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Front cover: Male Hierophis viridiflavus involved in a male to male combat at MUD section of the study locality at Chizé west France. Photographed by Xavier Bonnet. See article on page 142.

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REVIEW PAPER



Killing them softly: a review on snake translocation and an Australian case study

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Human-wildlife conflicts with 'nuisance' snakes are becoming more frequent around the world as urbanisation continues to encroach on remaining habitats. In an attempt to mitigate this issue, snakes are often translocated in an uncontrolled fashion, with little to no conservation value. To determine the most appropriate methods of translocation we reviewed the available primary literature on studies performing translocations of snakes. We found two types of translocation: long and short-distance. Based on the welfare of the animals involved and difficulty of achieving success with long-distance translocations, we deduced that short-distance translocations are the most favourable. We also reviewed the literature on a third method - repatriating wild populations of snakes with captive-bred or captive-reared individuals, the results of which were very similar to those of long-distance translocations. In conjunction with a mark recapture study carried out by snake catchers in Darwin, Australia, we use our findings to make suggestions on the most appropriate course of action for the mitigation-based snake catching activities in Australia. The difficulty of ensuring successful outcomes for long distance translocations along with a high mortality rate meant we cannot suggest this as an appropriate method for managing 'nuisance' snakes. Instead, we argue that short distance translocations are the most suitable for the welfare of the snakes involved. Nevertheless, no outcome will be more favourable for the snakes than to be simply released within their home range accompanied by a change in attitude of the general public towards a willingness to coexist. Although we focus primarily on Australia our suggested framework can be applied in any country where there is conflict with snakes. Furthermore, should our suggestions be implemented, they are merely a temporary solution to an ongoing problem and we are in desperate need for further research to devise a long-term management plan.

Keywords: serpentes, translocation, relocation, repatriation, human-wildlife conflict

INTRODUCTION

lobally, there is a fear of snakes. Often this fear is G somewhat warranted considering that in many parts of the world, snakes are a significant cause of mortality to humans: up to 80,000 deaths per year, mainly occurring in the world's more remote, and less developed tropical areas (Kingsbury et al., 2019; Williams et al., 2019). This fear often results in people killing snakes that they encounter (Narayanan & Bindumadhav, 2019; Whitaker & Shine, 2000). Even in Australia, home to some of the most venomous snakes in the world (Mirtschin et al., 2017), where the number of deaths attributed to snake bites is extremely low (average two deaths per year; Johnston et al., 2017), the attitude that "the only good snake is a dead snake" is a widespread view held by many Australians (Whitaker & Shine, 2000). As urbanisation continues to encroach on remaining habitats around the world, human encounters with snakes are on the rise, especially since many snakes exploit urban or suburban

environments (Schlauch, 1978; Zappalorti & Mitchell, 2008; French et al., 2018). In an attempt to mitigate these conflicts, thousands of 'nuisance' snakes are translocated every year (Craven et al., 1998; Shine & Koenig, 2001; Brown et al., 2009). A few generalist snake species are good examples of this, such as carpet pythons (Morelia spilota) in Australia and water snakes (Nerodia sipedon) in the USA which have successfully exploited the abundant new shelter and prey opportunities available in urban areas (Fearn et al., 2001; Pattishall & Cundall, 2009). At the same time, habitat destruction due to urbanisation is a major cause of species decline for many of the world's taxa and this holds true for reptiles, a severely understudied taxonomic group of which 20 % is at risk of extinction (Wilcove et al., 1998; Sala et al., 2000; Todd et al., 2010; Böhm et al., 2013).

Translocation can be considered either as conservation translocation or mitigation translocation. Conservation translocation, focused on particular species of concern (Armstrong & Seddon, 2008), is often followed by

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monitoring of the translocated population. Mitigation translocation, however, tends to be targeted at humanwildlife conflict, with the aim of reducing wildlife mortality and danger or nuisance to people (Sullivan et al., 2015), and is rarely followed by monitoring (Massei et al., 2010). Mitigation translocation has a history of high failure rates (Sullivan et al., 2015), and this is likely to be particularly true for reptiles and amphibians where translocation of all types has resulted in successful outcomes in only 41 % of attempts (Germano & Bishop, 2009). Translocated reptiles appear to suffer high mortality rates relative to resident individuals, and this is often attributed to aberrant movement patterns, stress, disease, and inability to survive winters for species where finding adequate hibernacula is a priority (Nowak et al., 2002; Brown et al., 2008; Massei et al., 2010; Harvey et al., 2014; Sullivan et al., 2015).

While much data for conservation translocation is available in the primary literature, the data for mitigationbased reptile translocations, which are often ad hoc, either simply does not exist or is largely inaccessible (Germano et al., 2015). In Australia, a huge number of largely uncontrolled, mitigation translocations take place every year with the intent to reduce human-wildlife conflicts. For example, one voluntary organisation in New South Wales Australia, 'rescued' over 22,000 squamate reptiles during a ten year period, with the majority of snakes translocated to another locality (Shine & Koenig, 2001). However, with the evidence from scientific articles indicating that snakes and other reptiles react poorly to being translocated, and with mitigationbased relocations occurring globally and serving little to no conservation purpose, it has been suggested that regulations should be changed to match conservation outcomes (Germano et al., 2015).

The aim of this review is to evaluate from the literature the viability of different methods for translocating snakes. There have been some recent reviews on translocation for mitigation purposes and translocation of herpetofauna but we deemed it important to focus on snakes due to the large scale mitigation translocations happening around the world (Germano & Bishop, 2009; Germano et al., 2015). For the purposes of this review, translocation of snakes, whether for conservation or mitigation, is defined as the movement of animals, either individually or as groups, by humans, from one part of their range to another (Brown et al., 2008). We also include studies of repatriating wild populations of snakes from captivity. Finally, we present data from a two year mark-recapture study collected by snake catchers in Darwin, Australia, and in conjunction with the results from the literature review provide suggestions on how snake relocation efforts should be carried out. Although these suggestions are aimed specifically at Australia, they have the potential for global application.

Review of the literature on snake translocation

We reviewed scientific literature on snake translocation and repatriation projects. We searched the first 10 pages of Google Scholar and the Curtin University library database using the following search terms: "snake translocation", "snake long distance translocation", "snake short distance translocation", "snake repatriation", "snake headstarting", "snake mitigation translocation" and "snake conservation translocation". We also used reference lists and personal contacts to find articles. This resulted in a database of 65 publications. We then removed non-empirical (e.g. literature reviews), duplicate publications (e.g. university theses and their subsequent publications) and non-peer reviewed case studies or pre-prints. Finally, we screened for methodologies that aimed to evaluate the viability of short-distance translocation (SDT), long-distance translocation (LDT), or repatriation for their study species or what impact these processes would have on the species biology (e.g. space use). Following this, we were left with 34 publications comprising 38 studies (publications that included, for example, both LDT and SDT were treated as two separate studies) on 24 snake taxa, across 9 countries (Table 1). For each project we recorded the taxa studied, type of translocation (mitigation or conservation), whether the donor population was wild or from captivity, and the success of the project. Translocation success is hard to determine and it has been suggested that a translocation can only be considered successful if it results in a selfsustaining population (Dodd & Seigel, 1991; Griffith et al., 1989). However, it can require several years of monitoring to make such an observation which is beyond the remit of many of the studies we have compiled. Hence, for studies that did not fit this criterion, we considered the translocation a success if 70 % or more of the translocated individuals survived for the duration of the study, or if the survival probability of the translocated individuals was not significantly lower than that of the resident population. Studies that did not estimate survival or did not monitor appropriately to estimate survival were classed as uncertain.

We reviewed 34 projects comprising 19 long distance translocation studies, 11 short distance translocation studies, and eight studies releasing captive-born or reared snakes into the wild. Of the 19 LDT studies 37 % were successful, 47 % failed, and 16 % had uncertain outcomes (Fig. 1). 73 % of SDTs (11 studies) had successful outcomes while 19 % failed and 8 % had uncertain outcomes (Fig. 1). There were only a small number of studies on repatriation or releasing captive-reared snakes into the wild (eight studies) of which 40 % succeeded, 50 % failed, and 10 % were uncertain (Fig. 1). The most commonly reported causes of failure were attributed to aberrant movement behaviour and reduced overwinter survival (Table 1). The majority of studies were conducted in the USA predominantly on rattlesnakes

Long and short distance translocation

Long distance translocation studies were generally driven by conservation goals; the primary goal of short distance translocation studies was to mitigate humanwildlife conflict although this still has a conservation function. LDT is defined as the transport of an animal beyond its home range (Hardy et al., 2001), or at least

Table 1 Snake	translocation studies with	summarised aims and out	comes			Constant of	Aim of study	0	Defenses
	translocation studies with				Country	Species Western diamond-backed	Evaluate the effects of	Translocated snakes	(Nowak et al., 2002)
Country Antigua and Barbuda	Species Antiguan racer (Alsophis antiguae)	Aim of study Describe the methods of the successful reintroduction of the	Outcome The efforts increased the population size from 51 individuals on one island	Reference (Daltry et al., 2017)		rattlesnake (Crotalus atrox)	nuisance rattlesnake relocation	moved considerable distances and experienced 50 % mortality. Hom- ing behaviour was also observed.	
	Woma python (Aspidites ramsayi)	critically endangered species to several offshore islands Test the efficacy of reintroducing captive bred	to >1100 on four islands All individuals were predated by mulga snakes	(Read et al., 2011)			Assess whether SDTs resolve the human-wildlife conflict as well as LDTs do	SDTs were successful in easing the fears of the public and the translocated snakes quickly resumed normal	(Sealy, 1997)
Australia	Tiger snake (Notechis scutatus)	woma pythons Determine the effects of translocation on the spatial ecology of tiger snakes	(Pseudechis australis) Translocated snakes had much larger home ranges and travelled greater distances	(Butler et al., 2005a; Butler et al., 2005b)		Timber rattlernake	Locate critical habitats, determine causes of decline and study reproductive and spatial ecology	behaviours. SDT had a 100% success rate with adults quickly resuming normal behaviours and breeding successfully	(Sealy, 2002)
	Dugite (Pseudonaja affinis)	Assess the impact of translocation to resolve human-wildlife conflict for dugites	All translocated snakes died and travelled great distances before doing so.	(Wolfe et al., 2018)		(Crotalus horridus)	Assess the impact of translocation on timber rattlesnakes	Translocated snakes had higher mortality and aberrant movements compared to resident	(Reinert & Rupert, 1999)
China	White-lipped pit vipers (Trimeresurus albolabris)	To determine if LDT is viable conservation option	Aberrant movement patterns of females, reduced reproduction. High mortality when compared to residents.	(Devan-Song et al., 2016)			Test whether relocating an entire population to suitable habitat post winter emergence would allow for establishment in	snakes Snakes regained normal activity and foraging patterns after the second full activity season	(Walker et al., 2009)
India	King cobra (Ophiophagus hannah)	Study the effects of translocation on king cobras.	The translocated king cobra moved a lot more, ate less frequently and did	(Barve et al., 2013)		Western rattlesnake (Crotalus oreganus)	a new home range Effects of LDT on stress levels in male rattlesnakes	Increased testosterone and corticosterone	(Heiken et al., 2016)
Jamaica	Jamaican boa (Chilabothrus subflavus)	Determine the suitability of Jamaican boa for SDT	Female boas appeared to be able to establish new home ranges following SDT Translocated snakes bad	(Newman et al., 2019)			Use radiotelemetry to determine effectiveness of SDT	Multiple SDT did not affect survivability but did influ- ence activity patterns and snakes often returned to	(Brown et al., 2009)
South Korea	(Elaphe schrenckii)	ecology of resident and translocated individuals to design translocation projects	higher mortality, aberrant movements, and used different habitat structures compared to resident snakes			Northern pacific rattlesnakes (Crotalus oreganus)	Test if repeated SDT and handling represent thermal stressors	point of capture SDT and repeated handling does not have major adverse effects on the thermal ecology	(Holding et al., 2014)
Sweden	Grass snake (Natrix natrix)	Evaluate movement and habitat use during egg laying period and if translocated snakes	Translocated snakes moved more than residents but used same habitats	(Elmberg et al., 2019)		Red diamond rattlesnake (Crotalus ruber)	Study the effects of translocation on movement and survivorship	If excluding deaths from complications with surgery there was a low mortality rate in both SDT and LDT	(Brown et al., 2008)
		have different movement behaviour compared to resident snakes				Eastern indigo snake (Drymarchon couperi)	Establish a viable population in Conecuh National Forest and test most suitable release	High mortality rate in the first year but survivability increased in the second year post release. Hard	(Godwin et al., 2011)
		Evaluate the adaptability of potentially gravid female grass snakes	Translocated snakes had larger home ranges and remained close to familiar habitat types	(Pettersson, 2014)			method	release had a higher survivability than soft release.	
UK	European adder (Vipera berus)	Ascertain the effects of translocation on the spatial ecology of adders	Translocated males on average moved more every day than resident males. Males also	(Nash & Griffiths, 2018)		Hognose snake (Heterodon platirhinos)	Assess viability of translocation for conservation	Translocated snakes had higher mortality and aberrant movements compared to resident snakes	(Plummer & Mills, 2000)
			exhibited uni-directional movements away from release site, and one male even displayed homing behaviour. Females tended to stay within 50m of release site			Northern water snake	Compare individuals translocated from wild population with individuals translocated from captive populations	Both showed low survivorship with captive reared snakes showing aberrant behaviours and both cohorts had low overwinter survival	(Roe et al., 2010)
	Barred grass snake (Natrix helvetica)	Establish what proportion of translocated reptiles remained within receptor sites	Negligible recapture rate.	(Nash et al., 2020)		(Neroula sipeaon)	Test if enrichment during captivity improves the success of captive reared snakes in the wild	Elaborate enclosures may not have affected survival of captive reared snakes but brumation in captivity may have	(Roe et al., 2015)
USA		Examine the utility of LDT for managing eastern diamond-backed rattlesnakes	High survival rate, limited aberrant movements because the source population had small home ranges	(Jungen, 2018)		Ratsnake (Pantherophis obsoletus)	Test if enrichment offsets negative effects of captiv- ity prior to translocation	Captivity negatively affected survival and enrichment did not offset this. Wild translocates temporarily had aberrant	(DeGregorio et al., 2017)
		Examine the effectiveness of LDT on eastern diamond-backed rattlesnakes.	Translocated snakes had larger home ranges but second year survival probability was not significantly different from non-translocated individuals	(Kelley, 2020)				movement patterns but did not affect survival	

