was 220.23 m, the dimensions of the sampling grid (100 x 100 m cells) yielded an appropriate and qualified accurate detection of the snake distributions.

Average Nearest Neighbour Distance

We conducted the average nearest neighbour distance test using the Spatial Statistical Tools extension of ArcGIS 10.1 to detect the distribution patterns of 20 snake species within the study area. This tool is expressed as the ratio between the observed distance among individuals and the expected distance. The expected distance is the mean distance between neighbours in a hypothetical random distribution. This analysis provides three values: the index of the nearest neighbour (R-value), the value of z , and the p -value. A clustered distribution has an R-value < 1 , while a dispersed distribution has an R-value > 1 . If the R-value = 1, the default is considered random distribution. A z -value is calculated, which is compared to a critical z for the appropriate N and determines whether or not to reject the null hypothesis that says that distribution among individuals of each species within the area is random. If the p -value is too small ($p < 0.01$), it is very unlikely that the observed spatial pattern is the result of random processes, and thus the null hypothesis is rejected.

The minimum number required for analysis is three individuals; thus, the species *Apostolepis cearensis* Gomes, 1915, Boa constrictor, *Drymoluber dichrous*, *Erythrolamprus almadensis*, *Sibon nebulatus*, *Spilotes pullatus*, *Spilotes sulphureus*, *Taeniophallus occipitalis*, which were also found in urban areas but had fewer than three individuals and were excluded from the analysis.

RESULTS

We recorded 291 specimens distributed among 28 species, 22 genera, and 4 families (Boidae, Colubridae, Elapidae, and Viperidae) from the urban area of Rio Tinto. The most abundant species were *Micrurus* aff. *ibiboboca* ($N = 43$), *Helicops angulatus* ($N = 41$), and *Bothrops leucurus* ($N = 36$). Snakes in this area were found in streets, houses, churches, at a university campus, streams, and even within local supermarkets. The venomous species that can cause lethal snakebites are from the families Elapidae and Viperidae. The points of occurrence of the snakes are shown in the map (Fig. 2). The significant results of the average nearest neighbour distance analysis indicated that *B. leucurus* and *Philodryas olfersii* had clustered distribution patterns, while *Epicrates assisi*, *H. angulatus*, *Erythrolamprus poecilogyrus*, *Oxyrhopus trigeminus*, *Philodryas patagoniensis*, *P. nattereri*, *Xenodon merremii*, and *M. aff. ibiboboca* presented dispersed distributions. Index values of the nearest neighbour (R), z -values, and corresponding p -values for each species are shown in Table 1.

The population map was prepared using the density of snakes, which ranged from 0–14 per grid (Fig. 3), and it demonstrated that the individuals' distribution area focused on three distinct points (in circles on the map). They are located in the centre of the urban area.

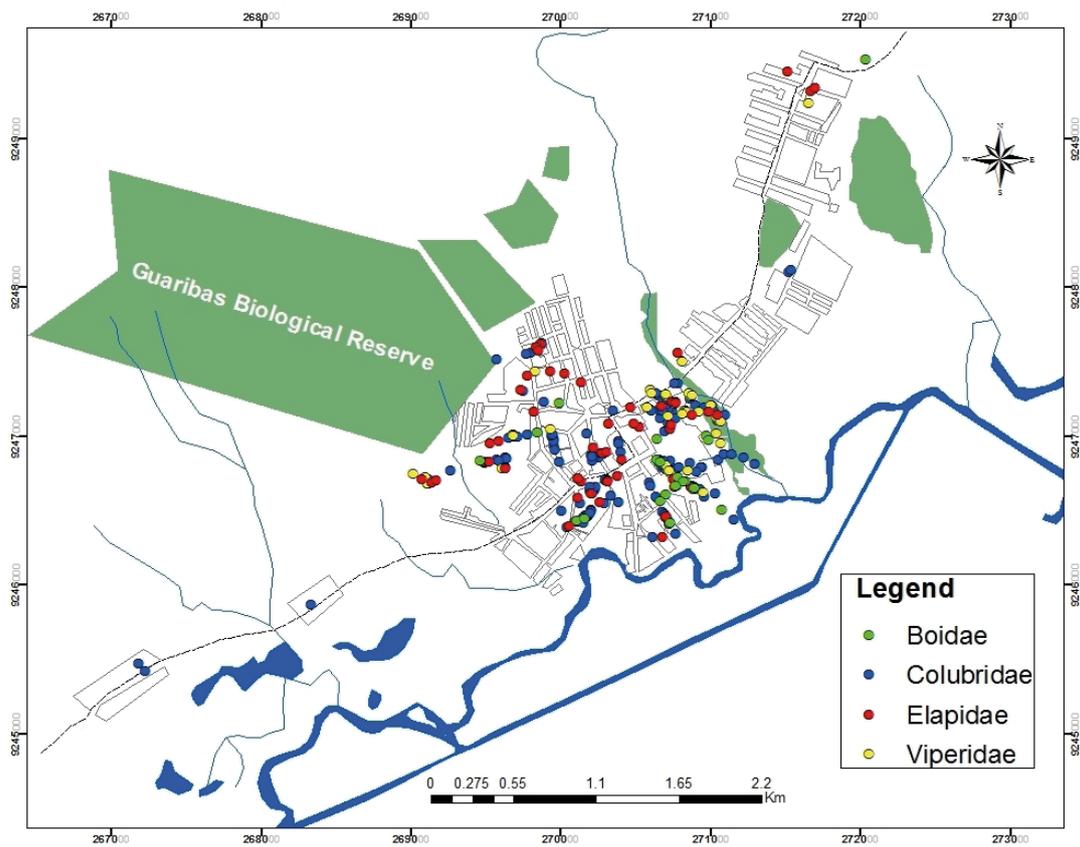


Figure 2. Distribution of snakes from the families Boidae, Colubridae, Elapidae, and Viperidae in the Rio Tinto urban area.

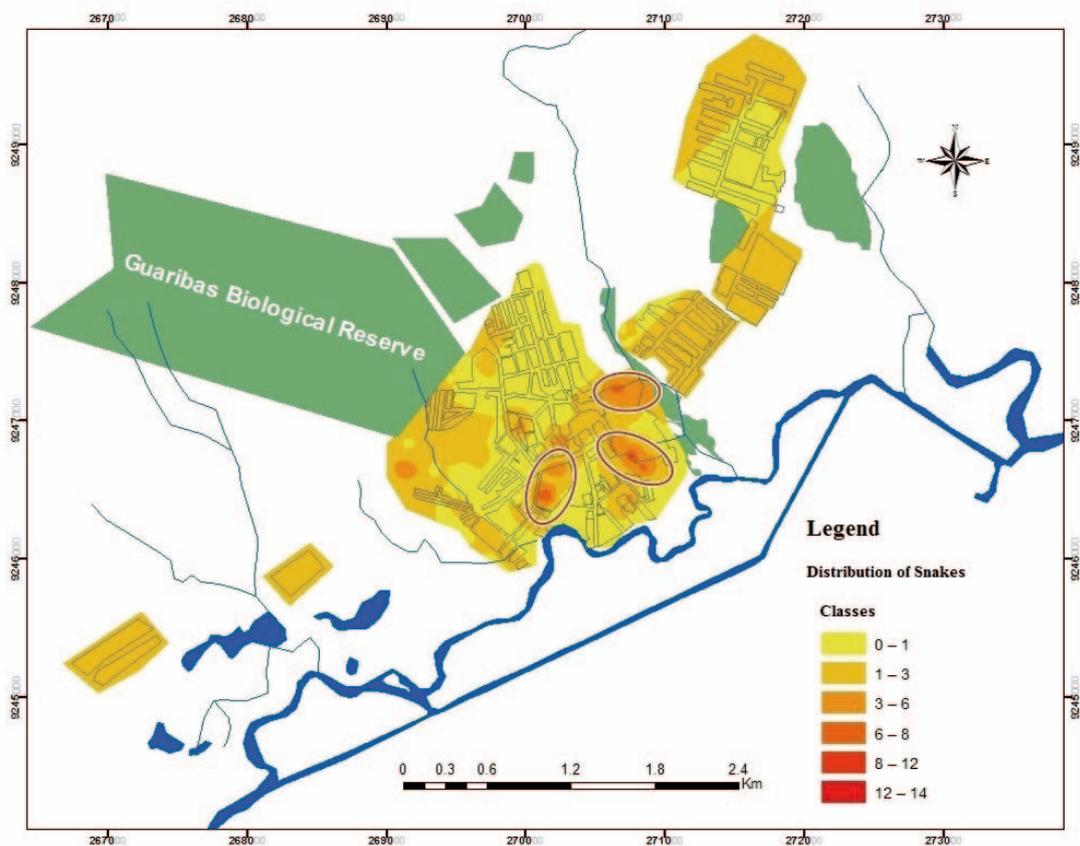


Figure 3. Spatial distribution of snakes in urban areas of the Rio Tinto municipality based on ordinary kriging. The darker colours represent points of higher probability of finding snakes, and the classes represent the number of individuals.

Table 1. Results of the average nearest neighbour distance analysis, where n = number of individuals, p = probability value, pattern = type of pattern shown by each species, R = the index of the nearest neighbour, and z = statistical significance

Family/Species	n	R	z	p	Pattern
Boidae					
<i>Epicrates assisi</i> (Machado, 1946)	22	29.37	254.57	< 0.01	Dispersed
Colubridae					
<i>Chironius flavolineatus</i> (Jan, 1863)	8	0.69	1.68	< 0.1	Clustered
<i>Dipsas mikanii</i> (Schlegel, 1837)	10	0.79	-1.27	< 0.1	Clustered
<i>Erythrolamprus poecilogyrus</i> (Wied, 1825)	9	2.11	6.39	< 0.01	Dispersed
<i>E. taeniogaster</i> (Jan, 1863)	6	0.46	-2.57	< 0.05	Clustered
<i>Helicops angulatus</i> (Linnaeus, 1758)	41	1.29	3.54	< 0.01	Dispersed
<i>Leptophis ahaetulla</i> (Linnaeus, 1758)	10	1.16	1	< 0.1	Random
<i>Lygophis dilepis</i> (Cope, 1862)	5	0.75	-1.08	< 0.1	Clustered
<i>Oxybelis aeneus</i> (Wagler, 1824)	4	0.69	-1.2	< 0.1	Clustered
<i>Oxyrhopus petolarius</i> (Linnaeus, 1758)	4	0.4	-2.31	< 0.05	Clustered
<i>O. trigeminus</i> Duméril (Bibron & Duméril, 1854)	10	1.64	3.88	< 0.01	Dispersed
<i>Philodryas patagoniensis</i> (Girard, 1858)	17	1.46	3.65	< 0.01	Dispersed
<i>P. nattereri</i> Steindachner, 1870	4	3.75	10.53	< 0.01	Dispersed
<i>P. olfersii</i> (Lichtenstein, 1823)	17	0.59	-3.21	< 0.01	Clustered
<i>Psomophis joberti</i> (Sauvage, 1884)	3	1.57	1.9	< 0.1	Dispersed
<i>Pseudoboa nigra</i> (Duméril, Bibron & Duméril, 1854)	12	0.81	-1.17	< 0.1	Clustered
<i>Xenodon merremii</i> (Wagler, 1824)	6	2.09	5.12	< 0.01	Dispersed
Elapidae					
<i>Micrurus</i> aff. <i>ibiboboca</i>	43	1.85	10.65	< 0.01	Dispersed
<i>M. potyguara</i> Pires, Silva, Feitosa, Prudente, Pereira-Filho & Zaher, 2014	11	1.15	0.97	< 0.1	Random
Viperidae					
<i>Bothrops leucurus</i> (Wagler, 1824)	36	0.65	-4.01	< 0.01	Clustered

DISCUSSION

In this study, we observed that the points of highest occurrence of snakes were near rivers. This fact might be linked to the abundance of food that these places offer. The rivers are surrounded by green areas, and these areas maintain other populations of animals, such as frogs and small mammals, which are potential prey for snakes. Conversely, forested areas concentrate a large number of species but decrease the rate of encounters with snakes by the human population, which is probably caused by the permanence of snakes in their microhabitats in the forests (Barbo, 2008).

Due to the constant loss of natural environments, squamate species have been forced to expand their home range into other areas, including urban areas (Mitchell & Brown, 2008). Groves, vacant lots, back yards of houses, and rivers are some of the sites that act as refuges for these animals, providing shelter and food (Barbo, 2008; Brites & Bauab, 1988; Puerto et al., 1991). Thus, encounters between people and snakes are more likely near these places. Most snake species found in the urban area of Rio Tinto are not venomous (França et al., 2012) and therefore do not present a risk to humans. However, there are dangerous species from the families Elapidae and Viperidae that can cause lethal snakebites (Cardoso et al., 2003). This scenario is aggravated because some of these species have high abundance in the urban areas (França et al., 2012).

The presence of snakes of genus *Bothrops* in urban areas of Brazilian municipalities has often been documented (Barbo et al., 2011; Brites & Bauab, 1988; Puerto et al., 1991; Carvalho & Nogueira, 1998; Costa et al., 2010; Marques et al., 2009; Sao Pedro & Pires, 2009). This can be due to their feeding habits, as most *Bothrops* species feed mainly on small rodents, which are abundant in urban areas. Individuals of this species were captured near residences but were generally more restricted to sites within the urban area near fragments of natural habitats. *Bothrops* individuals recorded by Puerto et al. (1991) and Barbo (2008) in urban areas of the municipality of São Paulo and by Brites & Bauab (1988) in urban areas of the municipality of Uberlândia were also associated with forest fragment. This indicates that although *Bothrops* species are often recorded in urban areas, these snakes are not as tolerant to anthropogenic environments, given that they appear in urban areas associated to patches of natural habitats. Thus, the distribution pattern found may be related to environmental conditions such as temperature, humidity, and vegetation cover, rather than solely because of prey availability.

The genus *Micrurus*, which is ecologically different from *Bothrops*, showed a wide distribution in the urban area of Rio Tinto, and some individuals were even collected within homes, churches, and local markets. This was also recorded by Brites & Bauab (1988), who found specimens deeper into the urban matrix. These snakes seem to have a high degree of environmental

plasticity, living well both in natural areas and in anthropogenic environments. The abundance of coral snakes in the wild is still poorly understood; however, it is certain that associative aspects of soil, vegetation, temperature, precipitation, and humidity influence their abundance, as well as the availability of food (Marques et al., 2006; Almeida et al., 2016). One reason that may favour the wide distribution of elapids in urban areas is their ophiophagous habit. Lema et al. (1983) noted that *Micrurus* are usually collected in places where an abundance of other snakes is found.