Country	Species	Aim of study	Outcome	Reference
	Burmese python (Python molurus)	Study the movement and activity patterns of pythons in Florida	Burmese pythons are capable of homing after being displaced at an incredibly large scale	(Pittman et al., 2014)
		Repatriate an area where massasaugas used to exist from a captive population and carry out short distance translocations to assess how this species responds to SDT	All captive born snakes did not survive the winter. SDT snakes moved more than resident snakes but were not negatively affected by SDT.	(Harvey et al., 2014)
	Eastern massasauga rattlesnake (<i>Sistrurus catenatus</i>)	Test if soft release improves survival rates for mitigation translocation	No difference in survival between soft release and hard release but had significantly lower survival than resident snakes	(Josimovich, 2018)
		Evaluate translocation of wild snakes versus repatriation with captive snakes	Snakes released in summer had a higher survival rate.	(King et al.,2004)
	Plains gartersnake (Thamnophis radix)	Evaluate the effectiveness of headstarting versus releasing neonates	Some offspring born in captivity succeeded in reproducing either when released as newborns or following headstarting	(King & Stanford, 2006)



Figure 1. Outcomes of translocation and repatriation projects for 37 studies. The number above each category indicates the number of studies.

twice the distance the animal could cover in a straight line over the course of one year (Nowak et al., 2002), but extensive translocation distances (e.g. 50 km) have been reported (Clemann et al., 2004; Holding et al., 2014). SDT is defined as the relocation of an animal within or near its home range (Hardy et al., 2001; Brown et al., 2009). For LDT studies, we found a trend of high mortality reported across a wide range of species including hognose snakes (Heterodon platirhinos; Plummer & Mills, 2000), Amur ratsnakes (Elaphe schrenckii; Lee & Park, 2011), whitelipped pit vipers (Trimeresurus albolabris; Devan-Song et al., 2016), dugites (Pseudonaja affinis; Wolfe et al., 2018) and many others (Table 1). These species include a wide range of ecological types - active hunters, ambush hunters - but they all experienced high mortality, sometimes within days of translocation. Many factors are likely to influence this observed increased rate of mortality; however, increased predation is probably the most common cause of snake mortality in LDT (Sullivan et al., 2015; Teixeira et al., 2007) which can be attributed to the aberrant movement behaviour (Devan-Song et al., 2016) displayed in almost every study. Snakes subjected

to LDT moved more frequently and covered greater distances (Jungen, 2018; Nowak et al., 2002), explored different habitat features (Lee & Park, 2011), and, in some cases, males and females displayed different movement behaviours (Brown et al., 2008; Devan-Song et al., 2016). Road mortality was also a common cause of mortality and is almost certainly related to aberrant movement after translocation (Sealy, 2002; Wolfe et al., 2018)

LDT comes with other problems: snakes distributed in temperate zones of higher latitudes require hibernacula to survive the winter (Shine & Mason, 2004) and when snakes are relocated to a completely unfamiliar area finding a suitable place to overwinter can be a limiting factor to their survival (Reinert & Rupert, 1999; Lee & Park, 2011; Shonfield et al., 2019). Disease is also likely to be an important consideration when translocating snakes outside of their home range as the introduction of a diseased animal to an area that already supports a population could be detrimental to many more individuals than just the ones being translocated (Nowak et al., 2002; Brown et al., 2008; Suarez et al., 2017). Introducing animals into an area with an existing population might also push the number of individuals in the already established population above their ecological carrying capacity (Germano et al., 2015). When translocating individuals from a disjunct population into an existing one there is the potential for introducing deleterious genetic effects and causing outbreeding depression if the two populations are genetically divergent (Whiting, 1997). Similarly, if translocating individuals into an area of suitable habitat but without an existing population, this new population may undergo a genetic bottleneck or inbreeding depression from a loss of genetic diversity (Gautschi et al., 2002; Újvári et al., 2002; Daltry et al., 2017).

Despite all these issues, LDT is the favoured option both for conservation, if managed appropriately posttranslocation (Daltry et al., 2017), and for mitigating human-wildlife conflict because the possibility of future conflict with humans is greatly reduced (Brown et al., 2009). Despite the high proportion of failed attempts with LDT's there are success stories from species such as eastern diamondback rattlesnakes (Crotalus adamanteus; Jungen, 2018) and timber rattlesnakes (Crotalus horridus; Walker et al., 2009) where the practitioners went to great lengths to understand the phenology and ecological requirements of the species and relocated them to suitable habitat absent of conspecifics. Western rattlesnakes (Crotalus ruber) also managed to locate dens, forage, mate, and establish new home ranges (Brown et al., 2008). Another example of a successful LDT is the reintroduction of the Antiguan racer (Alsophis antiquae) to offshore islands where the practitioners had removed all predators and the populations were continuously monitored (Daltry et al., 2017).

In comparison, at first glance SDT seems to be a much more favourable option than LDT when considering ecological, genetic and disease transmission concerns (Dodd & Seigel, 1991; Reinert, 1991; Sealy, 2002). However, snakes that undergo SDT still demonstrate an increase in movement and activity patterns which can, in part, be attributed to exploratory behaviour, but is often associated with snakes attempting to return to the point of capture (Brown et al., 2009; Germano & Bishop, 2009). This homing behaviour occurs in many species regardless of their ecology; e.g. European adder (Vipera berus; Nash & Griffiths, 2018), western rattlesnake (Crotalus oreganus; Brown et al., 2009), and tiger snake (Notechis scutatus; Butler et al., 2005a), all three of which appear to have otherwise relatively small home ranges. It has been demonstrated through LDT that some snakes will travel extreme distances in an attempt to return to their home range (Pittman et al., 2014). Homing behaviour is a problem in the context of SDT because it does not resolve the human-wildlife conflict when snakes have been translocated for mitigation purposes and return to the area of conflict (Germano et al., 2015; Sullivan et al., 2015). Even if the translocation itself did not negatively affect the survival of the snakes (Brown et al., 2009) the common lack of post-release monitoring for SDT does not allow us to draw robust conclusions on the long-term effectiveness in helping snakes. However, though SDT does disrupt the normal behaviour of snakes, some species have been shown to be capable of habituating to a new home range, e.g. Jamaican boas (Chilabothrus subflavus; Newman et al., 2019) and massasauga rattlesnakes (Sistrurus catenatus; Harvey et al., 2014) while timber rattlesnakes (Crotalus horridus) actively avoid contact with humans within their chosen habitats post-translocation (Sealy, 1997). SDT for many species could be a viable management strategy, at least in the short-term, but it requires extensive knowledge of species' requirements and the efforts put into successful release in a suitable area needs to be combined with a willingness of communities and the public to coexist with snakes (Sealy, 2002).

Reintroduction from captive population

Capturing wild snakes and breeding them in captivity

to reintroduce a population into the wild is of growing interest in conservation (Germano & Bishop, 2009). The primary aim of these studies is to use captive reared snakes to re-establish populations where they have been extirpated or to bolster existing populations (King et al., 2004; Roe et al., 2010). Several studies have attempted releasing snakes born in captivity after 'headstarting' - raising them to a certain age so they are less vulnerable than are neonates prior to release. Studies on woma python (Aspidites ramsayi; Read et al., 2011), eastern indigo snakes (Drymarchon couperi; Godwin et al., 2011), and massasauga rattlesnakes (Harvey et al., 2014; Josimovich, 2018) have even included soft-release techniques where snakes were released in temporary outdoor fenced areas to assist the captive-reared snakes. Interestingly, all the woma pythons were depredated and soft-released eastern indigo snakes and massasauga rattlesnakes fared worse than or the same as hard-released individuals. A lack of prior experience in captive-born and headstarted eastern water snakes impacts their behaviour as such snakes were incapable of thermoregulating or selecting adequate hibernacula to survive winter, had limited movements, and uncharacteristically spent the majority of their time in terrestrial environments (Roe et al., 2010). It has been suggested that the deleterious effects imposed on snakes during their time in captivity such as aberrant behaviour could potentially be mitigated by providing enrichment with naturalistic enclosures during the headstarting process (Roe et al., 2010) but this has proven to be ineffective, at least in the relatively short period enrichment was provided (Roe et al., 2015). Similar studies on ratsnakes (Elaphe obsoleta) showed that adult snakes captured in the wild and then held in captivity had a reduction in their ability to detect and react to prey after extended periods of time in captivity (DeGregorio et al., 2013) and that this deleterious effect was not mitigated with enrichment during captivity either (DeGregorio et al., 2017). Simulating an overwintering period in captivity may have contributed to improved overwinter survival for headstarted eastern water snakes in the wild; although the death of several snakes during this brumation process could have resulted in the eventual release of only individuals better suited to survive the winter (Roe et al., 2015).

Moderate success has been recorded in studies releasing headstarted individual tiger snakes (Aubret et al., 2004; Shine & Bonnet, 2009), massasauga rattlesnakes (King et al., 2004) and both neonate and headstarted plains gartersnake (Thamnophis radix) (King & Stanford, 2006), all of which had a relatively high survival rate. Releasing captive-born snakes while they are still neonates may be a better option as it is time and cost effective. Releasing neonates allows for natal habitat preference induction (NHPI; Davis & Stamps, 2004) where the released neonate snakes gain experience in selecting shelters, foraging for food and avoiding predators (Roe et al., 2010). Few studies have assessed the survival rate of neonates released immediately back into the wild after birth in captivity, but, this has been shown to be a

successful tactic with royal pythons (Python regius) where gravid females are caught in the wild and the eggs are collected in captivity; the females are then released and when the eggs hatch 10 % of the offspring are released to maintain the local population while the rest go into the wildlife trade (Aubret et al., 2003; Shine, 2009). Several studies conducting headstarting programs agree that when retaining young for captive rearing a proportion of neonates is to be returned to the capture site to minimise the effects on donor populations (King et al., 2004; Roe et al., 2010).

Australian case study

Australia is home to a plethora of snake species, some of which occur in high abundance in urban areas. As a consequence, human-snake conflicts are commonplace throughout Australia (Clemann et al., 2004; Shine & Koenig, 2001). Conflicts are generally mitigated by private snake catchers who obtain permits from their relevant state wildlife departments. These permits allow individuals to translocate 'nuisance' snakes at the request of the public on a fee for service basis (New South Wales Government, 2019). Such a license can be obtained with ease across most of the country; in most cases a person simply needs to be trained in first aid and complete a venomous snake handling course (Queensland Government, 2019), which is often a single day event using captive snakes that often do not reflect the behaviour of wild conspecifics (J.C., pers. obs.). In some cases, as per the recent amendment of the regulations in the Biodiversity Conservation Act, a permit is no longer required to take reptiles in certain situations in Western Australia (Western Australia Government, 2018).

In the Northern Territory (NT), snake catching services are jointly managed by the Parks & Wildlife Commission of the NT (PWCNT) and private contractors. For the NT's three main population centres (Darwin, Alice Springs and Katherine), snake catchers are employed under contract agreements with the PWCNT. These contracts are advertised to the private sector on a three-year basis with a suitable applicant chosen based on their experience, capability and the competitiveness of their quote (T.P., pers. obs.). This system is highly valued by the community because snake removal services are reliable, available at all hours and free of charge to local residents and businesses.

Darwin is the capital city of the Northern Territory, situated in the wet-dry tropics (Fig. 2). It is Australia's least populated capital city (132,054 people; ABS 2016) and boasts the highest diversity of snake species (35 species; Zozaya & Macdonald, 2017). The greater Darwin area covers a sprawling urban-rural gradient of approximately 550 km², and is bordered by a number of conservation reserves and crown land estates. Human-snake conflicts here are common: between 2011 and 2017, contract snake catchers attended between 631 and 851 callouts per year (Parkin et al., 2020). The vast majority (98%) of snakes caught in Darwin are not considered dangerous to humans, with dangerously venomous species such as

northern brown snakes (Pseudonaja nuchalis) accounting for only a small proportion of total callouts (2 %; Parkin et al., 2020). Venomous snakes captured in urban areas were translocated outside of town boundaries due to concerns about public safety, and as per the stipulations of snake catching permits (PWCNT, 2017). For most harmless snakes, translocations were often deemed unnecessary. Once a snake was identified as nonvenomous and posing no threat, the public were usually content to leave it alone. However, translocations did occur if the snake was, a) threatening domestic pets or poultry, b) found living inside a home or building, or c) injured or in immediate danger of being killed (i.e. from a dog, cat, or human). Non-venomous snakes were usually translocated to the closest uninhabited parcel of crown land or conservation reserve (typically under 1-2km). Release sites were chosen based on suitability of the habitat, and the presence of immediate sheltering opportunities. Between September 2016 and December 2017, Darwin Snake Catchers, a business contracted by the NT Government, undertook a mark-recapture study to assess rates of recapture for translocated snakes. Data from this preliminary study is presented herein.

During the mark recapture study, 464 snakes of five predominant species were individually marked with ventral scale clips, weighed, measured and sexed. The coordinates of the snake's original capture site, translocation site, and recapture site were recorded using Sightings (Macdonald, 2013), an ecological datacollection app for iPhone. To determine whether a translocation was SDT (within the species expected home range) or LDT, we examined previous radio-telemetry studies on the same or similar species to estimate expected home range sizes. Unfortunately, no data was available on the home range size of Children's pythons (Antaresia childreni), olive pythons (Liasis olivaceus) or common tree snakes (Dendrelaphis punctulatus). Some species of pythons e.g. water pythons (Liasis fuscus) and carpet pythons, have been found to undergo a seasonal shift in home range size associated with mate searching activity and migrations of their prey, while black-headed pythons (Aspidites melanocephalus) can move considerable distances (>500 m) in a single day (Heard et al., 2004; Johnson et al., 1975; Madsen & Shine, 1998; Slip & Shine, 1988). Slaty-grey snakes (Stegonotus cucullatus) at Fogg Dam on the outskirts of Darwin have been found to retain a relatively small home range throughout the year, despite their habitats becoming seasonally inundated in the monsoon season (Brown et al., 2005). Given the limited published data available on home range sizes for our focus species, we broadly defined translocations <1500 m as SDT and translocations of a greater distance as LDT. This method has clear limitations because home range sizes may vary intraspecifically (Madsen & Shine, 1998), and the available data was also collected from individuals living in natural rather than urban environments. Snakes living in urban areas may behave differently to conspecifics living in natural environments (Pattishall & Cundall, 2008) and therefore we must acknowledge that our



Figure 2. Map of Darwin with black lines indicating boundary of contracted snake call-out services (PWCNT, 2016).

Table 2. Number of snakes marked and recaptured

Species	Marked	Recaptured
Antaresia childreni	57	3
Aspidites melanocephalus	3	1
Boiga irregularis	2	0
Dendrelaphis punctulatus	79	2
Furina ornata	1	0
Liasis fuscus	94	8
Liasis olivaceus	27	5
Morelia spilota	119	17
Stegonotus cucullatus	78	2
Tropidonophis mairii	4	0

definition of what constitutes a 'short distance' is merely an assumption.

Of 464 marked snakes, only 8 % were recaptured (Table 2) either during call-outs for snake relocations or opportunistically while driving around Darwin. This included a total of 44 recapture events of 7 species and 38 individual snakes. The majority of the recaptured snakes had been subjected to SDT (92 %) of which a few were released on site within their home range (8 %). Very few recaptured snakes had been subjected to a LDT (8 %). 15 % of the recaptured snakes were either found dead or had to be euthanised and in one case. the snake was observed being killed by a member of the public (Table 3). All of the deceased snakes except for one had been subjected to SDT and all of them had been killed as a result of human activity, either run over by a car, attacked by dogs, or directly killed by a person. Considering all of these records were incidental, as none of the snakes were radio-tracked, the mortality rate of 15 % should be viewed as a conservative minimum and may indeed be much higher. 52 % of the recaptured snakes had displayed some kind of homing behaviour, defined as either heading away from their translocation point in the direction of the original point of capture or by being recaptured at the original point of capture (Table 3). All recaptured snakes that were translocated to nearby bushland subsequently returned to urban areas, with at least seven individual snakes returning to exploit anthropogenic prey sources such as domestic chickens or caged birds. One individual olive python was recaptured three times in the same suburb preying on caged birds. When examining homing behaviour by species it appears that primarily nocturnal, ambush hunting pythons (Children's python, water python, olive python, and carpet python) were likely to home when translocated (Fig. 3). Due to the small sample size of the remaining snake species (black-headed python, common tree snake, and slaty-grey snake) it is difficult to draw any conclusions concerning homing behaviour without speculating (Fig. 3), although it is unsurprising that the single specimen of black-headed python did not home considering this species can move large distances and are thought to be active foragers (Bedford, 1996; Johnson et al., 1975; Swan & Harvey, 2019). The low number of recaptured slaty-grey snakes also does not let us draw any conclusions on their tendency to home; however, due to their small home ranges at Fogg Dam, without a seasonal shift in range size, homing behaviour is probable. Furthermore, we did not omit the accidental release of a slaty-grey snake and the two potentially translocated carpet pythons where the identifying marks may not have been noticed; if we had removed them from the data, the trend of homing behaviour would have been even greater (Table 3, Fig. 3). The high proportion of snakes that displayed homing behaviour reflects the findings of many translocation studies around the world (Germano & Bishop, 2009) and is a strong indication that more information is required on the management of 'nuisance' snakes in Australia, and globally. The interspecific variation in homing behaviour also shows that regulations on translocated snakes need to be tailored to the ecological requirements of species. Although our sample size is small and conclusions broadly speculative, we would like at least to bring attention to the fact that the three recaptured snakes that were released within their home range had moved 100 m or less at the time of recapture, while all snakes that were translocated a 'short distance' still moved several hundred meters (Table 3). The data collected by snake catchers in Darwin has proven useful for examining patterns of human-snake conflict, as well as revealing information about the urban ecology of snakes. Their study could be used as an example to set the standard for other snake catching activities around Australia.

Summary and suggestions for the future

Globally, wildlife agencies have been largely unsuccessful at documenting the success or otherwise of mitigation translocations and consequently managing these activities with appropriate conservation outcomes



Figure 3. Percent of recaptured individuals that did or did not display homing behaviour. AC = Antaresia childreni, AM = Aspidites melanocephalus, DP = Dendrelaphispunctulatus, LF = Liasis fuscus, LO = Liasis olivaceus, MS = Morelia spilota, SC = Stegonotus cuculatus. Number above each category indicates sample size.

(Germano et al., 2015). In Arizona, USA, for example, thousands of rattlesnakes are translocated to the urban edge every year without surveying for habitat suitability, resident population viability, or post release monitoring (Sullivan et al., 2015), a situation which is mirrored with other species around the world. Mitigation translocations have been criticised for lacking robust evidence to support their use as an effective conservation management tool (Germano et al., 2015; Sullivan et al., 2015; Bradley et al., 2020), this can be attributed to the lack of published data on unsuccessful translocations (Berger-Tal et al., 2020) or the lack of appropriate data being collected in the first place (Nash et al., 2020). Human-snake conflict is also common issue in Africa, Asia and South America and the associated management tactics are broadly similar to those of Australia. The removal of nuisance snakes in these parts of the world are carried out by various actors, including non-profit organisations (Deshmukh et al., 2015; Hauptfleisch et al., 2020), independent snake catchers or 'rescuers' (Yue et al., 2019), dedicated government agencies (Teixeira et al., 2015) or combinations thereof. Unfortunately, a lack of training, equipment, staff, policy guidelines (Roshnath & Jayaprasad, 2017) or adequate pre and post-release monitoring (Teixeira et al., 2015) to carry out these mitigation services effectively is of major concern. Wildlife agencies in Australia are in a position to take a step in the right direction by collecting high quality data on regional human-snake conflict that could be reviewed and implemented in improved management practices, revised policies and permit conditions. By doing so these agencies can evaluate whether the current state of affairs is appropriately protecting the species they are responsible for and in turn any advancements made in management practices may be applied elsewhere. Based on some of the practices incorporated by snake catchers in Darwin, we have devised a framework that would address some of these issues:

1. If translocated snakes are marked, age is determined (juvenile, sub-adult, adult), sexed, coordinates recorded for point of capture, release and recapture, and all of this data was compiled in a database. we would be able to develop better management practices tailored to the ecological needs of species.

- 2. The accuracy of data collected by snake catchers could be greatly improved with the use of simple smartphone-based data collecting apps such as Sightings (Macdonald, 2013), which records an accurate GPS location of capture and translocation site, date and time the snakes were caught, and allows records to be easily uploaded to wildlife authorities. This would streamline the reporting requirements of snake catchers, allow state wildlife departments the ability to examine temporal and spatial patterns of human-snake conflict in regional areas, and monitor these mitigation translocations.
- 3. Likewise, state governments or local councils could take more responsibility for human-snake conflict as being a community and urban conservation issue. The situation may be improved by providing guidelines to snake catchers on appropriate translocation methods, improving reporting requirements and data management, making permit acquisition more competitive, and relieving the burden on the general public who pay for snake catching services by subsidising the role of professional snake catchers, such as in Darwin.
- 4. Together with a transition to a framework such as this, research institutions would ideally also conduct radio-telemetry studies on the most commonly translocated snakes such that we gain proper insight to the movement patterns and mortality rates of individuals that are released within their home range or undergo translocations and whether they are capable of establishing new home ranges.

Researchers attempting to determine whether LDT is a viable option for snakes need to have a comprehensive understanding of the candidate species before translocations take place. Practitioners of successful conservation LDT studies have gone to great lengths to ensure the survival of the species they were studying by understanding their ecological requirements and taking great care in selecting suitable release sites (Jungen, 2018); creating artificial shelter sites, baiting them to attract prey and only releasing snakes at the appropriate time of year to aid in overwinter survival (Walker et al., 2009). Once the animals were translocated they were monitored for extended periods of time and possible predators were managed (Daltry et al., 2017). With LDT it is almost impossible to choose an area that looks appropriate, release the snakes and simply expect them to survive without such research. Therefore, we cannot recommend LDT for mitigation purposes. If it became clear that translocation of any kind is not appropriate for managing certain species, taking these animals into captivity for breeding purposes and releasing neonates into the wild to maintain the population could be considered (Aubret et al., 2003; King et al., 2004; Shine, 2009). Despite the probable high mortality rates, which

is what would likely occur naturally, it would allow the surviving snakes to gain experience in their environment (Davis & Stamps, 2004). We suggest that in terms of the welfare of the animals being translocated, SDT is the most favourable option to mitigate human-wildlife conflict, although some authors of previous studies argue the contrary because of homing behaviour. Even so SDT should be considered as a last resort to be used only for snakes that are potentially dangerous. Evidently, snakes found within a residence or building, or those threatening domestic pets and livestock, should be removed to reduce conflict but we advocate that for species that pose no threat to humans, release should be within the immediate vicinity of where they were caught (e.g. at the back of the garden). For snakes that are deemed too dangerous to be left in the garden and are translocated, even if it is only a short distance, great care should be taken in selecting an area where the snakes do not need to cross any major roads should they attempt to return to their home range. We acknowledge that homing behaviour is an issue but snakes can be incredibly hard to find, even by trained and experienced herpetologists (Boback et al., 2020). If a high standard of snake relocation service were to exist that does not incur a cost to the public, relocating the same snake multiple times should not have a negative impact on the public or the snakes involved. Given the low number of recaptured snakes in Darwin, even if the majority of translocated snakes did return to the original conflict area, they appeared to remain out of sight and out of mind. The general public could further reduce the likelihood of encountering a snake by maintaining their garden and ensuring that their outdoor bird enclosures, should they have them, are snake proof (Bush et al., 2007).

Ultimately, public education and community engagement are probably the most effective tools to resolve the conflict between humans and snakes (Sealy, 1997), particularly for places like urban Australia where the risk of snake bite is relatively low, and the public are naïve or uninformed about snakes in their area (Wolfe et al., 2020). People need to be willing to accept that even though they live in an urban world "nature is not a separate domain hiding away in the wilderness" (Low, 2002) and animals live around them and don't need to be removed simply because their presence is unwelcome (Shine & Koenig, 2001). Easing this discomfort appears to be the main objective of the mitigation translocations snakes are subjected to, and very little attention has been given to the ecology, behaviour, and habitat requirements to ensure survival of translocated individuals (Sullivan et al., 2015). Although the success rate of reptile conservation translocations has improved (Germano & Bishop, 2009), translocations for mitigation purposes have received far less scientific scrutiny and few advancements have been made in their application (Germano et al., 2015). A significant improvement would be to uphold mitigation translocations to the same guidelines described by the IUCN for conservation (IUCN/SSC, 2013; Sullivan et al., 2015).

Although we have focused heavily on Australia, as this

Table 3. Distance travelled by recaptured snakes. Distance was calculated as a straight line between translocation and recapture points. ⁺ Deceased snakes, [‡] snakes displayed homing behaviour, [§] snakes were recaptured at original capture point, * snakes potentially relocated by park rangers without noticing marks. (HR=released in home range, SDT=short distance translocation, LDT=long distance translocation)

Species	ID	Translocations	Distance travelled (m)
Antaresia childreni	AC117a ^{+§}	1 SDT	350
A. childreni	AC127 ⁺	1 SDT	323
A. childreni	AC215 [±]	1 SDT	318
Aspidites melanocephalus	AM111	1 SDT	4817
Dendrelaphis punctulatus	DP114	1 SDT	430
D. punctulatus	DP117	1 SDT	399
Liasis fuscus	LM121§	1 SDT	466
L. fuscus	LM124 [‡]	1 SDT	802
L. fuscus	LM146§	1 HR	74
L. fuscus	LM152	1 SDT	560
L. fuscus	LM167a [‡]	1 SDT	339
L. fuscus	LM219	1 SDT	1249
L. fuscus	LM224 ^{†§}	1 SDT	1109
L. fuscus	LM80-30-4§	1 HR	0
Liasis olivaceus	L0112 [‡]	2 SDT	850
L. olivaceus	L0118 [‡]	2 SDT	737
L. olivaceus	LO124 ⁺	1 SDT	491
L. olivaceus	LO126	3 SDT	926
L. olivaceus	LO212 [§]	1 SDT	1454
Morelia spilota	MS114*	1 SDT	3802
M. spilota	MS128 [‡]	1 SDT	380
M. spilota	MS129	1 SDT	168
M. spilota	MS137 [‡]	1 LDT	1663
M. spilota	MS138 [‡]	1 SDT	668
M. spilota	MS179	2 SDT	875
M. spilota	MS213§	1 LDT	1449
M. spilota	MS222	1 SDT	375
M. spilota	MS226*	1 SDT	2465
M. spilota	MS228§	1 SDT	836
M. spilota	MS234 [‡]	1 SDT	1068
M. spilota	MS235	1 SDT	356
M. spilota	MS236 [§]	1 SDT	568
M. spilota	Ms237 ⁺	1 SDT	30
M. spilota	MS253	2 LDT	3000
M. spilota	MS254 [±]	1 SDT	837
M. spilota	MS268	1 LDT	625
Stegonotus cucullatus	SC123 ⁺	1 LDT	41
S. cucullatus	SC226 [§]	1 HR	100

is where our case study was conducted, the framework we have suggested is not limited to Australian application; any country experiencing human-wildlife conflicts with snakes is in a position to benefit. Furthermore, should our suggestions be implemented, they are merely a temporary solution to an ongoing problem as urbanisation will further encroach on the habitat of wild reptiles. To ensure these animals' survival amidst the destruction of their habitat, it is critical for us to develop tools and management plans that can suitably deal with this issue.