Despite the fact that it was more widely distributed in the urban area of Rio Tinto, the genus *Micrurus* did not exhibit aggressive behaviour, and this may explain the absence of accidents involving these snakes in the region. Still, their abundance and proximity to human population needs further attention, as it is a highly venomous species. *Bothrops* are more aggressive than *Micrurus*, which increases the occurrence of accidents (Strauch et al., 2018). In a study by Albuquerque et al. (2005), where records of 1098 patients bitten by *Bothrops* in the state of Paraíba was investigated, the municipality of Rio Tinto ranked second in number of notifications, with 1.5 % of snakebites in the state.

Although traditionally only species from the families Elapidae and Viperidae are called venomous, some cases of human envenomation by dipsadids, which have opisthoglyphous dentition, have been recorded in Brazil (Araújo & Santos, 1997; Bernarde, 2011). Some cases have involved species of the genus *Philodryas*, and a case of envenomation by *Philodryas olfersii* was even recorded in the urban area of Rio Tinto in 2015. Locals should take some care in dealing with these snakes, as that family is the most diverse in the area, and thus a better knowledge of snake species the region is warranted to avoid accidents with potentially dangerous snakes otherwise believed to be inoffensive.

Species of *Philodryas* (usually found in open areas) are the most frequently recorded snakes in the literature on urban areas in Brazil (e.g. Carvalho & Nogueira, 1998; Bernarde & Machado, 2002; Costa et al., 2010; Sousa et al., 2010; França et al., 2012). Intuitively, their adaptation to open areas may facilitate the use of urban and anthropic matrices. However, Hartmann & Marques (2005) compared the habitats of *P. olfersii* and *P. patagoniensis* and found the first species near forest fragments and the second mainly in open areas. Herein, both *P. patagoniensis* and *P. nattereri* present dispersed distribution patterns which contrasted with the clustered pattern observed for *P. olfersii*. While former two species have generalist diets, feeding on mammals, birds, lizards, and amphibians (Vitt & Vangilder, 1983; Hartmann & Marques, 2005), *P. olfersii* seems to prey exclusively on frogs (Hartmann & Marques, 2005), and hence is most commonly found near rivers and fragments of natural habitats. Also, due to the green colour pattern of *P. olfersii*, it is likely less perceptible to predators and humans in vegetation-covered environments.

Other common snakes found in Rio Tinto are grass snakes of genus *Erythrolamprus* and the water snake *Helicops angulatus*. According to Dixon (1981), snakes

of the genus *Liophis* (currently *Erythrolamprus* – see Graziotin et al., 2012) have generalist diets. However, some species seem to be frog specialists (Strussmann & Sazima, 1993). *Erythrolamprus poecilogyrus* showed higher abundances in areas near water bodies, where there should be higher densities of frogs. Also showing a dispersed distribution pattern, the water snake *H. angulatus*, an aquatic species that feeds on fish and frogs (Ford & Ford, 2002), was also found along rivers within in the urban area, especially during rainy months.

In general, we recognised three areas with riparian environments, including streams, rivers, and mangroves, which are the main places where snakes are found in Rio Tinto. One is a mangrove area used by local people for fishing and recreation, and thus the rate of casual encounters with a snake is higher, many of which are commonly harmful (Greene, 2000). Therefore, information on the sites where snakes are more likely to be found in and near urban areas is important not only for conservation (Brites & Bauab, 1988), but for society in general. Understanding where dangerous species are more likely to be found helps to reduce the likelihood of accidents, possibly contributing to a more harmonic interaction between people and snakes, also warning locals to avoid these areas.

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Incorporating habitat suitability and demographic data for developing a reintroduction plan for the critically endangered yellow spotted mountain newt, *Neurergus derjugini*

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In reintroduction programmes for amphibians, data on age structure in hosting populations and choices of life stage or age groups in releasing captive bred individuals are often missing. Similarly, employing site selection procedures for selecting appropriate reintroduction locations are often neglected. Here, we obtained data on longevity, age at maturation, and age structure from skeletochronological data in a free living population of the yellow spotted mountain newt, *Neurergus derjugini*. A maximum longevity of 13 years for males and 12 years for females showed that *N. derjugini* is a long living newt with a stable age structure. We also employed maximum entropy modelling, geographic information system, and multi-criteria decision analysis to obtain ranked suitability scores for reintroduction sites. Finally, we determined post-release survival rates for different life stage and age groups of *N. derjugini* including 30 eggs and 60 individuals of six-months old larvae, one and three-year old juveniles, and six-year old adults (15 each) born and raised in a captive-breeding facility and released into mesh enclosures in a selected stream. Over 10 visits to the site before and after overwintering, the survival rates for eggs, larvae, one and three-year juveniles and six-year old adults were 25, 80, 86.66, 93.33 and 53.33 % respectively. Applying survival rates obtained from current experimental reintroductions through a static life table suggest that an optimal release strategy to arrive at a numerical target of 100 adults aged three can be achieved by reintroduction of 650 fertilised eggs and fostering them in meshed enclosures in the selected stream.

Keywords: reintroduction, life table, GIS, survival rate, critically endangered species, *Neurergus derjugini*

INTRODUCTION

Captive breeding and reintroduction programmes are important conservation tools that are playing an expanding role in preserving endangered species. The number of captive breeding plans has increased rapidly in recent years. Harding et al. (2016) reviewed captive breeding programmes involving 213 amphibian species and showed a growing number of parameters resulted from life in captivity have an influence on a variety of characters of the amphibian species. These authors have reported an increase of 57 % in the number of amphibian species involved in conservation breeding and reintroduction programmes since 2007. Biega et al. (2017) reported 532 amphibian species (7 % of all amphibian species) held ex-situ, compared to 4 %, reported five years earlier. Moreover, research focusing on the reintroduction of captive-bred individuals to the natural environment concerns several important issues such as adjusting to the new environments, health control of released individuals, genetic management, and long-term monitoring of released animals (Armstrong & Seddon, 2008).

Despite the growing number of captive breeding and reintroduction programmes operated for conservation

purposes to rescue a number of endangered species, the consequences of these programmes have not always been satisfactory (Hedrick & Fredrickson, 2008). There are many reports demonstrating that these programmes have encountered various shortcomings. In amphibians, some captive breeding programmes have experienced poor nutrition (Pough, 2007), diminished natural behaviours (Burghardt, 2013), inactivity in the natural environment (Keulen & Janssens, 2017), inability to recognise natural foods (Olfert et al., 1993), a variety of diseases due to nutritional deficiency (Densmore & Green, 2007) and chytridiomycosis infection (Parto et al., 2013), failed reproduction (Browne & Zippel, 2007), loss of social interactions (Rabin, 2003), changes in morphology of natural skin colouration (Ogilvy et al., 2012), loss of anti-predator response to predators (Kraaijeveld-Smit et al., 2006), loss of predation ability (Salehi & Sharifi, 2019), a reduced immune response (Keulen & Janssens, 2017) and loss of genetic diversity due to inbreeding (Zippel et al., 2011).

There are also cases of pragmatic failures in various efforts of breeding and release of threatened species that may cast doubts on the value of captive breeding, and as a consequence, reintroduction as a conservation procedure for endangered species (Griffiths & Pavajeau,

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2008). However, there are many situations in which captive breeding is the only conservation choice available (Stuart et al., 2004). Meanwhile, efforts to expand current understanding of ecology and behaviour of reintroduced species are growing (Pough, 2007).

Questions regarding the importance of captive breeding and subsequent reintroduction of threatened species will continue to appear as more studies are carried out on these programmes (Armstrong & Seddon, 2008). In the view of the practical irreversibility of many current threats to amphibians in their natural environments, captive breeding and reintroduction are becoming an essential conservation tool (Griffiths & Pavajeau, 2008; Harding et al., 2016).

Owing to reintroduction efforts, individuals born and raised in captivity can help to restore threatened wild populations or prevent rapidly diminishing populations. There are several factors attributed to causing failure of reintroduced individuals to survive in a new environment, including the source of the founder populations (Kleiman, 1989), the demography (number, sex ratio and age structure) of the release group (Maran et al., 2009), genetic diversity of the released individuals (Godefroid et al., 2011), choice of the life stage (Canessa et al., 2014; Sarrazin & Legendre, 2000), failure to remove previous threats (Moseby et al., 2014) and selection of inappropriate habitat for resource provision (Cheyne, 2006; Osborne & Seddon, 2012). Inadequate population sizes and restricted distribution range can greatly increase the risk of species extinction (Converse et al., 2013). Griffiths and Pavajeau (2008) investigated 58 reintroduction projects involving different species of amphibians and reported 22 projects and 13 were considered highly successful. In an assessment on the degree of success and failure of 58 reintroduction programmes reported in the sixth Global Reintroduction Perspectives by the IUCN (Soorae, 2018), 23 projects were considered highly successful, 24 were successful, 17 were partially successful and 4 were listed as failures.

Our main goals in the present study are to (1) determine longevity, age at maturation, age structure for *N. derjugini* based on skeletochronological data, to (2) integrate species distribution modeling (MaxEnt), geographic information system (GIS) and multi criteria data analyses (MCDA) methodologies for selecting a suitable reintroduction site, and to (3) determine survival rates of various age class *N. derjugini* including eggs, larvae with six-months old, juveniles at one-year and three-years-old, and adults at six-years-old, within a mesh enclosure to determine optimum choice of age groups and life stage for a reintroduction programme.

MATERIALS AND METHODS

The species

The yellow spotted mountain newt, (*Neuregus derjugini* formerly known as *N. microspilotus*), is listed as Critically Endangered by the IUCN because of its very small area of occupancy in its breeding streams (<10 km²), fragmented habitats, continuing decline in the extent and quality of aquatic habitats, habitat degradation, drought, and

pet trade (Sharifi et al., 2009). Recent field studies on distribution and abundance (Afroosheh et al., 2016), activity pattern and home range (Sharifi & Afroosheh, 2014) and also laboratory experiment on growth and development (Vaissi et al., 2018; Vaissi & Sharifi, 2016a, 2016b), and captive breeding (Sharifi & Vaissi, 2014; Vaissi & Sharifi, 2018) shows that *N. derjugini* is a long-living mountain newt that reproduces in highland streams at low density and feeds on benthic macroinvertebrate communities in cold and clean water in highland first-order streams in the mid-Zagros range. The maximum linear distance between the most segregated breeding streams in the south and north parts of the species range is only 205 km. However, localities inhabited by *N. derjugini* are separated with nearest neighbour distances averaging 7.95 km. Surveys on the abundance of *N. derjugini* in 32 of the 42 localities within the Iranian range of *N. derjugini* resulted in the total visual count of 1,379 adults, juveniles, and larvae (mean/stream = 43; range, 1–601). Most of these observations (51 %) were found in just two of the localities, 44 % were found in 14 streams, and the remaining 5 % were scattered among 16 streams (Afroosheh et al., 2016).

Skeletochronology

The *N. derjugini* used in the skeletochronology study (37 males, 44 females) were all caught in the daytime on the 3rd June 2011, in Kavat Stream. The sex of each mature individual was determined according to Sharifi et al., (2012) as males having a fleshy protuberance at the base of the tail, whereas females have a prominent cloaca. A toe clip (i.e., second or third) was cut and kept in 70 % ethanol. *Neurergus derjugini* collected for tissue sampling were kept in small (30 cm×30 cm) pools for about two hours to see if toe amputation caused any visible side effects such as bleeding. No bleeding was seen and the newts were released at their collection site. The phalange was fixed with 10 % buffered neutral formalin, dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin wax, and sectioned serially at 8–10 µm with a rotary microtome. Ten slides were stained using Harris hematoxylin and observed with a light microscope (Leica, Galen III, Leica Microsystems, Wetzlar, Germany). Bone sections were photographed by a digital camera (Leica with Dinocapture 2). All photographs were taken at the same magnification, allowing for simultaneous comparison and facilitating the analysis of the bone growth pattern (Farasat & Sharifi, 2016). The age of newts was determined by counting the lines of arrested growth (LAGs).

Site selection for experimental reintroduction - Maximum entropy model (MaxEnt)

The site selection for the present experimental reintroduction of various age groups of the *N. derjugini* was initiated with a habitat suitability analysis based on a presence-only model (MaxEnt) to provide potential distribution for this species in Iran and Iraq (Sharifi et al., 2017). Within predicted habitats with suitability score of 0–100 %, the site selection operated using a GIS-MCDA decision analysis.

- Geographic Information System (GIS)

Spatial data for complete GIS analysis in the highlands of the mid-Zagros mountains, rural, and regional planning are prepared by various organisations. Sources and attributes of geospatial information used in the GIS process to select suitable areas for the reintroduction are shown in Table 1. GIS data obtained from different sources was used to perform spatial analysis via ESRI ArcGIS 10.2 software.

Table 1. Source and attributes of geospatial information used in the GIS process to select suitable areas for the *N. derjugini* area in western Iran and eastern Iraq. USGS: United States Geological Survey; IFRWMO: Iranian Forests, Range and Watershed Management Organization; IMO: Iran Meteorological Organization; SCI: Statistical Centre of Iran.

No	Data type	Data sources	Resolution
1	Digital elevation model (DEM)	USGS	10 m
2	Human settlement and village	SCI	1: 250000
3	Main road	IFRWMO	1: 250000
4	Floodplain, lake, dam reservoirs	IMO	1: 250000
5	Land use	IFRWMO	30 m
6	MaxEnt modeling output	Sharifi et al. (2017)	-
7	Newt localities	Afroosheh et al. (2016)	-

In the current study, 15 spatial attributes are involved in the site selection (Table 2 and 3). These attributes are distinguished as exclusionary (Table 2) and non-exclusionary (Table 3) criteria. In this study, exclusionary criteria are assumed as hazardous to the site selection and therefore are considered as decisive factors, which have been employed to keep away from the reintroduction site by assigning various buffers to these areas. These include stream density, connectivity of the localities reported for the newt, vegetation cover, land use, human settlements, village density, main roads and highways, flood plain, lake and dam reservoirs. Exclusionary criteria and their buffering values to identify reintroduction site are present in Table 2.

The second group of spatial data used to select reintroduction site for the yellow spotted mountain newt comprises non-exclusionary criteria relevant to environmental parameters and field observations. The non-exclusionary criteria used in this study include conservation integrity, suitable habitat based MaxEnt score (0-100), number of newt localities, stream density, distance to the protected area, villages density, distance to the main road. Exclusionary and non-exclusionary criteria are not necessary mutually exclusive. For example, although the village's density or main road is set as exclusionary criteria within the reintroduction site distance to the villages density or main road can act as a non-exclusionary criterion.

- Multi Criteria Decision Analysis (MCDA)

Here, multiple criteria decision analysis is considered as a family of techniques that aid us to compare and

Table 2. Exclusionary criteria and their buffering values used for the selection the suitable areas for reintroduction.

No	Criterion	Buffering
1	Stream density	Exclude stream density less than 0- 0.007 Km ²
2	Connectivity: ridge density	Exclude areas over 2200 m.a.s.l
3	Vegetation cover	Exclude unsuitable terrestrial vegetation cover
4	Land use	Excluded all arable lands
5	Human settlement	
	City between 1000-10000 population	1000 m buffer zone
	City between 10000-100000 population	2500 m buffer zone
	City between 100000-500000 population	5000 m buffer zone
6	Village density	Exclude areas with high village density (more than 3.68 Km ²)
7	Main roads and highway	1000 m buffer zone
8	Floodplain, lake and dam reservoirs	1000 m buffer zone

Table 3. The relative importance of non-exclusionary criteria obtained based on the nine-point rating system. These values obtained based on a pair-wise comparison of all criteria using the weighted linear combination described by Saaty (1987). To rate each pair-wise comparison and to fill in the matrix cells, the relative importance of the row variable to its corresponding column variable is considered.

No	Criteria	Weights
1	Conservation integrity	0.25
2	Habitat suitability score	0.20
3	Number of newt localities	0.16
4	Stream density	0.13
5	Distance to protected area	0.11
6	Villages density	0.09
7	Distance to main roads	0.06

evaluate the alternatives of land for development of a conservation-oriented goal. This technique has been used for about two decades with geographic information systems (GIS) to analyse spatial problems.

In the process of aggregation of all criteria, weights were assigned according to how important each factor was considered, as indicated in Table 3. The implemented technique of factor pair-wise comparison was used as defined by Saaty (1987) in the context of a decision-making process known as the analytic hierarchy process (AHP) (Saaty, 1987; Pavlikakis & Tsihrintzis, 2003). Also, for all non-inclusionary criteria rating curves were developed according to Sharifi et al. (2009) as indicated in Table 4. The overall suitability for a reintroduction site was obtained according to the following equation:

$$SI = \sum_{i=1}^n (WiXi)$$

where suitability index (SI) designates the suitability score for reintroduction site attained by *n*th alternative; the *W_i* is the weight of the factor calculated by using the

pair-wise comparison between various criteria (Table 3); and X_i is a suitability value obtained from rating curves (Table 4).

- Field studies

Following overlaying of the exclusionary layers, a series of field studies were conducted in order to provide more information for a suitability evaluation. Also, extensive use of Google Earth Pro and Ultimate Maps Downloader was employed in order to collect information about spatial data regarding land use alteration, development projects, and changes in vegetation cover that were not represented in the exclusionary spatial layers. Within patches of suitable areas obtained from overlaying of various layers, eight plots of land were selected by removing marginal areas to provide more interconnected area.

Finally, the suitability of different potential reintroduction sites in the patches with highest suitability values (15 water bodies) were assessed against several criteria including:

- 1- Habitat parameter including: altitude (m); position (l/s); food availability.
- 2- Water quality including: pH, salinity (EC; $\mu\text{S}/\text{cm}$), turbidity (NTU), dissolved oxygen (mg/L), water temperature ($^{\circ}\text{C}$).
- 3- Structural parameters including: permanence of water body and predator (snake, crab, buffalo).

These data were not used quantitatively in the process of evaluating of the suitability index, but were used to represent, qualitatively, various characteristics of the breeding streams.

Trial reintroduction

The captive breeding facility (CBF) at Razi University, Kermanshah Iran, established in 2010, when the Mohamed bin Zayed Species Conservation Fund helped to develop and implement a conservation management plan for the yellow spotted mountain newt. Details of the CBF and a trail reintroduction of the juvenile born in the CBF are explained in Sharifi and Vaissi (2014) and Vaissi and Sharifi (2018). All eggs, larvae, juveniles and adults used in the present study had been maintained in the CBF for all of their life and were siblings of individuals from a known locality. The larvae and adult newts chosen for the experimental reintroduction were arranged for a visual health inspection and behavioural examination (absence of skin slough and wound, viability and sensitivity to stimulus) to ensure they were healthy (Vaissi & Sharifi, 2018). In June to September 2017 across three occasions, 30 eggs, 15 six-months old larvae, 15 one-year old, 15 three-year old, and 15 six-years old adults were released inside 10 meshed enclosures (size of the mesh bag: length: 60 cm; width: 50 cm; height: 15 cm) in the Shelmav stream (N35 $^{\circ}$ 00'56.5"; E046 $^{\circ}$ 26'41.4") in Khaneghah villages in Paveh, Kermanshah province in Iran.

Releases into mesh bags were as follows; on the first occasion, for each age class (one, three, six-years old), except six-months old larvae, seven newts (total N=

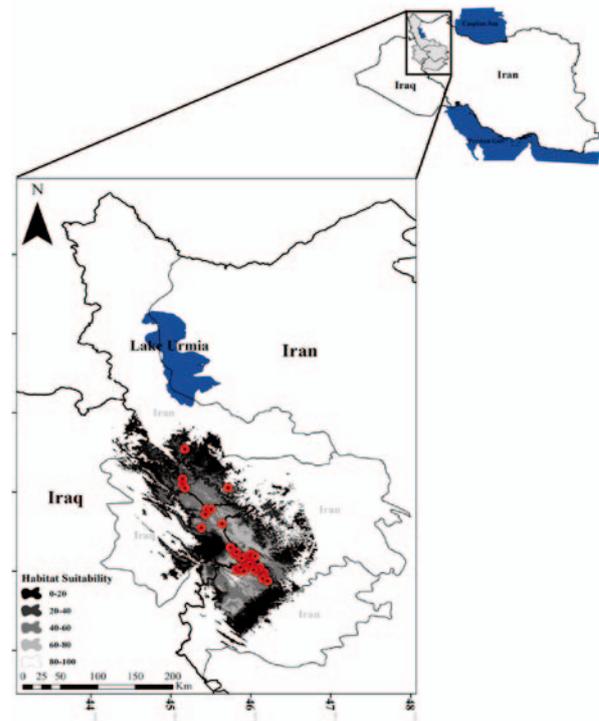


Figure 1. Habitat suitability map developed by Maximum entropy model (MaxEnt) for the *N. derjugini* based on known distribution (red-circle) in Kermanshah, Kurdistan and West Azarbaijan provinces in the western Iran and Sulaymaniyah province in eastern Iraq. Percentage probability of occurrence is pooled in five categories and expressed as different shades of grey shown in the legend.

21) and 15 eggs; on the second occasion, for each age class, except six-months old larvae, eight newts (total, N= 24) and 15 eggs; and on the third occasion, six-month old larvae (total, N= 15) were released. In addition, for five bags, in each bag, seven newts, while for five other bags, for each bag, eight newts were released. Each mesh bag was made of loose plastic with 5×5 mm grids allowing the flow of water and food in the stream. The grid was smaller (2×2 mm) for bags containing eggs. In order to standardise conditions inside the mesh bags and prevent direct contact of the newts, several stones and moss were placed inside the bags. On every visit, the stones and moss were replaced. Although we regularly provided mealworm as additional food, the flow of water through the mesh bags provided plenty of food as in each visit, we found food in the bags, including earthworms, red worms, gammarus and larvae of insect in the meshed bags and between moss. However, to ensure enough food, five mealworms (*Tenebrio molitor*) per newt, were added to the mesh bags.

The reintroduction site and mesh bags were monitored on 10 occasions: 2 June 2017; 16 June 2017; 30 June 2017; 21 July 2017; 6 August 2017; 1 September 2017; 27 September 2017; 30 October 2017; 19 January 2018; 9 March 2018. For each monitoring, the newts within each mesh bag were counted and survival rate determined as a percentage of live remaining.

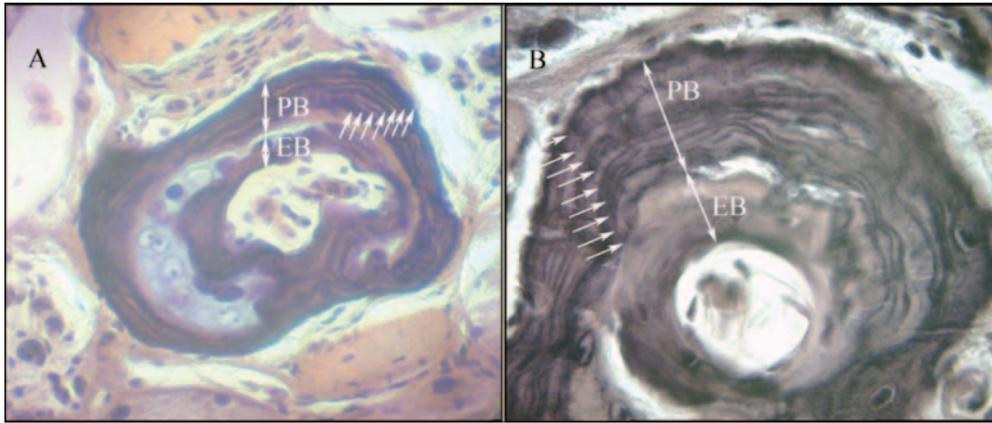


Figure 2. Transverse sections of phalange of *N. derjugini*, viewed with a light microscope, showing seven (A) and nine (B) lines of arrested growths LAGs (arrows). EB, endosteal bone; PB, periosteal bone.

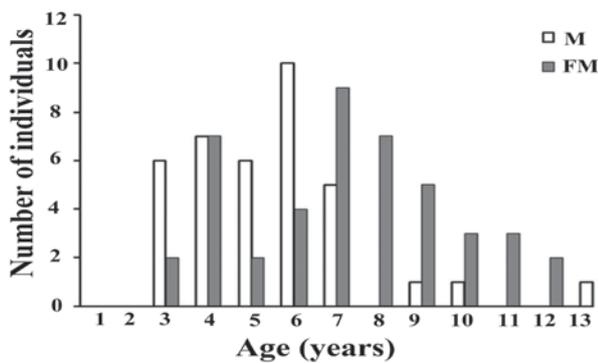


Figure 3. Frequency distribution of aged individuals of male and female *N. derjugini* sampled in Kavat stream.

RESULTS

The distribution of the yellow spotted mountain newt is limited to the conjunction of the western Iranian Plateau and the northern Mesopotamian Plain in western Iran and eastern Iraq. This area has a highly heterogeneous topography and climate. Within this area, the MaxEnt model identified substantial uninterrupted areas of geographic distribution (Fig. 1). In the remaining area, the model detected only very limited and scattered sites with low suitability. The model also detected some areas characterised by a high presence likelihood where records for the species are lacking (Fig. 1). This is particularly well pronounced in Iraqi territory at the middle of the distribution range. The model has shown a strong predictive ability, with the area under curve (AUC) of the ROC analysis providing a value of 0.92 (Sharifi et al., 2017).

Skeletochronology

Examples of sections prepared from toe clips of *N. derjugini* are illustrated in Figure 2. The age of each newt was determined by counting the lines of arrested growth in the compact bone layers in the outer and broader layer of periosteal bone (PB) and any line in the endosteal bone (PB) was neglected. Of the 81 newts over three-

years old (larvae and post-metamorphs are excluded) for which toe clips were available for age estimation, 37 were male and 44 female. Adult females made up 54.32 % of the samples with a close sex ratio (male: female =1:1.21). Percentage of both male and female *N. derjugini* increased from age three to six. Males and females of ages three to six years constitute 61.72 % of the total sampled *N. derjugini*. A consistent decrease in the number of males and females *N. derjugini* is seen in age 7-13 (Fig. 3; Table 5). The average and standard error of longevity for 81 male and female *N. derjugini* are 6.44 ± 0.27 years. The highest longevity reached was 13 years for males and 12 years for females. The minimum number of LAGs found in the mature newts shows that both male and female *N. derjugini* become sexually active at age three.

Site selection for experimental reintroduction

- Maximum entropy model (MaxEnt)

The potential distribution of *N. derjugini* in Iran and Iraq obtained based on a presence-only model (MaxEnt) model (Sharifi et al., 2017) is shown in Figure 1. All localities reported for *N. derjugini* are used to anticipated potential distribution of this species and in the site selection for reintroduction (Fig. 1, red circle).

- Geographic Information System (GIS)

In the basic map of the study area provided by the species distribution modelling (MaxEnt), the initial site selection for selecting reintroduction sites involved preparing the mask maps. Based on the exclusionary criteria, a map divides the study area into two suitable and unsuitable portions for *N. derjugini* illustrates in Figure 4. These mask maps include (1) exclude low connectivity among localities reported for *N. derjugini*, presence of high mountain georeferenced as areas with high ridge density (elevation > 2200 m.a.s.l); (Fig. 4A), areas with unsuitable land use including (2) agricultural lands (Fig. 4B) and (3) terrestrial vegetation cover (Fig. 4C), (4) all main roads and highways (Fig. 4D), (5) inappropriate distance to human settlement (Fig. 4E), (6) high village density (Fig. 4F), (7) lake, dam reservoirs and floodplain area (Fig. 4G), and (8) low stream density (Fig. 4H). Overlaying of these mask maps resulted in several groups of suitable