Conflict of interest statement:

We have no conflict of interests to declare.

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Potential distribution of the endemic Short-tailed ground agama Calotes minor (Hardwicke & Gray, 1827) in drylands of the Indian sub-continent

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The Short-tailed ground agama or Hardwicke's bloodsucker Calotes minor (Hardwicke & Gray, 1827) is known to occur in the Indian subcontinent and is largely confined to arid to semiarid environments, such as hard barren desert and abandoned fields. The precise distribution of this species is largely unknown to date, with few locality records spread biogeographically across Eastern Pakistan, Central and Western India. To improve on the existing spatial knowledge on this species and assess the ability to predict species distributions for taxa with few locality records, we studied the distribution of C. minor using a species distribution modelling framework. Our study allowed us to predict the distribution range of C. minor and help define a niche for this habitat-specific species. Highly probable habitats for C. minor were arid and semi-arid dryland habitats, characterised by plains or less rugged terrain with moderately narrow temperature range, lower aridity index, moderate to low vegetation index, and wide precipitation range. Furthermore, we report four additional occurrence records of C. minor from central Rajasthan.

Keywords: Agamidae; small sample size; environmental niche modelling; distribution range; arid environment

INTRODUCTION

The genus Calotes Cuvier, 1817 comprises 25 species, with the Indian subcontinent harbouring 11 of them (Uetz et al., 2020). The genus can be identified by its laterally compressed body, naked tympanum, presence of dorsal crest, equal-sized scales on back and sides of the body, well-developed gular sac, absence of femoral or preanal pores and round swollen tail-base in males (Günther, 1864; Smith, 1935). Xeric grasslands of the Indian subcontinent are home to two species of Calotes: the Short-tailed ground agama Calotes minor (Hardwicke & Gray, 1827) (Fig. 1) and the Oriental garden lizard Calotes versicolor (Daudin, 1802) (Patel & Vyas, 2019). Previously, C. minor had an unclear taxonomic position, which was disputed among the genera Agama (Hardwicke & Gray, 1827; Smith et al., 1935), Brachysaura (Blyth, 1856; Günther, 1864; Stoliczka, 1872; Moody, 1980; Manthey & Schuster, 1999; Das, 2003; Khan, 2006; Khan & Kumar, 2010; Ingle et al., 2012), Calotes (Blyth, 1856), Charasia (Boulenger, 1885), Laudakia (Das, 1994; Murthy, 2010), and Acanthosaura (Boulenger, 1885). The resolution of its taxonomic status was achieved by Deepak et al. (2015), who studied the morphology of C. *minor* including osteology and hemipenis preparations supported with molecular data to place the species in



Figure 1. Calotes minor: new records from central Rajasthan. 1A & 1B) C. minor (individual 2 from Nasirabad, Ajmer, Rajasthan) in an agricultural field. 1C) C. minor (individual 3 from Khamor, Bhilwara, Rajasthan) in an agricultural field.

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the genus Calotes (subfamily Draconinae; Deepak et al., 2015). They also ascertained the short tail length and relatively shorter fifth toe when compared to other Calotes as key identification features for the species (Deepak et al., 2015). Assessed as Data Deficient on the IUCN Red List of Threatened Species in 2009 (Böhm et al., 2013; Khan & Papenfuss, 2016), the species is currently undergoing reassessment (M. Böhm pers. comm.). Given the previously uncertain taxonomy and lack of records of C. minor, there is a notable gap in the understanding of its distribution and niche ecology. It is thus vital to determine the distribution and niche of this species to improve our knowledge of its conservation status.

It is known that several biotic and abiotic factors define a species' niche, and can thus be applied in the predictive distribution of species using an ensemble of techniques called Environmental Niche Modelling (Hirzel & Le Lay, 2008; Warren & Seifert, 2010). Also known as species distribution models (SDMs), niche models can predict the probability of species occurrence based on the life history and ecological needs of the species, expressed through the relevant environmental variables in the localities where the species have been recorded (Guisan & Thuiller, 2005; Elith et al., 2006; Elith et al., 2010; Singh et al., 2015). However, presence records and distributional information for secretive, cryptic, fossorial and difficult to observe species are often scarce (Pearson et al., 2007). For these species, species distribution modelling is challenging, given the dearth of presence records and general lack of reliable absence records which prevents the application of established methods to partition the data into testing and training sets and derive commonlyused test statistics of model performance (Pearson et al., 2007). Jackknifing (leave-one-out method) has been successfully used to model species distribution with a small sample size; here, each locality record in turn is removed once from the distribution model and model performance is assessed by the model's ability to predict the excluded locality (Pearson et al., 2007). In addition, test statistics based on jackknifing have been developed to evaluate model testing, thus allowing SDM evaluation under low sample sizes (Pearson et al., 2007).

Here, we use niche-based distribution models and jackknifing techniques to establish the potential distribution of Calotes minor, despite a limited numbers of presence records. Establishing the potential distribution of this species is important since following recent taxonomic work on the species (Khan & Kumar, 2010; Ingle et al., 2012; Deepak et al., 2015), the species' distribution remains poorly understood. Current information tells us that C. minor occupies a wide range of habitats, i.e., stony, sandy, and dark lava soil with grass, scrub, and thorny vegetation, in Pakistan, western and central India in subtropical regions (Khan & Kumar, 2010; Deepak et al., 2015). We developed a nichebased species distribution map for C. minor within the arid and semiarid regions of India and Pakistan, in the Indomalayan realm. We also discuss how this map can aid future survey effort for this species (and thus refinement of the distribution map) and inform current and future conservation assessment processes within the region.

MATERIALS AND METHODS

Study species and distribution records

Calotes minor is known to occur in grassland, agricultural land, and scrub-dominated landscapes in hot xeric (arid) and semiarid regions, with preferences for dry forest, barren and desolate land (Khan, 2006; Khan & Kumar, 2010; Deepak et al., 2015). The species is identified as both diurnal and crepuscular in habit, resides in burrows under the roots of bushes, and feeds on insects, leaves, and flowers (Khan, 2006). The species provides an important case study to examine the efficacy of jackknifing in niche-based species distribution modelling in the context of the tropical subcontinental and South Asian regions, as the species requires a specific range of temperatures, precipitations, and aridity (Deepak et al., 2015). Calotes minor has been documented from several Indian states, such as Gujarat, Rajasthan, Madhya Pradesh and Uttar Pradesh in India (Blyth, 1856; Günther, 1864; Stoliczka, 1872; Cockburn, 1882; Boulenger 1885; Smith, 1935; Vyas & Singh, 1998; Vyas, 2000; Vyas, 2002; GEER, 2014; Deepak et al., 2015; Ardesana, 2018); while substantial records were available from Sindh, Punjab and Balochistan in Pakistan (Khan 1999, Khan, 2002; Khan & Kumar, 2010; Deepak et al., 2015).

We collected species presence records along with their geographical coordinates from various sources, i.e., verified published records (n=17) by Khan & Kumar (2010), Ingle et al. (2012), Deepak et al. (2015), and Patel & Vyas (2019), personal observations (n=6), and personal communications with subject experts (n=1) (Table 1; Phillips et al., 2006; Pearson et al., 2007; Hosseinian Yousefkhani et al., 2016). Records from Banda and Allahabad, Uttar Pradesh, were not considered in the study, since there were no subsequent records after 1856 from these regions. We excluded the type locality of *C. minor* from the analyses since it is suggested to be erroneous (Khan & Kumar, 2010; Deepak et al., 2015). We also incorporated four recent locality records of C. minor from central Rajasthan, India (Fig. 2), recorded by the lead author. All reported individuals were sighted in agricultural fields dominated by green gram Vigna radiata, with an average crop height of around 15 cm (height range 3 - 20 cm). The sites were surrounded by landscapes dominated by the invasive shrub Prosopis *juliflora*. The species identity was confirmed using the key characters of sighted individuals, also suggested by Deepak et al. (2015), i.e., tail length less than snoutvent length; shorter fifth toe; two tufts of spines near tympanum. All observed characters of these new records are listed in Table 1. By merging all scrutinised records, we obtained a total of 24 locality records (from the years 1856-2019; Table 2).

Species distribution modelling Addressing cluster bias

To avoid cluster biases and spatial correlation, a 100 km² (10 km × 10 km) grid framework was used to eliminate double records from each grid cell, so that we included only one presence record per grid cell for further analysis



Figure 2. Map showing the previous records (n=20) and newly added records (n=4) of Calotes minor, scrutinised for predicting the distribution of *C. minor* (presented in current study).

Table 1. Recorded characteristics of four individuals of Calotes minor, recorded from central Rajasthan

	Individual 1	Individual 2	Individual 3	Individual 4
Place	Shokaliya, District Ajmer, State Rajasthan	Nasirabad, District Ajmer, State Rajasthan	Khamor, District Bhilwara, State Rajasthan	Shahpura, District Bhilwara, State Rajasthan
Location	26.21°N 74.84°E	26.24°N 74.68°E	25.77°N 74.80°E	25.49°N 74.71°E
Altitude	400m	448m	502m	393m
Period	July 2015	July 2017	August 2017	August 2017
Gender	9	8	5	6
Total length (Snout to Tail tip)	152 mm	163 mm	173 mm	171 mm
SVL length	81 mm	86 mm	91 mm	89 mm
Scales around midbody	49	54	58	57
Supralabials	13	14	14	14
Infralabials	11	13	13	12

Table 2. Information of predictor variables, used for modelling the species distribution for Calotes minor

Variable	Mean ± SD	Original spatial resolution	Resampled spatial resolution	Source
Aridity Index (AI)	0.18 ± 0.03	1000m	1000m	Global Aridity and PET Data- base (CGIAR-CSI)
Normalised Vegetation Difference Value (NDVI)	0.3 ± 0.18	1000m	1000m	Copernicus Global Land Service
Precipitation Seasonality (bio15)	127.62 ± 25.47	1000m	1000m	Worlclim version 2
Temperature Seasonality (bio4)	596.16 ± 148.4	1000m	1000m	Worlclim version 2
Terrain Ruggedness Index (TRI)	0.44 ± 0.11	30m	1000m	SRTM-USGS

(Pearson et al., 2007; Boria et al., 2014; Krishna Muliya et al., 2020). Since no multiple points were found in a single grid cell, we retained all 24 non-spatially correlated records for further analysis.

Variables selection

Selection of predictor variables was based on the known ecology of C. minor. We selected Aridity Index (AI), Normalised Difference Vegetation Index (NDVI), Precipitation Seasonality (bio15), Temperature Seasonality (bio4), and Terrain Ruggedness Index (TRI) (Table 3) as predictor variables. The AI is the degree of dryness, which is represented by the ratio of mean annual precipitation and mean annual evapotranspiration per year between 1970-2000 (Trabucco & Zomer, 2009). We computed mean NDVI across the months of July to October of each year over the time frame of 1999-2017 (Table 2), since grasslands and scrublands flourish in monsoon season in the tropical regions (Muratkar et al., 2015). Precipitation Seasonality and Temperature Seasonality for the timeframe of 1970 to 2000 were obtained from Worldclim version 2 (Fick & Hijmans, 2017). The Terrain Ruggedness Index (TRI) was derived from a digital elevation model (DEM) using the "Terrain Ruggedness Index" tool in QGIS version 2.14.3, which produces the mean difference in elevation between a pixel in the DEM and the surrounding eight neighbouring cells (Riley et al., 1999). We cropped all five variables to the laver extent of Thar and Indo-Gangetic biogeographic provinces (proposed by Udvardy, 1975) and the spatial resolution of the TRI data layer was resampled to 1,000 m using a bilinear resampling approach to match the resolution of the other variables. All data were transformed into the Asia Lambert Conformal Conic projection to perform spatial analyses. We extracted raster values of species presence locations for each predictor variable using the "Extract values to point" tool in ArcMap version 10.6. To avoid multi-collinearity, we computed Pearson's correlation coefficient (r) among our predictor variables using the "stats" package version 3.5.2 in R studio version 1.2.1335. Since no variable pair showed a strong correlation of > |0.7|, we retained all variables in further analyses (Chu et al., 2018).

Estimation of species occurence probability

We carried out the niche modelling for *C. minor* using a maximum entropy algorithm and presence-only framework in Maxent version 3.4.1 (Phillips et al., 2006; Norris, 2014). We implemented the jackknife evaluation approach proposed by Pearson et al. (2007) to assess model accuracy, given the small sample size of locality records for C. minor (n=24).