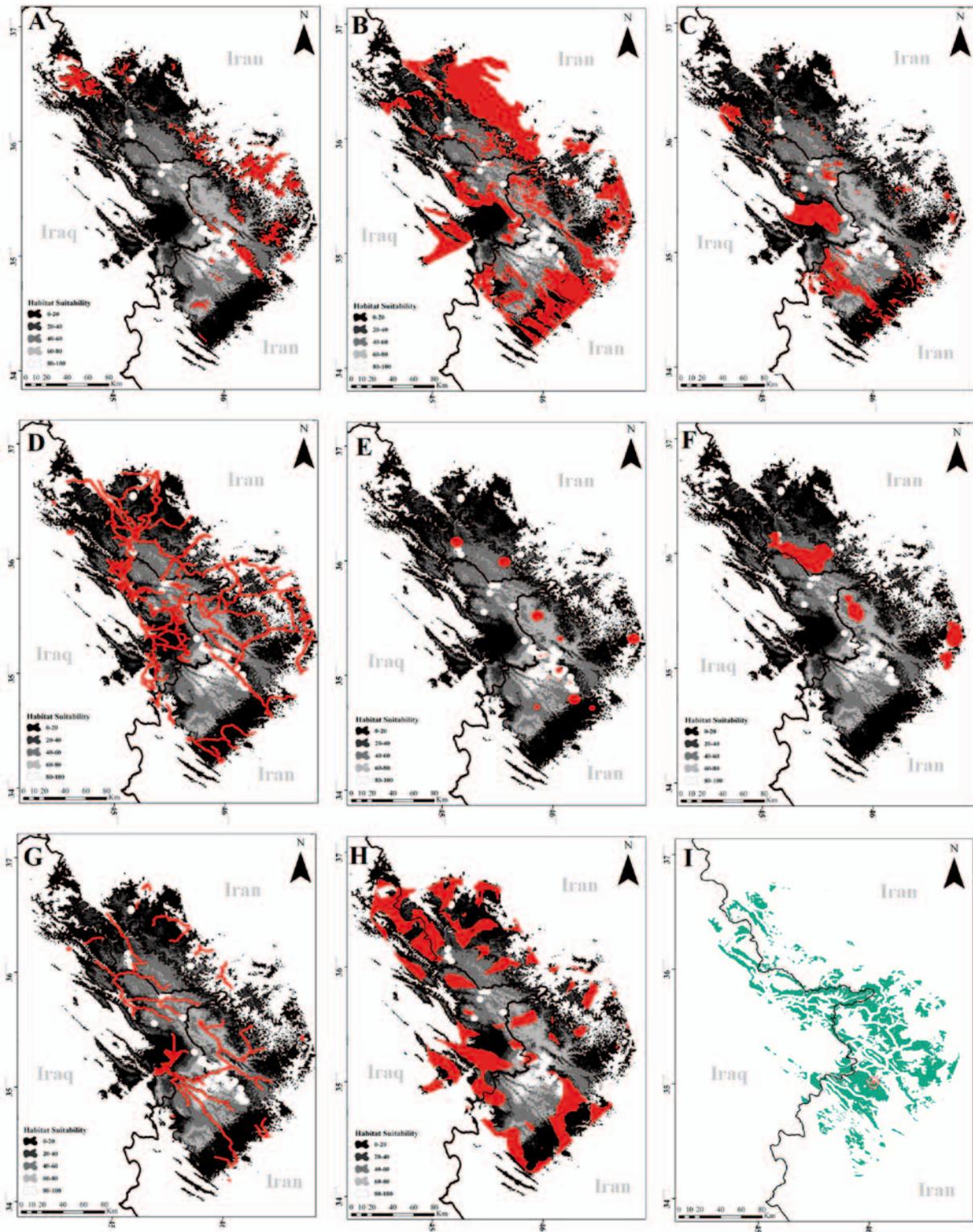


Figure 4. Mask map prepared within MaxEnt range to exclude (A) exclude low connectivity among localities reported for *N. derjugini*, presence of high mountain georeferenced as areas with high ridge density (elevation > 2200 m.a.s.l), areas with unsuitable land use including (B) all agricultural lands and (C) exclude terrestrial vegetation cover, (D) all main roads and highways, (E) inappropriate distance to human settlement (F) village density, (G) lake, dam reservoirs and floodplain area, and (H) low stream density. (I) Suitable habitat (green path) from overlaying of these mask maps. The red circle in Figure 3I: reintroduction site (Shelmav stream; Khaneghah). The buffering values as indicated in Table 2.

locations shown in Fig. 4I. Further field studies were conducted in order to provide more information for the suitability evaluation following the determination of the suitable sites by overlaying the eight spatial layers. In these field studies, several

new criteria and characteristics such as the presence of natural boundaries for the nominated area, vigour and vitality of vegetation cover, edge effect resulted from the shape of the nominated reintroduction site, and landscape appeal has been considered under a single

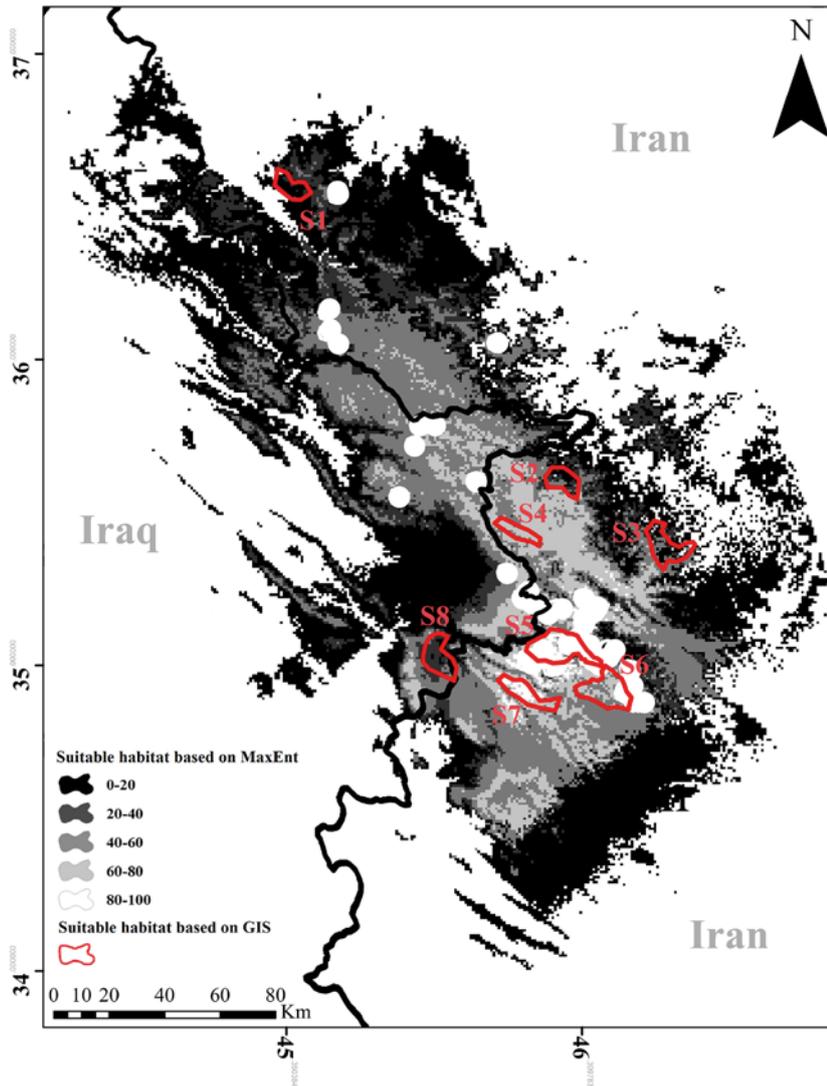


Figure 5. Eight most suitable sites for reintroduction obtained following overlaying exclusionary criteria, species distribution modeling (MaxEnt) and multi-criteria decision analyses (MCDA) of non-exclusionary criteria.

non-exclusionary criterion as "conservation integrity". The non-exclusionary criteria included in the second phase comprise conservation integrity; habitat suitability based on MaxEnt score, number of newt localities, stream density, distance to reintroduction site, village density, and distance to main roads.

- Multi Criteria Decision Analysis (MCDA)

All non-exclusionary criteria were assigned a weight calculated from pair-wise comparison of all criteria as described by Saaty (1987). Relative importance (weight) of seven non-exclusionary criteria used to evaluate the final suitability of each reintroduction site is shown in Table 3. Summarised values obtained from rating curves illustrating relationships between the quantity of the non-exclusionary criterion and a suitability score (0–1) are shown in Table 4. Final suitability values for eight different nominated reintroduction sites which are a product of weights and ratings of seven non-exclusionary criteria are shown in Table 6. This table shows the ranked suitability score for all sites based on numerical values obtained from the suitability assessment equation. The highest suitability

value belongs to S5 with scores of 0.89 (Fig. 5, Table 6).

- Field studies

Finally, in selected sites with ranked suitability values (Fig. 5), the suitability of different potential reintroduction sites for *N. derjugini* was further assessed against several criteria related to water quality (PH, DO, turbidity, electrical conductivity, water discharge), special heterogeneity (food availability, length and width of water body, permanence of water body, presence of potential shelter sites and type, visible pollution, predator, shade) and habitat parameters (altitude, land use, temperature) along various streams (Table 7). Fifteen water bodies (springs, first order streams and karst seepage) were initially examined for the reintroduction site. Shelmav stream (N35°00'56.5"; E046°26'41.4") in Khaneghah villages as a potential site for reintroduction of *N. derjugini* was selected based on a group of criteria to determine suitability for receiving founder stock (Fig. 5, Table 7). Most importantly, selection of this site was based on the degree of protection it afforded from predators, on its close vicinity to a macroinvertebrate community, its high altitude and cold water, isolation

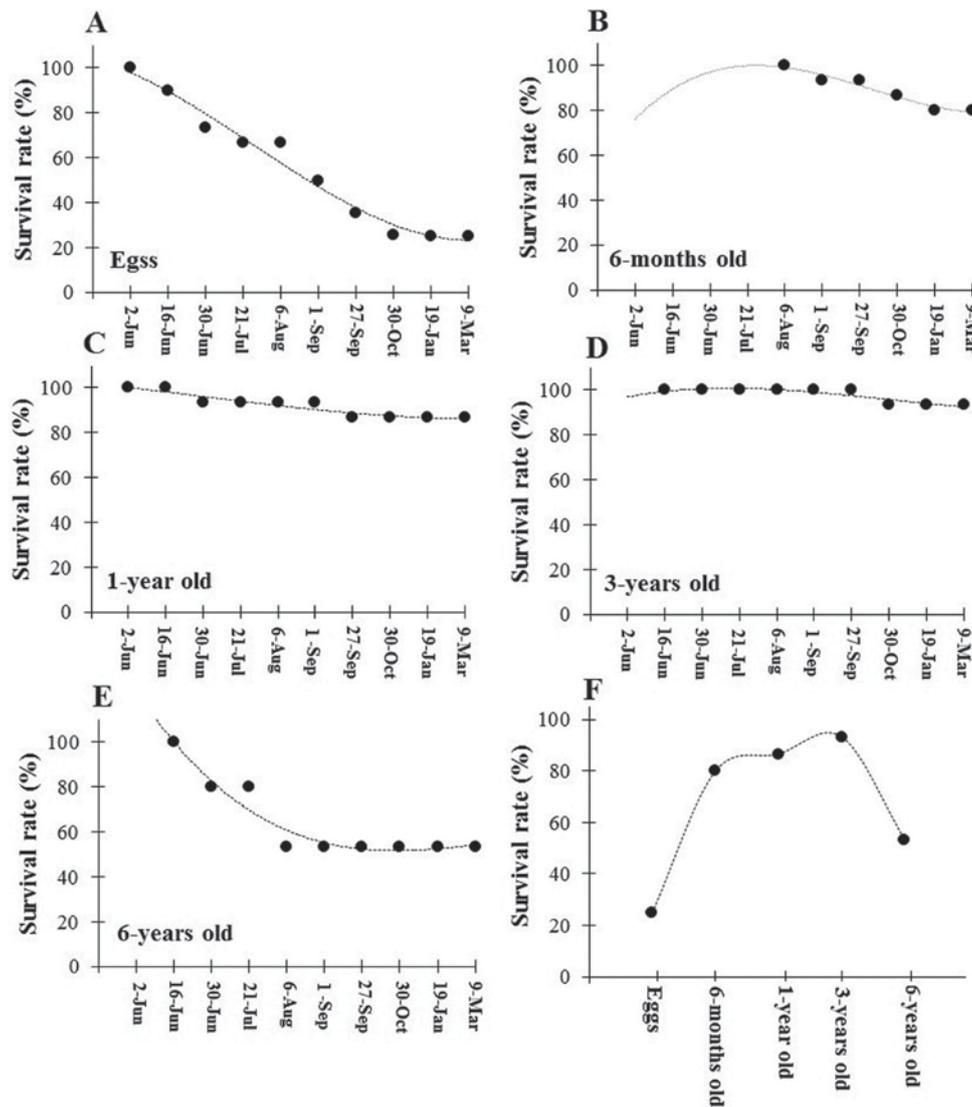


Figure 6. The survival rate (%) of different age class including (A) eggs, (B) larvae with six-months old, post-metamorphic with (C) one-year and (D) three-years old, an adult with (E) six-years old of *N. derjugini* reintroduced to the Shelhav stream during June 2017 until March 2018. The survival rate (%) at the end of the experiment (F).

from human disturbance, lack of pollution and relative security from current and future habitat threats which might result from land-use alteration (Table 7).

Survival rates

Following selection of Shelhav Stream as a suitable site for reintroduction, eggs and various age classes of *N. derjugini* reared in the captive breeding facility were introduced in meshed bags in the reintroduction site. A number of individuals released and size of freshly laid eggs, and the total length of larvae with six-months old, post-metamorphic with one-year and three-years old, and adult with six-year old *N. derjugini* are shown in Table 8. The survival rates of different age class of *N. derjugini* reintroduced to the Shelhav Stream on 2 June 2017 to 9 March 2018 are shown in Fig. 6 A-E and Table 8. Over 10 visits to the site before and after overwintering, the newts with the three-years old had the highest survival rates (93.33 %), (Fig. 6D). In contrast, the eggs had the lowest survival rates (25 %), (Fig. 6A). The survival rate for six-months old larvae (Fig. 6B), the one-year old newts

(Fig. 6C), and six-years old adults (Fig. 6E) were 80 %, 86 % and 53 %, respectively, at the end of the experiment (Fig. 6F, Table 8).

DISCUSSION

We assume that the ages of *N. derjugini* estimated indirectly from the number of the lines of arrested growth (LAGs) were accurate. This method has been used in an area with a cold temperate climate with over two months of freezing days in winter (Sharifi & Assadian, 2004). A similar method has been used by Uzum and Olgun (2009) and Kutrup et al. (2011) to estimate amphibian ages in Turkey close to the present study site. Several authors have supported the suggestion that one LAG equals one year by following known individual amphibians in the field (Buhlmann & Mitchell, 2000; Francillon-Vieillot et al., 1990; Gibbons & McCarthy, 2006) or under laboratory conditions (Kumbar & Katti, 2004). However, there are reports of inaccuracy in the skeletochronological estimates for oldest age class (13-15 years) when

Table 4. Grading values obtained from seven rating curves for seven no-exclusionary criteria used to evaluate relative suitability of selected areas by overlaying. Pattern is a general configuration of a rating curve describing relationship between each criteria and relevant suitability score ranging from 0 to 1.

No	Criteria	Pattern	Grading values
1	Conservation integrity	Linear	Weak=0.25-0.50, Average=0.50-0.75, Excellent=0.75-1
2	Habitat suitability score	Linear	0-20=0, 20-40=0.25; 40-60=0.50; 60-80=0.75; 80-100=1
3	Number of newt localities	Linear	0=0, 1-10=0.0.5; >10=1
4	Stream density	Linear	0-0.007 Km ² =0, 0.007-0.01 Km ² =0.50, 0.01-0.32 Km ² =1
5	Distance to protected area	Linear	>50 Km=0, 25-50 Km= 0.50, 0-25 Km=1
6	Villages density	Linear	3.68-10.94 Km ² =0, 1.18 -3.68 Km ² = 0.50, 0-1.18 Km ² =1
7	Distance to main road	Linear	0-10 Km=0.5, 10-20 Km= 0.75, >20 Km=1

Table 5. A static life table for *N. derjugini* population in Kavat stream in western Iran. Number of live newt sampled at different age x (n_x) and the proportion surviving at the start of age x (l_x) for both sexes of adult (over three years). Mortality rates (q_x) are obtained from the current reintroduction program.

Age class (x)	n_x	l_x	q_x
0-0.5	-	-	1-0.25
0.5-1	-	-	1-0.80
1-2	-	-	1-0.86
2-3	-	-	-
3-4	8	0.098	1-0.93
4-5	14	0.172	-
5-6	8	0.098	1-0.53
6-7	14	0.172	-
7-8	14	0.172	-
8-9	7	0.086	-
9-10	7	0.086	-
10-11	3	0.037	-
11-12	3	0.037	-
12-13	2	0.024	-
13-14	1	0.012	-
	81	1	

compared to actual age (Eden et al., 2007). The reduced accuracy of skeletochronology for age determination may be the result of extensive resorption that occurred throughout the amphibian's life, resulting in a gross underestimate of the age of older individuals and in long-lived species (Sullivan & Fernandez, 1999). Double LAG can also occur as a result of a double cycle of annual activity such as hibernation and aestivation (Caetano, 1990). Despite these drawbacks, skeletochronology is a quick and widely used method for determination of age in temperate regions. Eden et al. (2007) have reported that over 100 authors have used this method to determine

the age of various species of reptile or amphibian.

In this study, data obtained from skeletochronological analysis indicate that unlike its small body mass *N. derjugini* is a long-lived animal with a maximum age reported up to 13 years. Average and standard error of longevity for 81 male and female *N. derjugini* is 6.44 ± 0.27 years. The highest longevity reached to 13 years for males and 12 years for females. Analogous life spans have been reported in other amphibian species, such as *Ambystoma tigrinum nebulosum*, 15 years (Eden et al., 2007), *Neurergus kaiseri*, 14 years (Farasat & Sharifi, 2016). Also, the minimum number of LAGs found in mature males and females shows that both male and female *N. derjugini* are potentially able to reproduce in their third breeding season. Consistent with our results, Farasat and Sharifi (2016) reported the age of sexual maturity for *N. kaiseri* to be four year. Üzümlü (2009) has reported the age of sexual maturity for *Mertensiella caucasica* to be four year whereas in some populations of *Triturus karelinii* the age of maturation is three year (Olgun et al., 2005).

Assessing the suitability of a habitat prior to the release of animals is vital (Lawrence & Kaye, 2011; Van Liefferinge et al., 2018). This is particularly important for species or life stages that are unable to disperse long distances. For any species, the long-term consequences of habitat choice for survival and reproductive success define the ultimate quality of the habitat (William et al., 2007). Also, animals have adapted to consider habitat quality not only based on accessible resources but also by using secondary environmental or structural cues that have association with the final habitat quality, such as predation risk (Rantanen et al., 2010). Shelma Stream as a potential site for reintroduction of *N. derjugini* was selected based on a group of exclusionary criteria to determine suitability within an area defined by a presence-only model (MaxEnt) (Sharifi et al., 2017). The present study demonstrates that combining MaxEnt, GIS and MCDA provides a practical and effective framework for prioritising and ranking landscapes for a reintroduction site for *N. derjugini*. In particular, the MCDA developed for this study was useful in addressing various issues for which there were no available spatial data as well as to incorporate human judgment into the selection process. We conclude that the present study provides a practical approach toward the combination of MaxEnt, GIS and MCDA in providing a series of ranked areas and allowed managers and other stakeholders in creating a reintroduction site. New spatial data or hypotheses can be easily integrated into current GIS-MCDA exercise. This study, also, was able to provide a model study in an area with inadequate spatial data that could be used for other species where there is a need to establish a reintroduction site.

Sarrazin and Legendre (2000) studied a demographic approach to reintroduction of a long-living bird, Griffon Vultures (*Gyps fulvus*), and documented that the release of adults in comparison with young is the most effective. Contrary to long-live species such as Griffon Vultures, short-lived species release costs can be lower. It has been suggested that captivity until the age at sexual maturity

Table 6. Suitability scores for eight nominated reintroduction site (S1-S8) evaluated based on eight exclusionary and seven non-exclusionary criteria.

Criterion	S1	S2	S3	S4	S5	S6	S7	S8
Conservation integrity	0.06	0.12	0.12	0.12	0.25	0.25	0.18	0.12
MaxEnt suitability score	0	0	0.05	0.15	0.20	0.15	0.20	0.05
Number of newt localities	0	0	0	0	0.08	0.08	0	0
Stream density	0.06	0.09	0.06	0.06	0.13	0.13	0.13	0.06
Distance to protected area	0.11	0	0	0.05	0.11	0.05	0.05	0.05
Villages density	0.04	0.09	0.04	0.04	0.09	0.04	0.04	0.09
Distance to main road	0.03	0.03	0.03	0.04	0.03	0.03	0.06	0.03
Suitability score	0.30	0.33	0.30	0.46	0.89	0.73	0.66	0.40

Table 7. Several characteristics of aquatic environments in 15 first order streams nominated for reintroduction site including geographic position, altitude, food availability (adequate-inadequate), pH, salinity, DO, turbidity, dissolved oxygen water, temperature, discharge (Permanent/Ephemeral), and presence of natural predator (+).

Waterbodies	Position (I/s)	Altitude (m.s.l)	Food available	<i>N. derjugini</i> sighted (S)/ not sighted (N)	pH	Salinity (EC; $\mu\text{S}/\text{cm}$)	Turbidity (NTU)	Dissolved oxygen (mg/L)	Temperature ($^{\circ}\text{C}$)	Permanent (P) /Ephemeral (E)	Predator		
											snake	crab	bufo
Kavat	N34 $^{\circ}$ 52'41.2" E046 $^{\circ}$ 30'34.60"	1560	Adequate	S	7.8	0.372	1	7.70	12	P	-	-	-
Ghorighale	N34 $^{\circ}$ 53'12.5" E046 $^{\circ}$ 29'18.7"	1480	Adequate	N	7.58	0.40	18	5.8	13	P	-	-	-
Zali	N34 $^{\circ}$ 59'01.0" E046 $^{\circ}$ 28'41.0"	1777	Inadequate	N	7.26	0.36	1	6.7	12.5	P	-	+	-
Karvansara (up)	N34 $^{\circ}$ 57'41.4" E046 $^{\circ}$ 26'45.9"	1819	Adequate	N	7.27	0.34	3	7.4	14	P	-	+	-
Karvansara (down)	N34 $^{\circ}$ 57'29.5" E046 $^{\circ}$ 26'33.6"	1759	Inadequate	N	7.4	0.32	3	5.2	13	E	-	-	+
Gholani	N34 $^{\circ}$ 54'14.0" E046 $^{\circ}$ 27'27.9"	1461	Adequate	S	7.8	0.42	3	6.6	13.5	P	-	-	+
Baiangan	N34 $^{\circ}$ 57'55.4" E046 $^{\circ}$ 18'01.4"	1334	Adequate	N	7.26	0.39	1	7.26	14	P	+	+	+
Tazeh abad	N34 $^{\circ}$ 57'45.8" E046 $^{\circ}$ 26'01.3"	1761	Adequate	N	7.30	0.39	1	7.46	15	E	-	+	-
Shikahmad	N34 $^{\circ}$ 57'56.5" E046 $^{\circ}$ 27'01.4"	1882	Inadequate	N	6.88	0.30	1	7.22	14	E	-	-	-
Serajgah	N35 $^{\circ}$ 11'02.7" E046 $^{\circ}$ 14'43.8"	1469	Inadequate	S	7.13	0.37	2	6.09	15	P	-	+	-
Berno	N35 $^{\circ}$ 10'57.3" E046 $^{\circ}$ 14'47.0"	1464	Adequate	N	7.15	0.31	1	6.00	14	P	-	+	-
Kariz	N35 $^{\circ}$ 11'13.5" E046 $^{\circ}$ 14'37.7"	1558	Adequate	N	7.18	0.40	1	5.29	14	P	-	-	-
Shelmav	N35 $^{\circ}$ 00'56.5"; E046 $^{\circ}$ 26'41.4"	1713	Adequate	S	7.04	0.36	1	7.80	10	P	-	-	-
Nosod	N35 $^{\circ}$ 10'38.7" E046 $^{\circ}$ 12'06.2"	1355	Inadequate	N	7.62	0.32	1	5.70	16.5	P	-	+	-
Mivan	N34 $^{\circ}$ 57'29.5" E046 $^{\circ}$ 26'33.6"	1650	Adequate	N	7.20	0.36	1	7.69	12.50	P	-	-	-

Table 8. The number of individuals released and size of freshly laid eggs, and the total length of larvae with six-months old, post-metamorphic with one-year and three-years old, and adult with six-years old *N. derjugini* reintroduced into Shelmav stream.

Age class	No. released	Mean \pm SD (mm)	Survival rate (%)	SE	LCI-UCI %95 CI
Egg diameter	30	8.84 \pm 1.86	25	7.86	8.92-41.07
Larvae with 6-months old	15	78.63 \pm 6.81	80	10.69	57.07-102.92
Post-metamorphs with 1-year old	15	92.87 \pm 5.30	86.66	9.08	67.18-106.15
Post-metamorphs with 3-years old	15	139.43 \pm 4.20	93.33	6.66	79.03-107.63
Adult with 6-years old	15	150.28 \pm 8.18	53.33	13.33	24.73-81.93

CI= Confidence Interval, LCI= Low CI; UCI= Upper CI

can constitute a useful period in which the animal can acclimatise to the release area to achieve a comfortable release, and to reduce the stress causing short-term release costs (Bright & Morris, 1994). In the same way, this period can be used to complete the preparatory phase of habitat restoration, a crucial period for reintroduction success (Kleiman, 1989), and to educate local populations in order to increase conservation efficiency (Reading & Kellert, 2002). Also, another benefit of releasing mature individual is that the suitability of the selected habitat or the ability of released individuals to withstand and breed can be checked immediately after the release. This may not be the case for young individuals because of delayed maturity (Sarrazin & Legendre, 2000).

Generally, newly reintroduced individuals can suffer high mortality, as a result of the absence of natural selection during the captive phase or adaptation to life in captivity (Canessa et al., 2014). In several studies reintroduced adults have shown abnormally high dispersal ability (Le Gouar, 2012). Based on photographic identification method average minimum distance covered by recaptured individuals indicates that the home range of *N. derjugini* during the breeding season when the newts live exclusively in the water was estimated to be 230 m² (Sharifi & Afroosheh, 2014). This small home range indicates that the chance of dispersal of reintroduced newts may not be very high. Moreover, data obtained in present study suggest that early support of eggs and larvae in mesh enclosure can progressively increase the survival rates in early stages of *N. derjugini* in early stages of life.

There is some information available on reproduction and captive breeding of *N. derjugini* that may be useful in optimising the release strategies for reintroductions of this species. Based on a study in the captive breeding facility for *N. derjugini* female newts may produce 100-150 individual eggs (Sharifi & Vaissi, 2014; Vaissi & Sharifi, 2018). The egg stage lasts 3–4 weeks; according to the conditions of temperature, food levels, density and water level, the larval period 4–16 months (Vaissi & Sharifi, 2016a, 2016b), reaching metamorphosis (loss of gills) with SVL approximately 46.13 to 78.09 mm. At this stage young metamorphs leave the water (Vaissi & Sharifi, 2018). During larval growth, SVL increases in a linear fashion, with respective growth rates of 1.73 mm/day (Vaissi et al., 2018). The level of cannibalistic behaviour changed as the larvae grew, from a low level during the first four weeks, peaking from weeks seven to 12, and then dropped during weeks 14–52 (Vaissi & Sharifi, 2016b). Also, overwinter mortality in *N. derjugini*, in a small spring enclosure was estimated from July to September 2012. This study demonstrates that post-metamorph captive-bred *N. derjugini* released into the wild can survive to the second growing season (Sharifi & Vaissi, 2014; Vaissi & Sharifi, 2018).

Neurergus derjugini has a slow growth rate with sexual maturation at age three. This indicates that if it was found appropriate to release individuals at the adult stage, newts would have to be sustained in captivity for a longer period. However, maturation at age three slows down the build-up of a captive population available

for reintroduction and increases the expenditure per released newt. Moreover, if it was decided to release captive bred newts at higher age they live in the captive facility for a longer time, and adaptation to the captive life may cause negative impacts on the capability of the reintroduced individuals (Vaissi & Sharifi, 2018). The present experimental reintroduction in meshed enclosures demonstrated that six-months old larvae to three-year old captive-bred adult *N. derjugini* released into the wild can overwinter into the second growing season with high survival rate (≥80 %) and may be the best choice for a reintroduction plan.

CONCLUSION

In studying the reintroduction of the yellow spotted mountain newts in western Iran, we learned that integration of MaxEnt, GIS, MCDA and skeletochronology data with trial reintroduction results can provide scenarios for a typical reintroduction. In an area with a stable-age-structure of host population, or in similar habitat that have lost most of its population, reintroduction may involve releasing a captive population of similar age structure. This strategy requires the provision of adequate captive born individuals of different age classes, and is a long-term and expensive programme. A short-term and inexpensive programme involves a non-distributed captive bred population. The present study showed that with present surviving values some 650 fertilised eggs can produce 100 mature newts in mesh bags in three years. Reintroduction of 136 six-month old larvae, 116 one-year old juveniles, 107 two-year old, and 100 three-year old newts, the population can reach to 100 matured newts in 2.5, 2, 1 years, and directly at the same year, respectively.

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Local extinction of *Scinax caldarum*, a treefrog in Brazil's Atlantic forest

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Here we report the local extinction of *Scinax caldarum*, an endemic tree frog species of the Brazilian Atlantic Forest. We also report a reduction of the geographic range of this species to less than 15 % of the area in which it occurred 27 years earlier. We consider the excessive use of agrochemicals to be the main factor explaining the decline, including in farms with environmental certification. The local extinction of *S. caldarum* is a bioindicator of the severe impact of crops such as coffee and sugar cane, which heavily rely on agrochemicals. Stricter regulation of pesticides is needed to avoid damage to ecosystems and loss of biodiversity.

Keywords: Amphibia; Anura; conservation; endemism; Hylidae

The tree frog *Scinax caldarum* (Fig. 1) typically occurs in open areas. Reproduction takes place in standing water and the tadpoles are exotrophic (Haddad et al., 2013). The species was described in 1969 by Bertha Lutz with the type locality being the municipality (county) of Poços de Caldas in the southern part of the state of Minas Gerais, Brazil. In 1982, six specimens of *S. caldarum* were collected in Alfenas municipality, also in southern Minas Gerais (21°27'37.66"S; 45°56'54.35"W); the specimens were deposited in the herpetological collection of the National Museum in Rio de Janeiro (catalogue numbers: 64807 - 64812). However, recent surveys in the municipality have not reported the species (D'Anuniação et al., 2013; Ferrante et al., 2015, 2017), suggesting that it is locally extinct.