In total, we built 24 models, by leaving out one presence location at a time, so that each location is left out only once (Supplementary Figs. 5A-X; Pearson et al., 2007). We confirmed the settings of the modelling software as default, as follows: regularisation multiplier = 1: convergence threshold = 10^{e-6} : prevalence value = 0.5; and 500 maximum iterations. Regularisation values for linear, quadratic, threshold and hinge responses of variable features were computed using the default

settings (Pearson et al., 2007, Baldwin, 2009). Background points were configured to 200,000 since the raster has around 2 million pixels. The models' predictions of relative suitability were configured into presence probabilities (ranges between 0 - 1), wherein the value of a given grid cell is the probability of the presence of the species (Phillips et al., 2006). The 10 percentile logistic presence threshold values (LPT) were considered as the decision thresholds to classify the species occurrence probability into a binomial framework, in which pixel values higher than the 10 percentile logistic presence threshold were considered as "1" (high probability) and pixel values lower than the 10 percentile logistic presence threshold (10 percentile LPT) were designated as "0" (low probability) (Pearson et al., 2007). This is because other commonly used threshold-independent validation statistics (such as AUC) are considered unsuitable for presence-only SDMs (Boyce et al., 2002). This differs from the approach by Pearson et al. (2007) which utilised the lowest presence threshold and a second threshold which rejected the lowest 10 % of possible predicted values. This is because, after testing of different thresholds, the 10 percentile LPT produced a good balance between overprediction of suitable areas and conservative prediction of potential species range (Escalante et al., 2013). Using these classes (1/0), we further tested whether the particular *i*th model successfully predicted the higher species presence probability (higher than 10 percentile LPT) at the eliminated presence points (i) or not. If the model successfully predicted species presence, we recorded the prediction as "1" (success), if not, we coded it as "0" (failure). Using the program "pvalue" (Pearson et al., 2007), we calculated p-value to test the predictive ability of our jackknifed models.

Also, to give a sense of relative probability of occurrence per grid cell, we classified the pixel values of species occurrence probability into five equal categories, i.e., 0 – 0.2, 0.2 – 0.4, 0.4 – 0.6, 0.6 – 0.8 and 0.8 – 1. The pixel values >0.6 were considered as highly probable sites, in which priority surveys should be carried out for the species to elucidate its full distribution. These sites may also be focus areas for conservation actions for the species. Pixel values between 0.4 and 0.6 were considered as moderately probable sites.

To illustrate the size of the area where *C. minor* is potentially present, we calculated the area coded with "1" (higher presence probability than the 10 percentile logistic presence threshold) using ArcGIS version 10.6. We then plotted the relationship between predictor variables and probability of species occurrence for *C. minor* to describe climatic and topographical characteristics of the suitable species distribution.

RESULTS

Presence data

The locality points used in the environmental niche modelling are shown in Fig. 2 & Table 3. The first occurrence records for central Rajasthan (n=4), obtained by the lead author, were added to the 20 previously available and scrutinised records of C. minor, resulting in 24 locality records in total.



Figure 3. Species Presence Probability of *Calotes minor* in the study extent in India and Pakistan, using various thresholds. 3A) Five categories of species distribution probability, indicating the areas of importance with higher distribution probability. **3B)** Binomially classified probability of the species distribution for *Calotes minor*, with putting 10 percentile logistic presence threshold (LPT) as classifier.

Estimation of species occurence probability

Binomial pixel classification based on the 10-percentile logistic presence threshold disclosed that, on average, 30.72 % of the study area are classified as higher than the 10 percentile LPT (Fig. 3B). Assessment of the importance of predictor variables in describing relative suitability of areas for *C. minor* found that precipitation seasonality $(0.466 \pm 0.007 \text{ SE})$ makes the largest contribution, followed by NDVI (0.323 ± 0.005 SE), temperature seasonality (0.165 ± 0.004 SE), aridity (0.041 ± 0.002 SE), and terrain ruggedness (0.005 ± 0.0002 SE). Distribution probability maps indicate that the Gujarat state has the highest probable sites to hold C. minor, since most of the areas, i.e., Kutch, Saurashtra, and central Gujarat have grid cells with suitability values of more than 0.6 (Fig. 3). South-western, central and eastern Rajasthan, northwestern Madhya Pradesh in India, and southern Pakistan hold a moderate probability of species occurrence (0.4-0.6) (Fig. 3).

Precipitation Seasonality (bio15) has a positive relationship with predicted species distribution (Pearson correlationcoefficientr=0.92,p<0.05), wherein distribution probability of more than 0.6 is defined within the range of 140.53 mm to 187.81 mm precipitation seasonality (Fig. 4A). Normalised Difference Vegetation Index (NDVI) was found to be negatively related to species distribution probability with a sharp threshold in grassland and scrublands' NDVI values (r=0.74, p<0.05), and 0.05 - 0.26 units of NDVI indicate the areas where species distribution probability was more than 0.6 (Fig. 4B). There is a slightly negative relationship between temperature seasonality and predicted species distribution probability (r=-0.11, p<<0.05); areas with a temperature seasonality between 34.7 °C and 55.8 °C had a distribution probability of more than 0.6 for C. minor (Fig. 4C). Less arid areas, i.e., areas with high evapotranspiration and lower precipitation, are more suitable for the presence of C. minor (r=0.74, p<0.001); areas of predicted presence probability of more than 0.6 have Aridity Index values between -0.02 and

0.2 (Fig. 4D). TRI is negatively correlated with predicted distribution probability for *C. minor* (r=-0.74, p<0.001); areas of more than 0.6 probability of species distribution are found in areas with low TRI values between -0.05 and 1.05 (Fig. 4E).

The mean 10-percentile presence area was 0.303 (±0.006 SE), mean AUC was 0.89 (±0.001 SE), mean entropy was 11.32 (±0.01 SE) and mean prevalence was 0.02 (±0.002 SE).

The p-value estimation yielded that 83.33 % of jackknifed models successfully predicted the removed ith locality records, which was supported by the p-value < 0.001.

DISCUSSION

In this study, we used environmental niche modelling to define the species occurrence probability for a previously understudied species, C. minor. Our study showed that predicted suitable areas for C. minor had less rugged terrain (i.e., in Sindh in Pakistan; north-western Gujarat and south-western Rajasthan in India), in landscapes dominated by grasses or scrubs (throughout the western extent of study area; mainly covered by hot xeric parts of Pakistan; Gujarat and Rajasthan in India; avoiding higher ruggedness and forest areas of Indo-Gangetic province), with relatively moderate to low aridity (in Thar province, excluding Thar sand dunes, since areas of Thar desert have low precipitation seasonality as well; also found in Sindh, Balochistan and Punjab provinces in Pakistan; north-western Gujarat and south-western Rajasthan in India), moderate to low temperature variability (especially in mid-longitudinal areas of the study area; Sindh in Pakistan; Gujarat, Rajasthan, northern Madhya Pradesh and southern Uttar Pradesh in India) and higher precipitation variability (whole study area, except the north-western sides; Sindh region of Pakistan; Gujarat, Rajasthan, Madhya Pradesh and southern Uttar Pradesh in India).



Figure 4. Response curve of predictor variables, displaying relationship with species presence probability of Calotes minor. 4A) Precipitation Seasonality (bio15). 4B) Normalised Difference Vegetation Index (NDVI). 4C) Temperature Seasonality (bio4). 4D) Aridity Index (AI). 4E) Terrain Ruggedness Index (TRI)

Our models predict that the south-western extents of study area have a higher probability for the species distribution than both central and south-western biogeographic provinces combined. Politically, these high-probability areas are comprised by southern Sindh and southern Balochistan in Pakistan and Gujarat, southwestern, central and eastern Rajasthan, and northwestern Madhya Pradesh in India. This was found to be in accordance with the known ecology of this species (Khan, 2006; Deepak et al., 2015). Our results thus highly overlap with the known theoretical and observed ecology of C. minor, while allowing us to redefine its spatially explicit occurrence probability within this distributional range (Fig. 2). Responses of variables illustrate their crucial roles in defining the niche-based distribution model for C. minor. In addition, although the species was described in the year 1827 from the sandy plains of Chittagong

Terrain Ruggedness Index (TRI)

(Bangladesh), its presence in the area has been regarded as questionable, especially given the habitat differences. Chittagong has a narrow temperature range (ranges from 21.7 °C to 30.4 °C), high humidity (>75 %), and heavy rainfall in monsoon (~500 mm; Khatun et al., 2016). The species has not been reported from Chittagong since 1827 (after Hardwicke & Gray, 1827). Our study further suggests that, based on the climatic niche for this species elucidated in our study, the record from Chittagong is likely erroneous (also suggested by Deepak et al., 2015; Khan & Kumar, 2010; IUCN Bangladesh, 2015).

The occurrence of the species around its distribution margins is likely sparse and sporadic. While the species may be less probable to occur there, due to difference in climate and topography, and other range-effects, there is also a need for robust ground data from those understudied margin regions. Since our leave-one-out

approach accurately predicted the distribution of C. *minor* within the study extent (prediction capabilities of jackknifed models=83.33 %, p<0.001), it could be that the easternmost extents of the Indo-Gangetic plain distribution are characterised by differences in temperature and precipitation seasonality. In addition, topography also differs from the central and western parts of the distribution. Our results identify those areas for *C. minor*, where additional targeted surveys should be carried out to fill the gaps in our knowledge on the distribution of this particular species. Specifically, 10-percentile LPTs have been found favourable to uncovering potentially important distributional areas for understudied species (Pearson et al., 2007; Krishna Muliya et al., 2020). Each additional record is likely to enable better SDMs and will yield more precise distributional results.

Our findings highlight several priorities for field research on C. minor. Specifically, given the known ecology of the species and the suggested distribution of the species in south-western parts of the study area, extensive field research is required to validate the distribution of the species from east of Aravalli to the hill, since at present, the species presence from Uttar Pradesh is based on old records by Blyth, collected in 1856. Additionally, the niche models sparsely predicted the species occurrence in the central and southern parts of the study area, and this region would benefit from further field research. The grasslands and agricultural fields of southern Uttar Pradesh, western and north-western Madhya Pradesh, and north-western Maharashtra should be surveyed as they constitute potentially suitable habitat for this species. Finally, although the species has a higher probability to be distributed in Gujarat state (probability >0.6), records from Madhya Pradesh, Rajasthan and Pakistan play critical roles as well, because these records are maintaining a wide distribution extent of the species, which can buffer against declines and higher extinction risk of this species (Böhm et al., 2016; Joppa et al., 2016). Environmental and topographical characteristics of these regions are different to those of Gujarat, which is why the distribution is predicted to be sparse and patchy in these regions. However, further field surveys in these areas are required to establish species distribution limits, vital for the calculation of extinction risk metrics (e.g. EOO; IUCN, 2012), especially because our SDM approach should not be interpreted as accurately predicting range limits of the species (Pearson et al., 2007).

The ability of our modelling approach, and other SDM approaches, to predict probable areas of occurrence is highly dependent on the underlying locality data. Here, as in many other SDM approaches, we are focussing solely on identifying climatic and topographically suitable areas. Absences at potentially suitable localities may be caused by other underlying factors which have been omitted in our model but are important in driving species distributions, such as dispersal ability and biotic interactions. However, variable selection for environmental niche modelling essentially depends on the model species in question that reflect the species' ecology (Rödder et al.,

2009; Palaoro et al., 2013). Effective use of expedient environmental variables to model species distribution helps to collate ecological understanding of the species and prediction modelling, even when only few species localities are known; for example, Krishna Muliya et al. (2020) used a small sampling approach to identify new potential areas for further field surveys for another little-known species, Lycodon flavicollis. By adapting the habitat-based niche modelling for a small sample size (n < 25), we effectively predicted species occurrence probability for *C. minor* within its distributional range. Using jackknifing and evaluating the models with p-value analysis, we showed a high accuracy of model prediction despite small sample sizes (n=24).

However, despite high accuracy of model predictions, the probability of erroneous suitability outputs is higher under very small sample sizes, for example where omitted localities cannot be successfully predicted (e.g. a single occurrence is geographically remote from the other localities, with different environmental conditions; Pearson et al., 2007; Krishna Muliya et al., 2020). This in turn will impact the probabilities of suitability derived from the SDM approach. Thus, while probability maps may allow identification of highly suitable habitat patches, a focus on these should not come at the expense of lower probability areas which could be the result of the large influence each locality of a small sample has on model outputs (Pearson et al., 2007: Krishna Muliva et al., 2020). In this case, presence-absence maps (binomially classified using 10-percentile logistic presence threshold; Fig. 3B) can be a decision making output to inform about the conservation priority and distribution extent for the species.

Given that C. minor is perceived to be rare, it is important that we focus on obtaining the best possible information on the species in order to proceed to conservation assessment. The species was assessed as Data Deficient for the IUCN Red List of Threatened Species in 2009, owing to its rarity, patchy distribution, and our gap of knowledge in this ground agamid's ecology (Khan & Papenfuss, 2016). There was a need to assess the distribution of the species so that more accurate range-based metrics of extinction risk (extent of occurrence EOO and area of occupancy AOO) can be produced and appropriate conservation action can be defined. Additionally, this species is part of the random sample of reptile species which make up the sampled approach to the Red List Index (sRLI), a global biodiversity indicator which aims to track changes in extinction risk of species-rich but understudied species groups (like reptiles) over time (Baillie et al., 2008, Böhm et al., 2013). However, given its current Data Deficient status, it does at present not contribute to the index, as its extinction risk is unknown.

Environmental niche modelling can provide the first step to ensure the accurate listing of species extinction risk on the IUCN Red List and can work alongside efforts already undertaken to predict the true status of Data Deficient species (Bland & Böhm, 2016). However, it should be noted that for conservation applications, a

more conservative decision threshold may be required, such as lowest presence threshold (Pearson et al., 2007), to avoid overprediction of distribution area in conservation assessments. Given that no known speciesspecific conservation measures are in place and that it is perceived to face human-made threats like urban development with the conversion of grasslands for agriculture and infrastructure establishments (Khan & Papenfuss, 2016), there is a dire need to conserve grassland ecosystems so that not only C. minor but other grassland dependent species can be protected. Further research on the habitat, threats, and population monitoring of *C. minor* is mandated.