Splitting of terrestrial and aquatic habitats with barriers imposed by land-use change represents one of the strongest drivers of amphibian declines, including those in the Atlantic Rainforest (Becker et al., 2007, 2010), and the quality and type of the agricultural matrix surrounding rainforest fragments constitutes an aggravating factor (Lion et al., 2014). Water bodies available for reproduction of anurans in the south of the Minas Gerais region are mainly located in forest



Figure 1. *S. caldarum* in Poços de Caldas municipality, Minas Gerais, Brazil (Photo by Renato Gaiga).

remnants that are often surrounded by inhospitable matrices with intense use of agrochemicals. This could be an insurmountable barrier preventing individuals of the species from reaching their reproductive environment.

A comprehensive study carried out in the municipality found that water bodies in forest remnants surrounded by coffee and sugarcane matrices have few or no habitat generalist species (Ferrante et al., 2017), reinforcing the hypothesis of local extinction of *S. caldarum* by agrochemical use in this landscape.

To verify the occurrence of *S. caldarum* in the Alfenas region, we sampled 20 forest fragments and 22 agricultural areas surrounding the fragments. The sampling followed the methodology described in Ferrante et al. (2017). The sampling covered the neighbouring municipalities of Areado and Alfenas, which are also locations that were within the area of

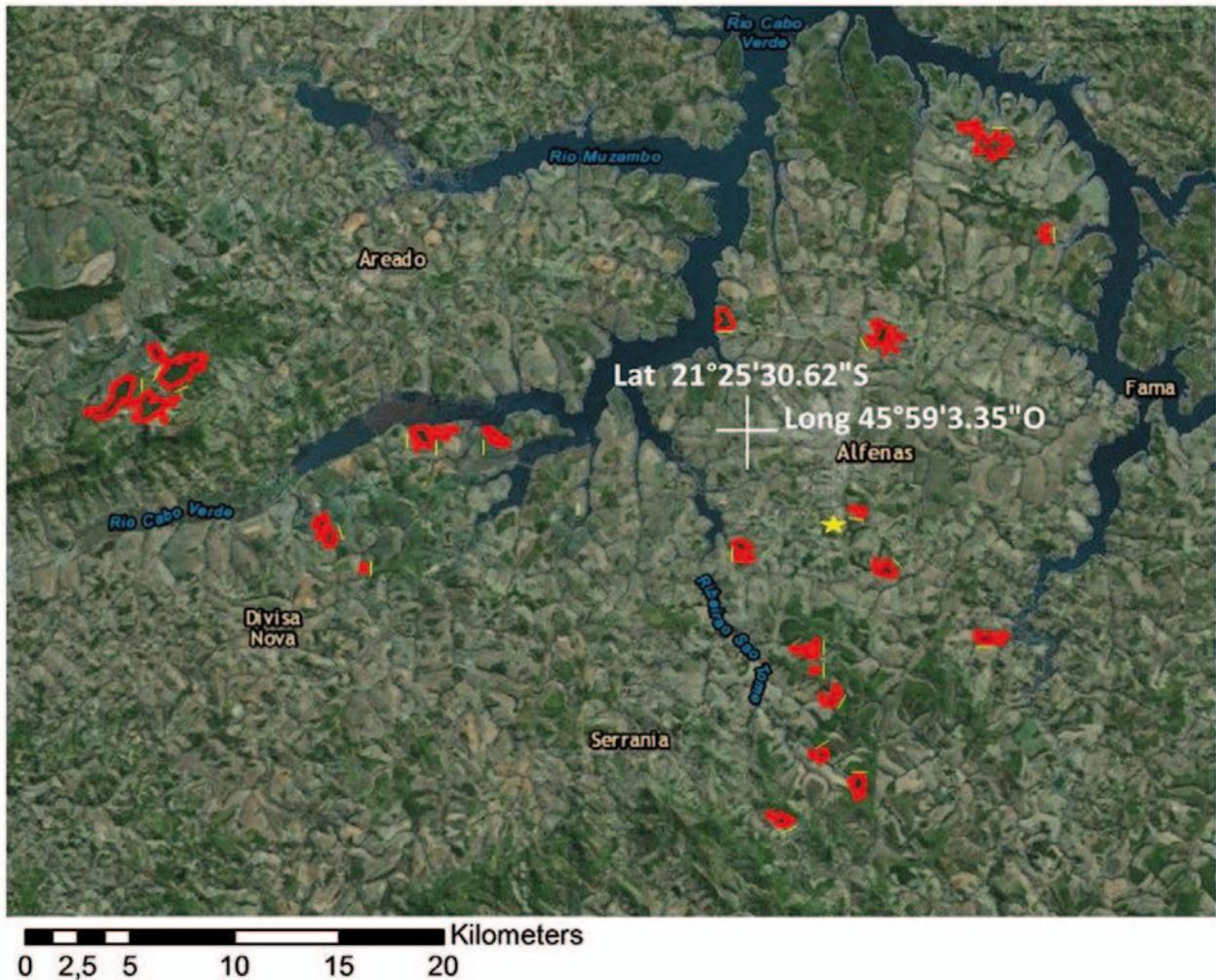


Figure 2. Landscape sampled in the Alfenas region: yellow star represents the collection point of *S. caldarum* in 1982; fragments circled in red represent sampled sites; yellow transects represent areas sampled in cultivated area.

occurrence of *S. caldarum* in 1982 (Fig. 2). The surveys were conducted between December 2011 and March 2012, which comprises the local rainy season (Roldão et al., 2012) and is the main amphibian breeding season in the Atlantic Forest (Haddad et al., 2013) including *S. caldarum* based on observations by ACML and RG in the type locality. Lutz (1973) shows *S. caldarum* has constant calling activity in all months of the year. Many of the sampled sites are located in farms growing sugarcane, coffee and pasture. Some of the farms have Rainforest Alliance and UTZ certifications, whilst others are not certified. Both sugarcane and coffee are crops with intensive use of pesticides and other agrochemicals. Only considering the certified farms, thirty-seven different pesticides are used in the sampled areas: Aureo, Blitz, Cantus, Abamectin, AzaMax, Lorsban 480 Br, Flumyzin, Glifos Plus, Glifosato Atanor, Galiagan, Goal, Zartan, Nimbus, Opera, Premier Plus, Prioriextra, Amistar WG, Durivo, Cuprozeb, Roundup, Sphere, Altacor, Warrant 700 WG, VERDADERO WG, Authority, Klorplan, Fegatex, Rovral, Aliete, Folicur, Stinger, Ally, K- Othrine 2P, Jaguar, Planador, Padron and Plenum.

No specimens of *S. caldarum* were found in Alfenas municipality despite a large sampling effort, including sampling in both forest remnants and in agricultural and

other modified areas. We therefore consider this species to be locally extinct in Alfenas municipality. Although we have observed degradation and loss of native vegetation throughout the southern portion of Minas Gerais, including in certified farms (Fig. 2), generalist species, such as species of *Scinax* of the ruber group to which *S. caldarum* belongs (Haddad et al., 2013), have benefited from the loss of vegetation in forest remnants and have disappeared from fragments that are surrounded by matrices with a high proportion of agricultural crops with heavy agrochemical use (Ferrante et al., 2017). This strongly suggests that the local extinction is due to the harmful effect of the pesticides used in the landscape. The hypothesis of local extinction due to use of agrochemicals is further corroborated by the fact that other likely threats, such as mining, are restricted to the municipalities where the species still occurs (Caldas & Poços de Caldas).

The total distribution area of *S. caldarum* in 1982 was 1228 km², considering the population in Alfenas. However, we can consider that the current range of the species as only 184 km², with the species occurring exclusively on the Poços de Caldas plateau (Fig. 3). These area measurements were performed based on polygons encompassing the recorded occurrences,

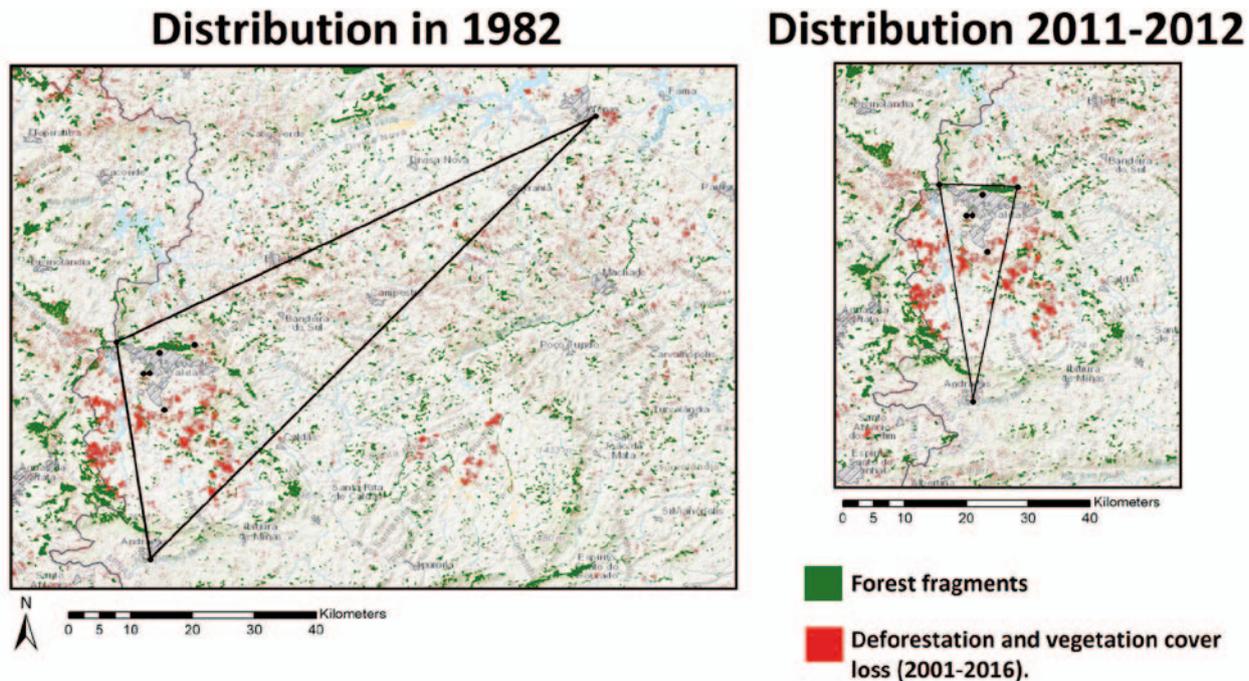


Figure 3. Range of occurrence of *S. caldarum* in 1982 and 2011-2012 according to the most recent census.

following the methodology of the International Union for the Conservation of Nature (IUCN) and Brazil's Chico Mendes Institute of Biodiversity (ICMbio). This plateau is a circular formation with a mean diameter of about 33 km; it is a result of orogenesis of volcanic origin in the Mesozoic era (Schorsche & Shea, 1992). This relief favoured the isolation of several species of anurans that now are endemic, such as *Bokermannohyla vulcaniae* and *Proceratophrys palustris* (Cruz & Feio, 2007; Neves et al., 2018). Although *S. caldarum* occurs in open or altered areas, imminent threats exist within the current range of this species on the Poços de Caldas Plateau. These include storage of toxic and radioactive mine tailings, new mining initiatives, and expansion of agricultural crops with heavy use of agrochemicals. In addition, habitat fragmentation is still a threat, and this could be aggravated by severe climate change in south-eastern Brazil. The region has experienced continuous water stress, with prolonged dry periods and absence of a rainy season (Geritana, 2016). Continued deforestation in the Amazon could exacerbate these changes by reducing the water recycling that is essential to providing water vapour that is transported by winds to Minas Gerais and other parts of south-eastern Brazil (Zemp et al., 2014; Ferrante & Fearnside, 2018). Indeed, climate change over the last decade in the southern portion of Minas Gerais (Getirana, 2016) may have also contributed to this extinction. However, while these events have been evident for more than 20 years since the last sighting of the species in the municipality of Alfenas, the same weather events have also been occurring in the municipalities where the species still occurs.

The IUCN listed *Scinax caldarum* as Least Concern (LC) in view of its wide distribution in the past, mistaken records of its geographic distribution, the tolerance of species to habitat modification, presumed large

population, and because the population would be unlikely to be declining fast enough to qualify for listing in a more threatened category (IUCN, 2019). Based on the current threats being significantly greater than the threats that exterminated the population in Alfenas in less than 27 years, we consider that *S. caldarum* should be re-categorised as Endangered (EN) under criteria B1ab(i+iii) of the IUCN RedList (IUCN, 2012). The arguments are: the range is now less than 5000 km²; the population is endemic to the plateau; there is only one location of occurrence (sub criterion B1a); a continuous decline has been observed in the range of the species and in the quality of its habitat. Due to the harmful effects of mining on anurans (Sasaki et al., 2016), our results alert us to two needs: mining activities should be restricted through zoning in the area of occurrence of *S. caldarum* or other threatened species, mine tailings should be treated, and affected areas should be recovered. In addition, species in the state of Minas Gerais must be continuously monitored. This should be a responsibility of any entrepreneur who performs activities harmful to species within their area of occurrence, taking full responsibility for population declines or local extinctions owing to agricultural or mining activities. Creation of a protected area (conservation unit) in Poços de Caldas is urgently needed, and this should include both forest areas and the natural grasslands that are the habitat of generalist species that are endemic to the region. More rigid laws are needed to control the use of agrochemicals near permanent preservation areas ("APPs") in the Atlantic Forest Biome.

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Wild diet of the critically endangered mountain chicken (*Leptodactylus fallax*)

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In this study, we provide the most complete review to date of the diet of the critically endangered mountain chicken *Leptodactylus fallax* in the wild, describing for the first time the composition of the diet from Montserrat. To do this we report the results of two studies carried out on Montserrat that investigated *L. fallax* diet based on the content of frog gastrointestinal tracts. We found diets on Montserrat to be similar to that recorded for Dominica, typified by opportunism and catholicity, including a wide range of invertebrate prey (dominated by orthopterans) with some small vertebrates eaten too.

Keywords: amphibian, diet, mountain chicken,
Montserrat, Dominica

The mountain chicken *Leptodactylus fallax* is the largest endemic amphibian of the Caribbean, the largest living members of the genus *Leptodactylus* (Kaiser, 1994), and one of the world's largest frogs (Adams et al., 2014). As such, the species is an important part of Caribbean ecosystems, as one of the largest native terrestrial predators on the islands on which it occurs. *Leptodactylus fallax* once occurred across seven Caribbean islands, it has since been extirpated from all but two (Dominica and Montserrat) (Adams et al., 2014; Breuil, 2011; Fa et al., 2013; Kaiser, 1994; Malhotra et al., 2007). *Leptodactylus fallax* was widely distributed on Dominica until the early 2000s when the arrival of the pathogen chytrid fungus *Batrachochytridium dendrobatidis* (*Bd*) caused an epizootic of the disease chytridiomycosis leading to rapid and severe population declines (Adams et al., 2014; Magin, 2004; McIntyre, 2003). On Montserrat, *L. fallax* was restricted to the Central Hills of the island until a series of volcanic eruptions beginning in 1995 reduced the species range (Daltry & Gray, 1998; Adams et al., 2014). This was followed by the arrival of *Bd* in 2009 leading to functional extinction of the species on the island in a matter of months (Garcia et al., 2009; Adams et al., 2014).

The only quantitative study of the diet of *Leptodactylus fallax* in the wild is that of Brooks (1982) based on gastrointestinal tract (GIT) contents data of 397 *L. fallax* collected from December 1965 to December 1966, describing a diet characterised by "opportunism and catholicity of choice..." (p.306, Brooks, 1982). Further dietary studies consist of only anecdotal data describing instances of ophiophagy and consumption of the large theraphosid spider *Cyrtopholis femoralis* (Buley, 2003; Rosa et al., 2013).

In this study we report for the first time dietary data for *Leptodactylus fallax* from Montserrat, detailing the results of (1) a study in 1979-1980 before population declines, and (2) a study carried out during the response to the emergence of *Bd* on the island based on post-mortem data of frogs killed by *Bd*. Combining our data with that from Dominica (Brooks, 1982), we provide the most complete review to date of the wild diet of *L. fallax*. We sampled *Leptodactylus fallax* on Montserrat from May 1979 to August 1980. We acquired *L. fallax* specimens from local hunters in the town of Salem who supplied local restaurants with the species. We purchased fresh specimens at dawn (captured the night before) from hunters at above the asking price at restaurants (averaging \$3.50 in 1979 and \$5.00 in 1980 per frog).

We sampled 206 specimens in this way. We removed the contents of the GIT of all specimens purchased on a given day and analysed the contents whilst still fresh. It should be noted that this sampling took place prior to major declines in the *L. fallax* population on Montserrat and prior to overharvesting being identified as a potential threat to the species. Such a protocol would not be appropriate or ethical for studying the diet of any threatened species, as this procedure has the potential to increase unsustainable hunting pressure on populations. We also sampled *L. fallax* on Montserrat from 1 March 2009 to 27 April 2009. At this time, the authors G.G. and J.L. were dispatched to Montserrat leading the rapid response team to determine the cause of the mass mortality of *L. fallax* reported by the Montserrat Forestry Department on 14 February 2009 (Garcia et al.,

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2009). This survey determined that the cause of mass mortality was *Bd* (Garcia et al., 2009). We carried out surveys in 14 localities within the known distribution range of *Leptodactylus fallax* on Montserrat. All *L. fallax* we encountered were sampled for veterinary analysis (Garcia et al., 2009). We sampled a total of 349 individuals, of these 124 were dead. We carried out post-mortems on all dead specimens. We removed the contents of the GIT of all specimens that had not begun to decay (N=32), storing the GIT contents of each individual in 75 % ethanol in separate plastic specimen tubes for later analysis.

For both data sets (1970-80 and 2009), we inspected GIT contents under stereomicroscopes and identified all prey items with appropriate taxonomic keys (Barrientos, 1988; Chinery, 1993) down to the lowest possible taxonomic levels (Table 1). We recorded the number of prey items of each taxonomic group (N) in both data sets. For the 1979-80 dietary samples we calculated the volume (V) of each prey category from each *Leptodactylus fallax* specimen by measuring the displacement of water in a graduated cylinder upon insertion of prey remains from the GIT (Hyslop, 1980). In the case of the 2009 dataset, we measured the length of intact prey items from the front of the head to the tip of the abdomen (L) and width at the widest point (W). For partially digested prey items we noted the dimensions and then used the formula proposed by Hirai and Matsui (2001) to calculate L and W. We then calculated the volume of each prey item using the formula proposed by Solé et al. (2009):

$$\text{Volume} = (4\pi/3)(L/2)((W/2)^2)$$

Size (mass or volume) measures represent a more accurate representation of diet than frequency of occurrence data as the latter may over-represent small quantities of prey if the prey is small, and under-represent if the prey is large (Deagle et al., 2007; Schmid & Tucker, 2018). In the data we collected (dataset M1 and M2), volume was used as a size measure, however, in Brooks (1982) study (dataset D) dry weight was used as a measure of size. We directly compare proportional measures of volume and dry mass, referring to both collectively as “size” measures. We also recorded occurrence of each prey item (O) as the number of *L. fallax* specimens with given prey category found in the GIT.

Due to the historic nature of the datasets, some data had been lost before analysis could take place and some key aspects of experimentation could not be repeated to make data collection consistent between datasets. As such, GIT contents could not be attributed to individuals, instead only totals of each measure were available from each dataset. This limited our use of any informative statistical analysis.

A complete list of all food items found in *Leptodactylus fallax* GITs from the Brooks, 1982 Dominica dataset (D), the 1979-1980 Montserrat dataset (M1), and the 2009 Montserrat dataset (M2) is given in Table 1. The total dataset represents the GIT content of 609 specimens of *L. fallax*, 371 from dataset D, 206 from dataset M1, and 32 from dataset M2.

Leptodactylus fallax diet in the wild is summarised for all datasets in Fig. 1. Diet was dominated by orthopterans (25.3 % by frequency, 42.1 % by size), with Opiliones, myriapods, hymenopterans, gastropods, coleopterans, and vertebrates also constituting core components of the diet (>5 % by frequency and/or size). *Leptodactylus fallax* diet is summarised for each dataset in Figure 2, displaying some variation in frequency (Fig. 2A) and size (Fig. 2B) contribution of different prey categories to overall diet between datasets. There is a greater proportional occurrence of prey items of each category in dataset D compared to M2 in all prey categories except hymenopterans, acariformes, dipterans, and hemipterans (Fig. 2A). This indicates that *L. fallax* sampled in dataset D had a more varied diet compared to those in dataset M2.

Seasonal dietary data for *Leptodactylus fallax* was only recorded for datasets D and M2. Two seasons prevail on Dominica and Montserrat; dry (December-April) and wet (June-October) (Brooks, 1982). May and November are considered transitional months, so data from these months was not utilised. Proportions of prey categories by frequency in diet for each season are displayed in Fig. 3A. Differences between seasons were minor, notably an increase in frequency of orthopterans (28.4 % to 32.4 %) and coleopterans (7.9 % to 14.3 %) from dry to wet season, and a decrease in frequency of Opiliones (24.0 % to 15.9 %) and vertebrates (5.1 % to 1.4 %).

Proportions of prey categories in diet by size (mass and volume) for each season are displayed in Fig. 3B. As with frequency of prey items in diet, size composition of prey items in diet also changed little between seasons, however changes that did happen occurred in different prey categories. Notable changes involved an increase in proportional size contribution of gastropods (9.8 % to 14.6 %), myriapods (6.2 % to 11.7 %), and coleopterans (5.3 % to 9.0 %) from dry to wet season, and a decrease in proportional size contribution of Araneae from 6.3 % to <1 % from dry to wet season.

Our study supports the finding of Brooks (p.306, 1982) that “opportunism and catholicity of choice best describe prey capture for *Leptodactylus fallax*”. We add additional dietary data to published datasets on GIT contents, providing the first dietary data from the population on Montserrat.

Given that *L. fallax* is an opportunistic generalist, differences in diet between the datasets are likely a result of differences in abundances and availability of prey items between the different islands and time periods. Differences between the Montserrat datasets could also be a result of ash falls caused by volcanic events between 1995 and 2009. Studies on Montserrat found that arthropod populations declined following ash fall events (Marske et al., 2007). Marske et al. (2007) hypothesized that although arthropod numbers as a whole recover rapidly from ash fall-induced declines, total community structure may be altered in the long-term. As such, ash fall induced changes in prey community structures may explain the shifts in diet observed on Montserrat between 1980 (M1) and 2009 (M2). Alternatively, all frogs in dataset M2 were infected with *Bd*, as such, disease may have influenced diet. Weakened frogs may

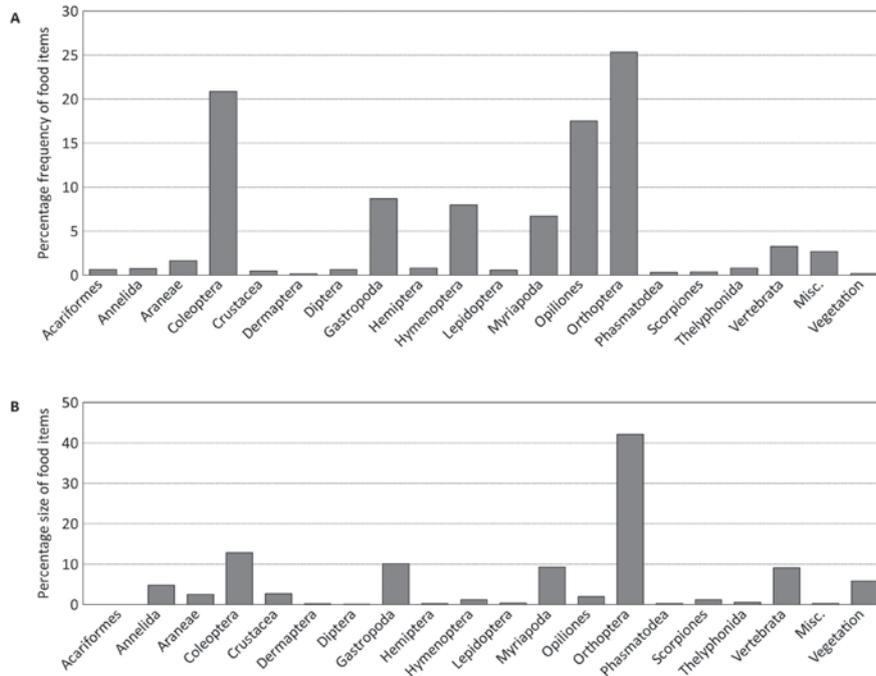


Figure 1. (A) Percentage of total frequency of food items of each prey category, and **(B)** Percentage of total size (proportional volume and proportional dry mass) of food items of each prey category for *L. fallax* across datasets from Dominica (1965-66), and Montserrat (1979-80 and 2009).

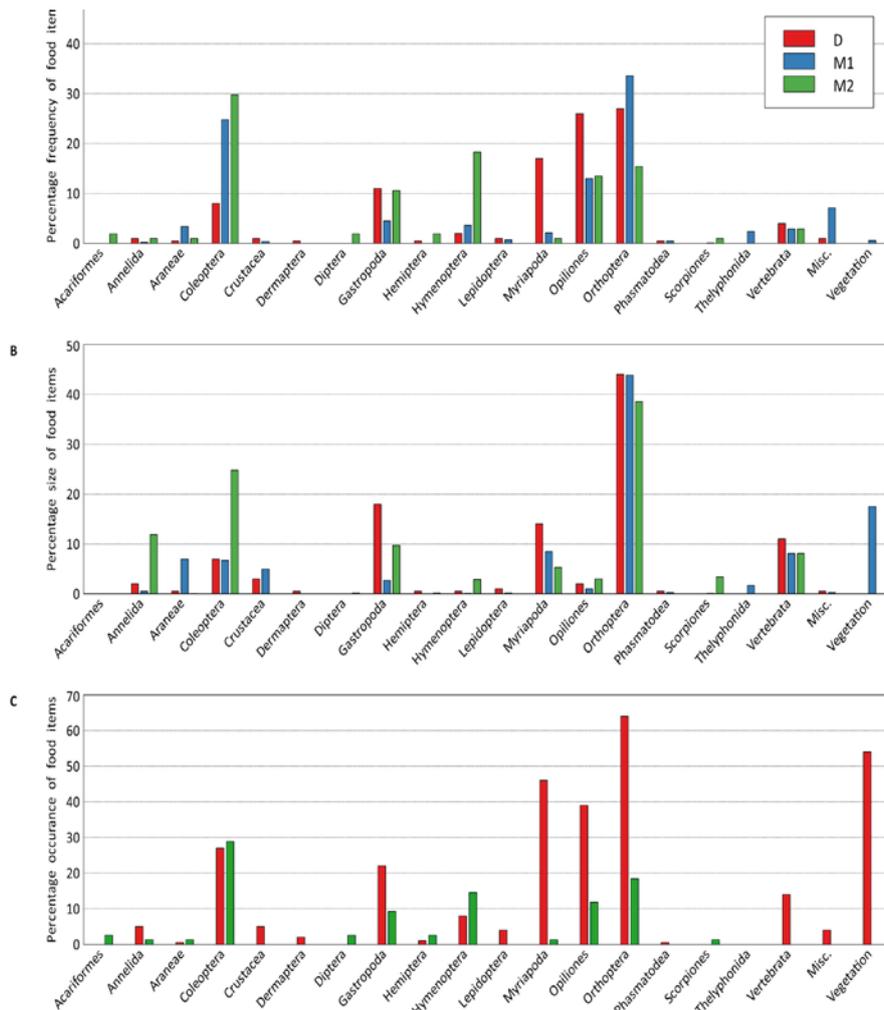


Figure 2. Proportional composition of diet by prey category of *L. fallax* for datasets collected on Dominica 1965-66 (**D**), and Montserrat 1979-80 (**M1**)/ 2009 (**M2**). **(A)** Percentage of total frequency of food items for each prey category. **(B)** Percentage of total size of food items for each prey category (percentage of total dry weight dataset D, percentage of total volume from datasets M1 and M2). **(C)** Percentage of total occurrence of food item per gastrointestinal tract of each prey category.

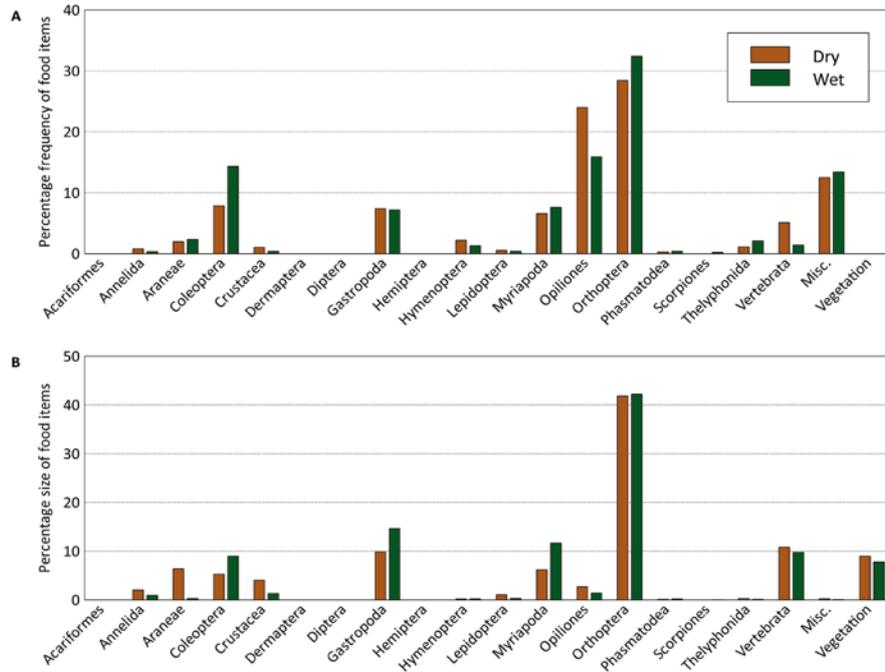


Figure 3. Proportional composition of diet by prey category of *L. fallax* in the dry and wet season by **(A)** percentage of total frequency of food items for each prey category and **(B)** Percentage of total size (percentage of total dry weight dataset D, percentage of total volume from datasets M1 and M2) of food items for each prey category.