CONCLUSION

We tested the jackknife evaluation method accompanied by the maximum entropy algorithm for predicting the species presence for the little-known ground agamid lizard Calotes minor, following Pearson et al. (2007). Our results show that through the inclusion of recently obtained data records from central Raiasthan. the known distribution for C. minor in the Indian subcontinent is extended. Our results also suggest the distribution range of C. minor in the dryland regions of the Indian subcontinent, mainly covered by Gujarat, south-western, central and eastern Rajasthan and northwestern Madhya Pradesh in India and southern Sindh and southern Balochistan region in Pakistan. Also, our study shows that both environmental niche modelling and field study can improve our knowledge of littleknown species with unexplored range limits. Specifically, environmental niche models can focus research activity to areas where a species is predicted to occur and can help to inform conservation assessments and develop targeted conservation action and research.

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FULL PAPER

Repeated use of high risk nesting areas in the European whip snake, Hierophis viridiflavus

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Oviparous snakes deposit their egg clutches in sites sheltered from predation and from strong thermal and hydric fluctuations. Appropriate laying sites with optimum thermal and hydric conditions are generally scarce and are not necessarily localised in the home range. Thus, many gravid females undertake extensive trips for oviposition, and many may converge at the best egg laying sites. Dispersal mortality of neonates post-hatchling is also a critical factor. Assessing the parameters involved in this intergenerational trade-off is difficult however, and no study has succeeded in embracing all of them. Here we report data indicating that gravid females of the highly mobile European whip snake, Hierophis viridiflavus exhibit nest site fidelity whereby they repeatedly deposit their eggs in cavities under sealed roads over many decades. These anthropogenic structures provide benefits of relative safety and suitable incubation conditions (due to the protective asphalted layer?), but they expose both females and neonates to high risk of road mortality. Artificial laying sites constructed at appropriate distances from busy roads, along with artificial continuous well protected pathways (e.g. dense hedges) that connect risky laying sites to safer areas, should be constructed.

Keywords: Hierophis viridiflavus, road mortality, cost of reproduction, reptiles, anthropogenic landscape, forest

INTRODUCTION

Although females of several snake species are able to excavate their own nest in soft ground (Burger & Zappalorti, 1991; Cunnington & Cebek, 2005), most exploit existing cavities in rocky areas or in trees to deposit their eggs. The attraction of certain sites for nesting can be considerable and many females, sometimes from different lineages of squamates, converge toward communal nesting places (Graves & Duvall, 1995; Filippi et al., 2007). Empirical and experimental studies, as well as intuition, suggest that these repeatedly used sites offer excellent environmental conditions for embryonic development (review in Doody et al., 2009). Guided by environmental and social clues (e.g. empty shells of previous hatchlings, Brown & Shine, 2005a; Meek, 2017) and using their spatial memory, gravid females search underground tunnels or cavities for localities with stable ambient temperatures of around 30 - 35 °C and high moisture levels, which produce optimal conditions for embryo development (Shine et al., 1997; Aubret et al., 2005; Brown & Shine, 2005b; Löwenborg et al., 2010, 2011; Ramesh & Bhupathy, 2010; Stahlschmidt et al., 2011; Meek, 2017). Such conditions can be represented by large partly rotting logs, hollow trees, thick leaf litters,

underground insect nests or the burrows of various animals (Carman-Blazquez & Villafuerte, 1990; Madsen & Shine, 1999; Pearson et al., 2003; Velásquez-Múnera et al., 2008; Ramesh & Bhupathy, 2010; Nagy et al., 2017; Meek, 2017; Alexander, 2018).

However, in landscapes shaped by anthropogenic activity, oviparous reptiles frequently use artificial structures as laying sites. The selection of artificial structures by gravid females is likely underpinned by adaptive mechanisms that operate in natural settings, and it is probably driven by the rarity of suitable natural nesting sites (Doody et al., 2009). For example, in cool climates, incubation success in the grass snake (Natrix helvetica) depends strongly on fermenting compost or dung heaps associated with livestock farming, especially in northern Europe (Löwenborg, 2012) to the extent that the disappearance of open manure heaps during the past decades has resulted in snake population declines across Sweden (Hagman et al., 2012). Besides this well documented example of the reliance of a snake on artificial laying sites, there are many other instances where gravid females use human constructions for oviposition, even in tropical countries. Egg laying sites under roads have been associated with snakes in Australia (Covacevich & Limpus, 1972), Europe (Capula et

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Figure 1. Google Earth map showing the village of St Denis du Payre and the location of the drain where hatchling H. viridiflavus emerge (inserted photograph).

al., 1997; Meek, 2017 and unpublished data) and Brazil (Braz & Manço, 2011). Additionally, snakes reportedly nest in railroad sleepers, artificial snake dens, in hollow retaining walls, old partly demolished buildings and in walls in a cement culvert (Albuquerque & Ferrarezzi, 2004; Filippi et al., 2007; Zappalorti & Mitchell, 2008; Shine & Bonnet, 2009; Hanslowe et al., 2016). Indeed, there are more documented examples of artificial than of natural sites, but there are few reports of long term repeated nesting in anthropogenic structures (Filippi et al., 2007).

We do not know if females prefer anthropogenic to natural laying sites or vice-versa; but it is likely that in anthropogenic environments, the scarcity of suitable natural sites constrain females to use artificial sites. Answering these questions is important for conservation managers, especially to instigate mitigation measures to restore or improve habitats, for example to remove those laying sites that attract gravid females but that are actually deadly traps for huge numbers of snakes. Conversely, it might be useful to construct artificial laying sites at suitable distances from high-risk high traffic roads (Bonnet et al., 1999). In the context of habitat fragmentation, sprawling urbanisation, and global snake decline, it is therefore crucial to attract or retain snakes in well protected areas instead of directing them toward dangerous zones. In oviparous species, road mortality may heavily impact reproductive females during round trips to laying sites, and males tracking females often suffer the same fate (Bonnet et al., 1999; Meek, 2015). Therefore for snake conservation in anthropogenic landscapes we need key information (e.g. mortality assessment, site preference) to assess the risk of artificial

laying sites situated in high risk zones, roads or urbanised areas.

In eight zones, long term-monitoring of living snakes and of road-kills provided a means to address the following questions: Are natural versus artificial nesting areas associated with differential long term frequenting by gravid females? Is there any evidence that neonate mortality is particularly high in anthropogenic areas? Would high usage of artificial laying sites, when associated with high neonate mortality, suggest a possible lethal trap effect?

METHODS

Study species

The European whip snake Hierophis viridiflavus is a medium-sized European oviparous colubrid snake with a maximal recorded snout to vent length (SVL) in the study localities of ~139 cm in males (163 cm total length) and ~108 cm (145 cm total length) in females. It inhabits a range of environments with an extensive home range and a life span that may reach 20 years (Scali et al., 2008; Lelièvre et al., 2011; Fornasiero et al., 2016). Mean egg clutch size varies geographically and broadly ranges from 4 to 8 eggs (Luiselli, 1995; Zuffi et al., 2007a). In the study areas, on average, mating occurs from mid-May to mid-June, oviposition from late June to early July, and then hatching in late August to September; this phenology is influenced by climate and latitude across the distribution range of the species (Bonnet & Naulleau, 1996; Capula et al., 1997; Filippi et al., 2007).

Study areas and surveys

Two main areas were monitored in central west France; respectively situated in Vendée and Deux-Sèvres districts and separated from each other by a straight-line distance of ~74 km.

Vendée: The nest site was located in a roadside drain in the centre of a village of St Denis du Payré (42°19`N, 1 °16W; Fig. 1). The locality is almost 300 m from the nearest natural area of woodland (Fig. 1) and for a snake accessing the site the route is almost devoid of cover - for example there are no hedgerows or other low risk movement pathways (Fahrig, 2007). During the active season (April – October), the locality was observed regularly with a minimum of between 4 and 6 monthly surveys undertaken at around 4 day intervals by a single observer (RM). Surveys were normally mid-week and at weekends. Road traffic volume recorded on the main route entering and leaving the village (D60) was just over 60 vehicles per hour during daylight hours (Meek, 2009). Data collected at the den was mostly hatchling mortalities (SVL<30 cm) and 2 adult females (SVL=67 cm) found at or within 4-5 m of the den entrance. All snakes were measured for SVL along with the dates they were found.

Deux-Sèvres: Seven sites were compared in this study area in the forest of Chizé (46°07'N, 0°24'W; Fig. 2). Five sites are situated in relatively natural areas in the integral biological reserve (RBI). Two other sites are in relatively urbanised areas next to busy roads (D1 and D53) that transect the forest. One (LABO) is a small 4 ha enclave at the northern limit of the RBI that contains a research campus (e.g. CNRS and University). The other (MUDB) is in the garden of a private property adjacent to the exploited forest. The seven sites differ in total area (from 0.3 to 50 ha; Fig. 2), terrain and degree of openness of the habitat (open meadows vs mature forest; Fig. 2).

Long-term mark-recapture monitoring from 1990 to 2019 (+episodic data collected from 1981 to 1988) showed that very few individuals move between sites, and that these exchanges are largely limited to adjacent zones (e.g. ZOSE vs CHEP, Fig. 2). Consequently, the seven sites were considered as independent, at least in terms of population functioning. In addition to the main sites, other natural sites and scattered locations in the RBI provided data on reproduction of female whip snakes and hatchlings (data pooled).

The networks of roads that connect the sites were also systematically surveyed for road killed snakes. In the



Figure 2. Modified Google Earth map showing seven sites monitored in the forest of Chizé. South of the D53 and D1, the forest is strictly protected (integral biological reserve, RBI) with no public admittance (RBI limits not visualized to limit overload). The north-eastern part of the forest (partly visible here) is open to the public and exploited for wood. Five natural sites are in the RBI (PAITO, SNAP, OUIL, CHEP and ZOSE). The two urbanised sites (LABO, MUDB) not included in the RBI are respectively adjacent to two busy roads, D53 and D1. The grey area shows a village (Chizé). Enlargements are provided for LABO and MUDB (grey arrows) to identify the main nesting site entrances in orange (ditch and stone wall). The dense network of roads inside the RBI is not visible at this scale, busy roads (all out of the RBI are highlighted); the thick yellow sections indicate where >70% of road kill snakes have been collected.

RBI, the roads are rarely used; but D1 and D53 are busy. Roads were surveyed by car driving at low speed (<50 km/h), cycling, and walking. The two main busy-roads (D1 & D53) were surveyed cycling and/or driving (XB). Additional roads (e.g. D106) were mostly surveyed by normal speed driving (70 to 90 km/h).

In the seven sites, snakes were searched for presence under corrugated slabs (>800 slabs deployed; fibrocement, roughly 120x80 cm, undulation height 6 cm) where if present they were captured by hand, measured (SVL), marked (individual code using heatbranded scales with medical cautery units: Winne et al. 2006) and released. Sex was determined by inspection of the tail. Females were considered as adults if larger than the smallest individuals with enlarged follicles (SVL=67 cm). Females do not reproduce every year (Capula et al., 1997), thus populations contain both reproductive and non-reproduce individuals. In order to determine the reproductive status of each female, we restricted analyses to the period during which abdominal palpation can reveal the presence of eggs (enlarged follicles), or evidence of recent egg-laying (extensive skin folds, distended sub-cloacal scale). Following emergence from hibernation, from March to mid-April, reproductive females cannot be distinguished from non-reproductive females by palpation. Similarly, from late summer until hibernation (August to late October), post-laying females cannot be distinguished from non-reproductive females. Consequently, analyses were restricted to the periods from 25th April to 30th July. Data from all years was pooled, but we included only one observation per individual per year.

Individuals were considered as neonates when SVL<30 cm, even if they were measured weeks or months after the hatching period (e.g. following hibernation). All roadkills were collected and measured for body size (SVL). For several snakes, information was incomplete (e.g. date or exact location), generating slight variations of sample size in the analyses (contingency table statistical tests were performed with Statistica 13.5.0.17, 2018 TIBCO Software).

RESULTS

Vendée

The entrance of the Vendée nest was first detected in 2005 in the centre of the village of St Denis du Payré in a roadside drain (42°19`N, 1°16W; Fig. 1). Observations of nesting activity were made in 12 of the 15 (80 %) years between 2005 and 2019. Hatchling emergence was between 26th August (recorded 2016) and 27th September (recorded 2017) with annual hatchling count over the 12 years ranging from 1-9 (mean = 4.1 ± 2.4 std. dev.). Additional hatchlings were found between 2nd and 22nd April at the entrance during the springs of 2015 (n = 2), 2017 (n = 2) and 2018 (n = 1). These were likely the previous year's hatchlings that over wintered at the site. Only two adult females were found at the site, on 17th and 30th June of 2010 and 2013 respectively. Figure 3 shows the annual counts. The broken line represents an expected probability of equality of annual counts derived



Figure 3. Annual numbers of hatchling H. viridiflavus found between 2005 and 2019 at St Denis du Payré. Cross hatched areas represent snakes found in April of the following year but here shown as year of hatching. The broken line represents expected snake numbers if annual counts were equal. See text for details.

from: Expected = $1/N_1 * N_2$, where N_1 is number of years and N_2 the total sample size. This gave an expected value of 3.3 hatchling snakes per year when years with zero counts are included in the calculation. This indicated that snake numbers at the site in 2011 and 2019 were 2.73 and 2.42 times greater than expected.

Deux-Sèvres

The proportion of reproductive versus non-reproductive females varied significantly among the seven sites $(\chi^2=103.96, df=6, p<0.001; Fig. 4)$. Compared to the 5 natural sites, the 2 urbanised sites (LAB & MUDB) showed the highest proportion along with highest absolute numbers of gravid females (Fig. 4). Each year, approximately 50 % of the individuals observed at natural sites were reproductive, this proportion reached 70 % and 90 % in the urbanised sites.

Gravid females were observed spatially scattered and mixed with non-reproductive females in natural sites. But, in the two urbanised sites, almost all females were gravid and were captured in highly localised places: under the tiles covering a small stone-wall that borders the D1 road in the MUDB site: under few corrugated fibrocement slabs placed in a ditch at the LAB site. The MUDB stony wall is directly connected to under road cavities resulting from partial land filling before asphalting; the ditch is connected to deep cavities under the D53 sealed road via a pipe partly obstructed by rocks. The clutches have not been discovered (it is impossible to excavate public roads). Yet, many gravid females were found at the entrance of the sites and they were recaptured a few days later with an empty abdomen and extensive skin folds indicating recent oviposition. We also found one male whip snake killed on the road just above this site with recently ingested conspecific eggs in the stomach; this species being occasionally known for oophagy and cannibalistic behaviour (Schmidlin et al., 1996; Capula et al., 2014). No adult female was found road-killed inside the RBI. Several reproductive and a few non-reproductive females were found roadkilled on the roads in the exploited part of the forest and in other roads of the district (n = 54 reproductive vs 8 non-reproductive females; 9 vs 1 focusing on the forest





Figure 4. Proportion of reproductive females (gravid or shortly after egg laving) compared to non-reproductive females in different populations (i.e. site) of H. viridiflavus. Absolute numbers are provided above each bar. Light grey bars indicate sites situated in natural environments (fully protected natural reserve); dark grey bars indicate urbanised sites adjacent to busy roads.

Figure 5. Annual numbers of hatchling whip snakes (H. viridiflavus) observed between 2000 and 2018 in the forest of Chizé. Living snakes were found under corrugated slabs; road kills were found on asphalt covered roads, essentially the D1 and D53.

Table 1. Live hatchling whip snakes and road killed hatchlings found in different sites on the road network in the forest of Chizé and surroundings areas. In addition to the 7 main sites used in the analyses (Fig. 2) we pooled observations collected in various other places in the forest of Chizé (n=13). Five sites situated in the fully protected part of the forest (RBI) were considered as natural. Two urbanised sites (LABO and MUDB) are juxtaposed to the roads D1 and D53. Other busy roads are on the south and east part of the forest.

Site	Status	Live	Roadkilled	Total
PAITO	Natural	70	1	71
OUIL	Natural	2	0	2
SNAP	Natural	8	0	8
CHEP	Natural	38	0	38
ZOSE	Natural	23	1	24
Other Natural sites	Natural	108	2	110
LABO	Urbanised	87	3	90
MUDB	Urbanised	9	0	9
D1	Road	0	50	50
D53	Road	0	17	17
Other local busy roads	Road	0	14	14
Sum		345	88	433

section of D1 and D53 roads).

Many living neonates were found under corrugated slabs in all the seven sites (Table 1), especially in a large meadow (PAITO, a natural site) and in one urbanised site (LABO). Most live hatchlings (62 %, n = 340) were observed following emergence from hibernation, from late March to late May, while most road-killed hatchlings (63 %, n = 82) were observed after nest emergence from late August to late September; generating significant temporal difference between the two categories of hatchlings (χ^2 =106.2, df=1, p<0.001). Living and roadkilled hatchlings were observed continuously over a prolonged time period, 6.5±6.7 per year on average (Fig. 5). Data collected before 2000 are not shown in Figure 5 because the D1 and D53 roads that transect the forest were not regularly surveyed by cycling. Cycling survey intensified over time, corrugated slab surveys also increased over time. In combination with demographic

variations (unpublished data), these factors caused interannual fluctuations. We did not analyse such annual changes because different data sources are involved (e.g. increasing but variable searching effort); disentangling their respective effect was outside the scope of this study.

DISCUSSION

The two study areas (Vendée and Deux-Sèvres) provided complementary information. Some results were confirmatory for example, the frequent use of certain sites associated with long-term fidelity. Others were novel: our analyses indicate Hierophis viridiflavus female preference for artificial sites and evidence of associated increased mortality risk. Together, these results suggest high and continuous mortality associated with artificial nesting sites and thus a possible behavioural trap effect

that requires adequate management options to mitigate mortalities.

1) Long term nest site fidelity: In Vendée, gravid females used under the road sites in the centre of St Denis du Payré, for a period of at least over 15 years and continuously for at least 10 years (Fig. 1). Similarly, in Deux-Sèvres, gravid females used the LABO and MUDB under road sites during 30 and 12 years of monitoring of each site respectively. Moreover, in both areas, other snake species used under road artificial sites. In Vendée, this observation is supported by data from a roadside nesting area around 3 km from the study site that is repeatedly used by wide foraging grass snakes Natrix helvetica (Meek, 2017). In Deux-Sèvres, Aesculapian (Zamenis longissimus) and grass snakes, regularly used LABO and MUDB artificial sites during the entire monitoring period of this study (unpublished data).

Long term monitoring of individual snakes (e.g. decades) indicate that different cohorts of females are involved and that nest site fidelity crosses generations: reproductive lifespan of females is relatively short (usually 3 to 6 years, unpublished data) and does not exceed 10 years (max observed = 9 years, unpublished data). Long term nest site fidelity and communal nesting has previously been documented in the western whip snake H. viridiflavus in Italy (Capula & Luiselli, 1995; Filippi et al., 2007; Rugiero et al., 2012); our paper presents additional observations in two localities in central west France, close to the northernmost limit of the species range. The observation that H. viridiflavus may also enter urban areas for nesting is of interest and makes a valuable comparison with nest site fidelity in more natural habitats. However, in the absence of recaptures of marked individuals we do not know if individuals exclusively used a preferred site or alternated between different sites.

2) Mortality risk of artificial nesting sites: For different reasons, mortality risk assessed with road mortality severely underestimates actual numbers of animals that are victim of a collision, including snakes (Slater, 2002; Santos et al., 2011; Antworth et al., 2005; Hastings et al., 2019). Firstly, persistence of carcasses is low, especially in forest sites where scavengers are numerous (e.g. various birds, foxes and cats) that patrol roads in search of fresh road kills (Schwartz et al., 2018). Unsurprisingly, almost all hatchlings we collected were flat and dry, probably because they were killed during traffic peaks, which limits the opportunities for scavengers to pick them up (Slater, 2002). Secondly, small animals are difficult to spot and hence the associated underestimation is particularly strong for very small individuals, <8 g for H. viridiflavus hatchlings (Teixeira et al., 2013). Nevertheless we accumulated large numbers of road killed hatchlings, which represent minimal counts.

Disregarding the underestimate issues, the numbers of annual hatchling mortalities we recorded were small, maximal annual count was nine hatchlings in Vendée and 25 in Deux-Sèvres, which compares with a broad average

clutch for a single female (6-8 eggs) or for three females in some years. This may suggest either a low-cost impact by road traffic if numerous females are using the site at the same time or, conversely, substantial mortality of only a few gravid females at each site each year. In the first case, many hatchlings must have escaped road traffic, in the second case many did not and were killed. In Vendée, only two females were found road killed over a 15 year period at a cross roads in a village centre providing support to the second possibility. In Deux-Sèvres, road mortality was concentrated at two artificial nesting sites (Table 1). The mean annual number of reproductive females at these sites (calculated from 2000 to match the Fig. 5 timescale) was 9.0±8.6, leading to a crude estimate of 63 hatchlings per site per year. Given that hatching success is not generally 100 %, and that annual numbers of road mortalities are strongly underestimated, our results suggest that road mortality was significant in the surveyed areas, impacting a high proportion of hatchlings from urbanised artificial sites (e.g. ~25 %). Even limited road traffic can generate substantial snake mortalities (Hartmann et al., 2011). Thus, the very small number of road-killed hatchlings found on the rarely used roads connecting natural sites (over four decades) suggests that the strictly protected reserve (RBI) was a low cost environment in terms of road mortalities.

Frequent use of artificial laying sites

The high number of reproductive females found close to artificial road sites strongly suggests that reproductive females are particularly likely to use these sites in the absence of good egg laying sites elsewhere, often resulting in high neonate road mortality during the post-hatchling dispersal phase. Adult males follow the pheromone trails of females, and hence they may converge to the best laying sites (Bonnet & Naulleau, 1996; Zuffi et al., 2007b). This was particularly apparent at MUDB, which is too small to sustain any population of medium sized snakes. Many gravid females from the adjacent forest visited the stone wall specifically during the mating and laying period, and they were accompanied by high numbers of adult males during the mating season (unpublished data).

Why do gravid females persistently and intensively nest under roads? In oviparous snakes, where offspring develop independently from the female, the location of egg-laying sites with optimal thermal and hydric conditions, that also limit accessibility to predators, are key factors in their ecology (Ackerman & Lott, 2004; Birchard, 2004; Filippi et al., 2007). It is probable that these artificial nesting areas offer easy access to appropriate conditions for incubation, for example the protective cover of road surfaces limits dehydration (no roots and thus evapotranspiration), indeed stable and warm ambient temperatures have been recorded in under road sites (Lelièvre et al., 2010). Asphalt is not only resistant to evapotranspiration but it is also periodically renewed and hence artificial sites under roads probably offer favourable and stable conditions over decades. If such sites are also minimally disturbed during times when not used for egg development, they may be used repeatedly. Locating optimal nesting sites may require well-developed orientation abilities in species that operate a wide ranging foraging strategy. In Europe several species of snake are known to employ this lifestyle, including the European whip snake H. viridiflavus (Luiselli, 2006).

Management proposal

In this paper we have shown that gravid females tend to use artificial sites situated next to busy roads over prolonged periods of time, which entails high risk both for adults (Bonnet et al., 1999) and for their offspring (this study). Animal abundance is known to be impacted by altered levels of predation risk in fragmented anthropogenic landscapes (e.g. Evans, 2004), especially when wildlife enters urban areas and faces increased risk from domestic predators. Frequent use of artificial nesting sites means that Hierophis viridiflavus often enter these areas where they suffer mortalities from dogs, cats and humans (Bonnet et al., 1999; Rugiero & Luiselli, 2004: Meek, 2012). It is conceivable that females may not be able to fully evaluate all these risks. Similar data are available for Aesculapian, grass and viperine snakes: many were collected as road kill or killed by humans and domestic pets during the hatchling season, along with many reproductive females and large males during mating and laying seasons (Bonnet et al., 1999). Thus the phenomenon observed with whip snakes may well apply to many oviparous snakes especially those species operating a wide foraging movement strategy.

Following hibernation, we observed many small snakes (i.e. hatchlings, n=249) in the natural sites. Many juveniles (SVL>30 cm) were also observed in the RBI (unpublished data). This suggests that those snakes living in the well-protected areas of the forest, situated away from the busy roads, can achieve their reproductive cycle in relatively safe places.

Importantly, our data do not show that artificial nesting sites are not useful for the maintenance of endangered snake populations, especially when no alternative exists. Instead, our results reveal high mortality risks; thus, appropriate management should target these risks.

From an evolutionary perspective snakes are probably able to evaluate that open patches present a high mortality risk, and thus might be reluctant to use them while preferring to follow sheltered pathways (e.g. hedgerows) when available (e.g. Fahrig, 2007; Naulleau et al., 1997). Therefore, we suggest three main nonexclusive actions: (1) Building suitable artificial laying sites in well protected areas where many snakes are observed (Bonnet et al., 1999), especially because such sites offer excellent refuges during hibernation and aestivation (Zappalorti & Reinert, 1994; Shine & Bonnet, 2009; Burger et al., 2012); (2). When possible, moving the highest risk sites (e.g. MUDB) to safer places (i.e. long distances from busy roads); and (3) Building low risk movement corridors to channel gravid females and hatchlings away from high risk laying sites toward safer locations, for example thick hedgerows with abundant ground shelters (e.g. stone piles), represents another

option. In addition, management planning should pay attention to preserving older artificial sites, for example old building, roads, esplanades that are often perceived as undesirable by managers for aesthetic reasons and demolished. These structures often offer valuable refuges to a wide range of animals, notably reptiles (Lecq et al., 2017).

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Contribution

RM and XB initiated the study, performed the analyses and wrote the first version of the manuscript. JMB and GB provided comments and approved the successive versions. All authors intensively participated to the acquisition of field data.

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Reproductive characteristics, diet composition and fat reserves of nose-horned vipers (Vipera ammodytes)

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Long-term ecological studies are usually both time-consuming and costly, particularly when conducted on species with low detectability, such as vipers. An alternative, non-expensive method for obtaining detailed information about numerous important ecological traits, e.g. size at maturity, reproductive output, diet composition and amount of body reserves, are dissections of museum specimens. We dissected 237 museum specimens (125 males and 112 females of all age classes) of the nose-horned vipers Vipera ammodytes from the central and western regions of the Balkan Peninsula. Their reproductive and digestive systems were examined, and fat stores estimated. Relative testes volumes were significantly higher in adults than in subadults. Also, we found seasonal variation of testes volumes in adult males. Females became mature at around 43.8 cm snout-to-vent length (SVL); 38.1 % of adult females were pregnant (i.e. had developed eggs/embryos, suggesting bi- or even triennial reproduction frequency). The average number of developed eggs/embryos was 9.1 (range 5-17). As expected, there was a positive correlation between maternal body size (SVL) and the number of embryos. The nose-horned vipers fed predominately on lizards (64.7 %) and mammals (31.9 %); only a few remains of different prey were found (birds, snakes and centipedes). Analyses of inter-sexual differences showed that males more frequently consumed lizards than mammals, while in females both types of prey were equally present. A subtle ontogenetic change in diet was recorded, with a shift from lizards towards mammals. Adult individuals had more fat reserves than subadults, but there were no inter-sexual differences, and gravid females had similar amounts of fat reserves as non-gravid individuals. The data about reproductive output and dietary specialisation demonstrate the vulnerability of the species, generally regarded as "quite common" and non-threatened. This study might help in the establishment of future conservation studies, and management of the impacts of anthropogenic factors on populations of V. ammodytes in the central and western Balkans.