Table 1. Percentage of frequency (% N), volume (% V), occurrence (% O), dry mass (% DM), and size (combined DM and V data- % S) for food items of *L. fallax* sampled in Montserrat in 1979-80 (M1) and 2009 (M2), and in Dominica in 1980 (D).

Category/Taxa			M1		M2			D		
	% N	% S	% N	% V	% N	% O	% V	% N	% O	% DM
Acariformes	0.6	<0.1	0	0	1.9	2.6	<0.1	0	0	0
Annelida	0.7	4.8	0.2	0.5	1.0	1.3	11.9	1.0	5.0	2.0
Araneae	1.6	2.5	3.4	7.0	1.0	1.3	<0.1	0.5	0.5	0.5
Coleoptera	20.9	12.8	24.8	6.7	29.8	28.9	24.8	8.0	27.0	7.0
Crustacea	0.5	2.7	0.4	5.0	0	0	0	1.0	5.0	3.0
Dermaptera	0.2	0.2	0	0	0	0	0	0.5	2.0	0.5
Diptera	0.6	<0.1	0	0	1.9	2.6	0.2	0	0	0
Gastropoda	8.7	10.1	4.5	2.7	10.6	9.2	9.7	11.0	22.0	18.0
Hemiptera	0.8	0.2	0	0	1.9	2.6	0.2	0.5	1.0	0.5
Hymenoptera	8.0	1.2	3.6	0.1	18.3	14.5	2.9	2.0	8.0	0.5
Lepidoptera	0.6	0.4	0.7	0.2	0	0	0	1.0	4.0	1.0
Myriapoda	6.7	9.3	2.1	8.5	1.0	1.3	5.3	17.0	46.0	14.0
Opiliones	17.5	2.0	13.0	1.0	13.5	11.8	3.0	26.0	39.0	2.0
Orthoptera	25.3	42.1	33.6	43.8	15.4	18.4	38.5	27.0	64.0	44.0
Phasmatodea	0.3	0.3	0.5	0.3	0	0	0	0.5	0.5	0.5
Scorpiones	0.4	1.2	0.1	0.1	1.0	1.3	3.4	0	0	0
Thelyphonida	0.8	0.6	2.4	1.7	0	0	0	0	0	0
Vertebrata	3.3	9.1	2.9	8.1	2.9	n/a	8.1	4.0	14.0	11.0
Misc.	2.7	0.3	7.1	0.3	0	0	0	1.0	4.0	0.5
Vegetation	0.2	5.8	0.6	17.5	0	0	0	n/a	54.0	n/a

have only been able to consume prey items requiring minimal energetic output to capture and process, leading to a shift in diet compared to that seen in dataset M1.

Seasonal differences in diet for Dominica and Montserrat show the same pattern of changes in prey groups. These changes likely reflect changes in abundance and therefore availability of different prey categories between the wet and dry season (Brooks,

1982) and a change in the size of individuals and/or the presence of different sized species between seasons.

Further long-term studies of diets combined with studies of prey communities would be required to test these hypotheses. We recommend that future studies of amphibian diets incorporate prey community data into their analyses.

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Photo-identification of horseshoe whip snakes (*Hemorrhois hippocrepis*, Linnaeus, 1758) by a semi-automatic procedure applied to wildlife management

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Photo-identification is an increasingly used method for the study of animal populations. Natural marks such as coloration or scale pattern to identify individuals provide an inexpensive and less invasive alternative to conventional tagging methods. Photo-identification has previously been used to distinguish individual snakes, usually by comparing the pileus region. Nevertheless, this method is seldom used in capture-recapture studies. We show the effectiveness of photo-identification in snakes using specific software for individual recognition applied to a wildlife control study of horseshoe whip snakes. Photos were analysed with Automatic Photo Identification Suite (APHIS), which allowed us to compare the variability of head scale patterns surrounding the parietal shields instead of the traditional method of using large scale groups of the pileus. APHIS correctly identified 100 % of recaptures of snakes. Although further studies are needed, the variability of the surrounding scales of the pileus region seems a robust method to identify and differentiate individuals.

Keywords: Photo-identification, capture-recapture, scale patterns, invasive snake, wildlife control

The management of natural populations relies on robust estimates of demographic parameters such as population size, individual survival probability or per-capita fertility (Williams, Nichols & Conroy, 2002). These estimates can be obtained by the monitoring of individuals' fates through capture-mark-recapture (CMR) techniques (Southwood & Henderson, 2009). A common assumption in CMR techniques is that marks do not harm the animal or influence its fate, and marks are not lost; for practicality, the marking method should not be expensive to ensure that a large number of animals can be identified. In this respect, reptiles, and Squamata in particular, constitute a challenge (Ferner, 1979; Fitch, 1987). The continuous growth and the complete skin moult render most external tagging methods temporary for lizards and snakes. Moreover, external marks can affect the behaviour, growth or

probability of recapture of marked animals, and can impair survival (Murray & Fuller, 2000). For example, snakes were traditionally marked by freeze branding or scale-clipping (Lewke & Stroud, 1974; Spellerberg, 1977). These marks had a variable permanence (Shine et al., 1988) and could affect behaviour of the animals (Weary, 1969). The use of medical cautery units (Winne et al., 2006) and of subcutaneous Passive Integrated Transponder (PIT) tags provided an alternative to the previous marking methods (Jemison et al., 1995; Christy et al., 2010; Oldham et al., 2016). PIT tags have near 100 % reliability if correctly implanted (Gibbons & Andrews, 2004) but the implantation of the device in small species can be complicated and, when many animals have to be marked, expensive.

With the advent of digital photography, attention has focused on using natural marks such as spots, scale patterns or colouration for individual recognition of reptiles (Sacchi et al., 2010; Rotger et al., 2016) as successfully done on other taxa (Katona & Whitehead, 1981; Van Tienhoven et al., 2007; Díaz-Calafat et al., 2018). Photo-identification techniques have the advantage of reducing handling time (Reisser et al., 2008; Gardiner et al., 2014; Sannolo et al., 2016), and in some cases, to preclude physical capture (Gatto et al., 2018). The scale pattern of individual snakes as unique was recognised in the last century, but manual techniques of comparison were time-consuming and limited the study to a small number of individuals. Nowadays, visual identification of individual snakes is based on the colouration pattern (Albu et al., 2008) or more commonly by the scale pattern of the pileus region (i.e. apicals, canthals, intercanthals, parafrontals, frontal, parietals and interparietals) (Benson, 1999; Stoyanov & Tzankov, 2017; Bauwens, Claus & Mergeay, 2018). However, the variability of these few large scales is limited, constraining the number of distinguishable identities. Here, we describe an alternative and more variable region of snake head scalation and illustrate a semi-automatic procedure (APHIS; Moya et al., 2015) used to successfully identify individuals of horseshoe whip snakes, *Hemorrhois*

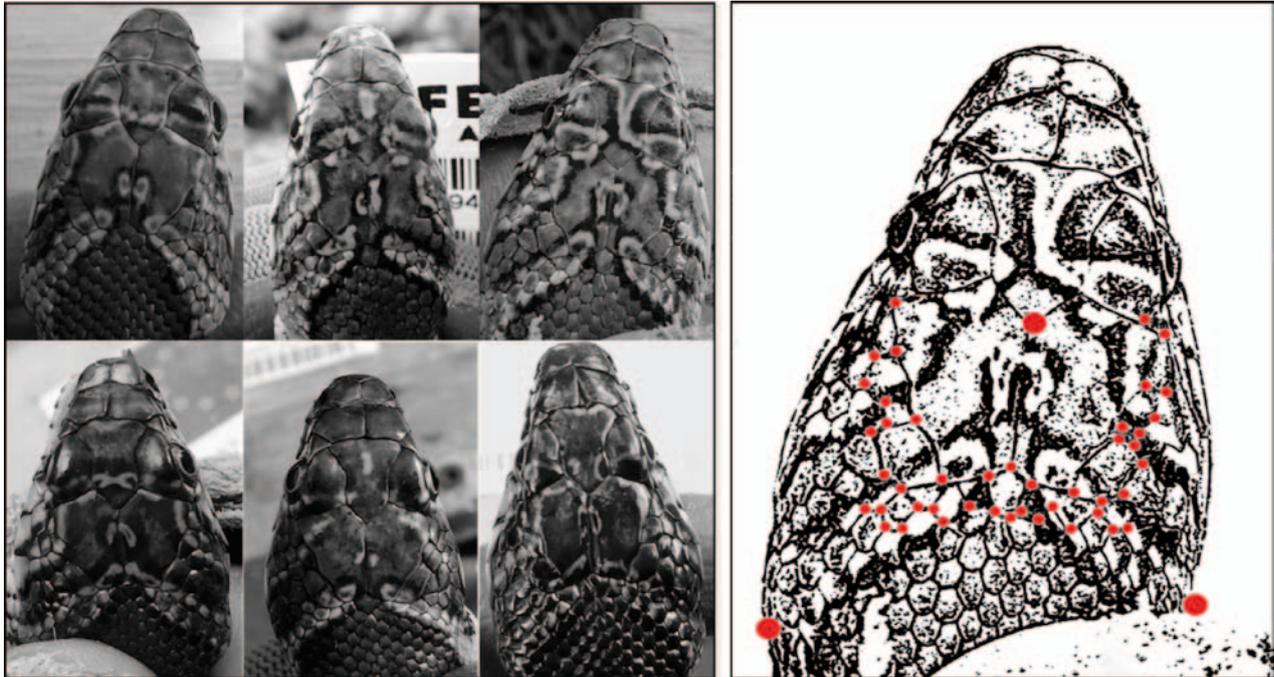


Figure 1. On the left, some photos of the head of horseshoe whip snake (*H. hippocrepis*) individuals where one can observe the scale pattern of the pileus region. On the right, picture showing the 3 reference points used (big dots) and the placement of the other points marking the intersections between scales.

hippocrepis. To our knowledge, this is the first time that a computer aided procedure has been used to identify snakes and may constitute an alternative to PIT tagging and scale marking.

The horseshoe whip snake is a long and slender-bodied colubrid, with a total length of up to 1800 mm, and no sexual dimorphism in body size. Populations are distributed in warm Mediterranean habitats of North Africa (northern Tunisia, Algeria and most regions of Morocco), and the eastern and southern half of the Iberian Peninsula. The invasive species was first reported on the island of Ibiza (Balearic archipelago, Spain) in 2003 inside Iberian olive trees (Álvarez et al., 2010). At present, this alien species represents one of the main threats to the native biota of Ibiza and several control campaigns have been carried out by the autonomic government. In 2018, a capture-mark-recapture study was conducted with the aim to quantify snake detectability and dispersal distance to optimise the control campaign. The study took place between the 17th of September and the 9th of November in 2018, where 50 traps were installed in five ha of a study area. Every three to five days, traps were checked at midday and all snakes trapped during the study period were photographed (head picture) with a digital camera, and marked with PIT tags to validate the photo-identification methodology. Finally, each snake was released near the trap in which it was captured.

Images were analysed using the I3S procedure in software APHIS (Moya et al., 2015), in which we delimit an area of interest with three reference points, then we use several additional landmarks inside this delimited area (in this study, at pre-defined scale intersections) to draw the scale pattern (see Van Tienhoven et al., 2007). The three reference points that we selected discarded

many of the large scales of the head (pileus region) commonly used in photo-identification, after we found that this region varied little (Supplementary Material; Fig. S1), and we included the first row of scales posterior to the two parietal scales for its more variable pattern. As a first reference point, we used the intersection of the frontal scale and the two parietals. The second and third reference points were the most distal scales of the left and right diagonal row below the parietals (Fig 1). Once the three reference points were marked, we placed 30–50 spots on scale intersections within this area of interest. These additional points define the pattern of scales of the area that will be used as a ‘fingerprint’ by APHIS (Fig. 1). An initial photo of each individual snake was entered into APHIS before any comparisons could be made. These photos comprise the repository folder that APHIS uses as a reference when matching new photos. Additional photos entered into APHIS will then contrast the resulting patterns with those in the repository and present users with a ranking of possible matches sorted by a score. APHIS presents a ranking of matches of all the individuals saved in the repository (up to 100 images). However, we considered that APHIS successfully matched two photos of the same individual if the match was within the top 10 candidate images (Gatto et al., 2018). After the analysis, we inspected the images identified as possible scored matches by APHIS and confirmed as either a recapture or a new individual. Photos were then saved in the APHIS repository, allowing APHIS to accumulate multiple photos for each individual when they are recaptured over time. This results in multiple photos of the same individual that may be used as comparison photos for future analysis. In order to avoid duplicates of the same individual in the scored matches list, APHIS only shows the best scored

photo of each individual in comparison with the new processed image.

A total of 26 captures were made during the study period, of which seven were recaptures from five different individuals. These seven recaptures were the photos that APHIS matched. All images were correctly matched (100 %) and in 85.7 % of cases, the match was within the first 10 ranked images proposed by APHIS (see Supplementary Material, Table S1). The average score was 0.366 and rankings for all pictures are out of 19 snakes (see Supplementary Material, Fig. S2). Scores were very tight indicating that defined patterns with points of two different images of the same individual are very similar to each other, although pictures were taken at different times (see Gatto et al., 2018). To sum up, head scale patterns in snakes provide a powerful tool to identify individuals and this method may be extended many species of snakes that combined with the use of specific software allow to optimise the identification process. The possibility to handle batches of images makes APHIS a good candidate but other software can be considered for the same purpose (see Sacchi et al., 2016).

In conclusion, photo-identification proved to be a reliable and non-invasive method to identify snakes based on scale patterns. Although this study has its limitations due to the small sample size, and further studies are needed to test correctly the reliability and accuracy of APHIS identifying individuals, it is a first step in the use of photo-identification software as a main identification method in snakes. Further development is needed and conventional methods may be still more reliable, especially when identifying many individuals. However, we show that scale patterns display potential as a robust identification method that should be considered when field studies are conducted; it is cheaper and less invasive than conventional PIT tag methods, in addition to involving minimal animal handling and null negative effects.

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