Keywords: testes volumes, follicles and embryos, prey spectrum, fat bodies, herpetological collection, Viperidae

INTRODUCTION

r or many common snake species, there are no data on population status, diet and reproduction, or these data are out of date. Such data are essential for assessment of conservation status and threats. Venomous snakes such as e.g. nose-horned vipers (Vipera ammodytes) are often not the subject of systematic studies, and are under great pressure due to intentional killing, hunting for anti-venom production (Jelić et al., 2013) and the illegal pet trade (Filippi & Luiselli, 2000). However, in some countries in the Balkans, V. ammodytes is not protected.

Vipera ammodytes is a medium-sized venomous snake with a relatively wide distribution, from Italy and Austria through the Balkans to the Caucasus (Heckes et al., 2005; but see Freitas et al., 2020). Although inhabiting elevations from 0 m to > 2,500 m, it is usually found in hills and low mountains, at 400-800 m (Crnobrnja-Isailović & Haxhiu, 1997; Jelić et al., 2013). The taxonomy, biogeography, and morphological and genetic

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diversification of V. ammodytes are well documented (Tomović, 2006: Ursenbacher et al., 2008: Tomović et al., 2010; Freitas et al., 2020), as well as its activity patterns (Crnobrnja-Isailović et al., 2007). However, information regarding its diet is meagre (Beškov, 1977; Dushkov, 1978; Saint Girons, 1980; Bea et al., 1992; Luiselli, 1996) compared to its congeners (e.g. Saint Girons, 1980; Bea & Braña, 1988; Luiselli & Agrimi, 1991; Bea et al., 1992; Luiselli et al., 1995; Brito, 2004; Santos et al., 2007, 2008). Members of the Viperidae family are almost exclusively sit-and-wait predators (Bea et al., 1992; Santos et al., 2008; Burbrink & Crother, 2011).

Data about reproduction of V. ammodytes is mainly derived from captive individuals (Gulden, 1988; Kutrup, 2000; Kariş & Göçmen, 2016; but see Beškov, 1977; Dushkov, 1978; Luiselli & Zuffi, 2002). Like other viviparous snakes, V. ammodytes is considered a capital breeder (Bonnet et al., 1998). Individuals of this species mate during April – May, and the young are born in late August - September (Luiselli & Zuffi, 2002; Tomović,

2015). In different parts of its distribution range females reproduce annually or bi-annually (Luiselli & Zuffi, 2002; Tomović et al., 2015). Litter size varies from four to 20 (Beškov & Nanev, 2002; Luiselli & Zuffi, 2002).

Given the fact that long-term field-based studies are logistically challenging, i.e. they are time-consuming (e.g. capture-mark-recapture population studies) and/or quite expensive (e.g. telemetry), dissections of museum specimens provide an uncomplicated alternative for obtaining detailed information about important ecological traits such as size/age at maturity, reproductive output, diet composition and body reserves (Shine et al., 2014). Although Luiselli & Amori (2016) made an important point that studies of snake diet made on museum specimens may have several drawbacks, such studies are inexpensive and avoid ethical and conservation problems related to collecting and killing of animals (Shine et al., 2014).

In this study we examined museum specimens of V. ammodytes from the central and western regions of the Balkan Peninsula to address the following questions: 1) At what body size does the species attain sexual maturity? 2) Are there differences in relative testes volumes between age groups and seasons? 3) What is the average number of offspring produced? 4) Is there a correlation between maternal body size and litter size? 5) Are there inter-sexual differences and ontogenetic shifts in diet composition? 6) Do seasonal differences in dietary preference exist? 7) Do fat reserves differ between sexes, age groups and seasons?

MATERIAL & METHODS

The herpetological collection of the Institute for Biological Research "Siniša Stanković", University of Belgrade, contains 460 specimens of Vipera ammodytes from the central and western parts of the Balkan Peninsula (Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, North Macedonia and Serbia, as well as few individuals from Bulgaria), collected between 1957 and 2007 (Džukić et al., 2017). According to the current taxonomic revision of Freitas et al. (2020), our samples belong to V. ammodytes sensu stricto.

Of the stored specimens from the Institute "Siniša Stanković", many could not be fully processed. In small individuals, we did not analyse reproductive organs, while many additional specimens were very old and in poor condition. For this study, we selected 125 males and 112 females that were non-systematically collected from February (males) and March (females) to September, i.e. during the active season. For 70 animals (29.5 %) location and/or the date of capture was not available (the "unknown date" group onwards) and they were excluded from some analyses.

Specimens were classified into the adult category if their snout-to-vent length (SVL) was larger than 43.8 cm for both sexes. This corresponded to a minimum of 49.3 cm total length (TotL) for males (the smallest male recorded mating in nature: Dushkov, 1978), and to 49.0 cm (TotL) for females (our results; the smallest female with embryos). Animals of < 30 cm TotL were classified as juveniles (following Luiselli (1996)). In our case, these corresponded to 26.7 and 26.3 cm SVL, for males and females, respectively. The individuals of intermediate body sizes were regarded as subadults. We measured body width (BW at mid-body), SVL, and tail length (TL) of the animals (with a tape measure and digital callipers); from the latter two, we calculated TotL.

In males (N = 117) we measured the dimensions of testes (length and width; with digital callipers, precision 0.01 mm). In females (N = 99), we measured the sizes of follicles/eggs and divided them into two groups: undeveloped follicles (< 10 mm of length) and developed eggs/embryos (> 10.0 mm) (Trauth et al., 1994; Shine et al., 2014). We counted all undeveloped follicles between 2 mm and 10 mm in the left and right ovary+oviduct. Length and width of embryos (not extending), were measured. Volumes of the entire body (for males), testes (TV) and developed eggs/embryos (> 10 mm, EV) were estimated from the SVL, length (L) of testes and developed eggs/embryos and width of the body (BW), and of testes and developed eggs/embryos (W). We used the equation for the volume of an ellipsoid: EV = 4/3 $\times \pi \times (L/2) \times (W/2)^2$ (Pleguezuelos & Feriche, 1999). As the testes volumes are correlated with body volumes, in some analyses we calculated relative values, considering the body (excluding tail) of the snake as a cylinder.

Gut content was checked in 125 males and 112 females. Due to the bad preservation quality of a large part of the sample, prey items were only identified to class level and categorised into the following prey types: mammal, lizard, bird, snake, and centipede. The presence of food remains was also separated according to their position in the stomach or intestine. Due to the small sample size for juveniles, in analyses of diet composition, we pooled them with subadults (i.e. immatures). In analyses of seasonal differences of diet composition, data were pooled into two seasons: spring (February to June) and summer (July to September). In 116 males and 96 females, the amount of the stored fat (fat reserves) was visually assessed following Pleguezuelos & Feriche (1999): 0 – no traces of fat; 1 – small traces of fat among the intestine loops: 2 - fat bodies cover less than half of the intestinal surface; 3 - fat bodies cover more than half of the intestinal surface; 4 – a continuous fat layer in the ventral zone of the abdominal cavity. Bearing in mind the effects of ethanol on the stored material (Vervust et al., 2009), we did not weigh any of the sampled animals or their internal organs.

Before any analyses, we tested the data for normality of distribution. The differences in relative testes volume between left and right testes in subadults and adults, the volume of developed eggs/embryos between left and right ovary+oviduct, and the number of undeveloped follicles and developed eggs/embryos between left and right ovary+oviduct for subadults and adults were analysed with the Wilcoxon Matched Pairs test. The differences between subadults and adults in relative testes volumes were analysed with the Mann-Whitney U test. Monthly variation (from February to September) in right testes volume (residual values of absolute testes volumes against SVL, both values log10-transformed) in

Table 1. Dimensions of testes in millimetres (average ± standard deviation, min-max), and the results of Wilcoxon Matched Pairs tests for differences in relative testes volumes between left and right testes within subadult and adult males of nosehorned vipers. LTL = left testes length, RTL = right testes length, LTW = left testes width, RTW = right testes width, RTV = relative testes volumes.

Age category	LTL	RTL	LTW	RTW	RTV
	24.6 ± 6.65	32.1 ±8.57	2.9 ± 0.92	2.4 ± 0.89	Z = 0.19
Subadults	(14.6-37.7)	(17.2-52.4)	(0.9–4.8)	(1.2–4.6)	p = 0.849
	N = 27	N = 26	N = 27	N = 26	
	34.5 ± 9.26	47.7 ± 10.69	4.3 ± 0.99	4.0 ± 1.10	Z = 3.49
Adults	(17.5–60.7)	(24.3–72.8)	(2.0–6.0)	(1.8–6.5)	p = 0.001
	N = 78	N = 80	N = 78	N = 80	

adult males was analysed by One-Way ANOVA test and Post-hoc (Fisher LSD) tests. The differences in volumes of the developed eggs/embryos among months were tested with the Kruskal-Wallis test. Linear regression was used to test the relationship between maternal body size (SVL) and the number of developed eggs/embryos; both values were log10-transformed.

Differences in diet composition between sexes, age groups (immatures and adults) and two seasons (spring and summer) were tested by χ^2 tests and contingency tables. Differences in the amount of fat reserves between sexes, subadults and adults (sexes separated), gravid and non-gravid females, as well as among months were analysed with non-parametric statistics (Mann-Whitney U test and Kruskal-Wallis test). Statistical analyses were performed with Statistica v. 10.0.

RESULTS

Reproductive characteristics Males

The relationship between the relative volume of the right testes against SVL showed that enlarged testes could be found only in individuals larger than 43 cm SVL (Fig. 1a). Mann-Whitney U tests showed that relative testes volumes were significantly higher in adult than in subadult males for the right organs (Z = 2.85, p = 0.004), but not for the left (Z = 1.63, p = 0.102) testes. In both subadult and adult males, the right testes were longer than the left (Table 1). Results of Wilcoxon Matched Pairs tests showed differences between left and right relative testes volumes only in adults (Table 1). We found seasonal variation in residual values of absolute right testes volumes in adult males: testis growth began in March-April, peaked in May, dropped during the summer, and then increased again in September (Fig. 2). Results of One-Way ANOVA showed that this variation was not significant (F_{148} = 1.12, p = 0.367). However, the results of post-hoc (Fisher LSD) tests showed that residual values of right testis volumes from June to August were significantly lower than in September (Table 2).

Females

We plotted the largest developed eggs/embryos length against the SVL of females to obtain female body size at maturity: enlarged follicles (> 10 mm) were observed only in individuals larger than 43 cm SVL (Fig. 1b), supporting



Figure 1. Graphic representation of body sizes at which enlarged testes (in males) or follicles/embryos (in females) occur: a) males - relative right testes volume against the body size (SVL); and **b**) females – length of the largest developed eggs/embryos against the body size (SVL)

that females achieve sexual maturity at this size. These females had between six and 39 follicles in both ovaries (14. 0±6.42 on average, 6.4±2.83 and 7.6±3.99 in left and right ovaries, respectively). In 75 adult females, we also found undeveloped follicles of different sizes (Table 3). Adults had between four and 35 undeveloped follicles in both ovaries (16.8±6.50 on average, 7.8±3.18 and 9.1±3.96 in left and right ovaries, respectively). Wilcoxon Matched Pairs tests showed significant differences in the number of undeveloped follicles between left and right ovaries only in adult females (Z = 3.20, p = 0.001).

Table 2. Results of Post-hoc (Fisher LSD) tests of residual values of right testes volumes in adult males of nose-horned vipers among months. * p < 0.05.

Month	February	March	April	May	June	July	August	September
February								
March	0.393							
April	0.430	0.827						
May	0.217	0.741	0.424					
June	0.536	0.628	0.725	0.195				
July	0.452	0.718	0.863	0.200	0.799			
August	0.532	0.646	0.751	0.225	0.981	0.831		
September	0.073	0.248	0.094	0.225	0.040*	0.040*	0.047*	

Table 3. Numbers of individuals of nose-horned vipers with undeveloped follicles and developed eggs/embryos / numbers of all females of the respective class caught in the given month.

	Individuals with un	developed follicles	Individuals with developed eggs/embryos
Month	Subadults	Adults	Adults
March		2/2	1/2
April		2/2	1/2
Мау	4 / 4	14 / 14	7 / 14
June	3/3	7/8	5 / 8
July	10/10	23 / 23	4 / 23
August		9/9	4 / 9
September	2/2		
Unknown	4 / 4	18/18	7 / 18
Sum	23 / 23	75 / 76	29 /76

In 29 of 76 (38.1%) adult females which were collected between March and August, we found developed eggs/ embryos (Table 3). The distribution of frequencies did not differ from a theoretical triennial reproduction frequency $(\chi^2 = 0.38, df = 1, p = 0.536)$. In two individuals, a single developed egg, and in one individual two developed eggs were found. Since they represent outliers we did not use them in analyses. The average litter size was 9.1 ± 2.5 (n = 26, range 5–17). There were significantly less (Z = 2.69, p = 0.007) developed eggs/embryos in left oviducts (3.8±1.5) compared to the right (5.3±1.87). Also, there was a statistically significant difference in sum volumes of developed eggs/embryos between left and right ovaries+oviducts (Z = 2.48, p = 0.013). Volumes increased significantly from March to August: Kruskal-Wallis test: $H_{(5 N = 198)} = 44.81$, p < 0.001 (Fig. 3). We found a statistically significant positive correlation between maternal body sizes (SVL) and the numbers of developed eggs/embryos they were carrying (r = 0.395, p = 0.046).

Diet

The majority of individuals with prey (97.5 % of the sample) had only one type of food, and only three specimens had two different types (lizard and mammal). Of individuals with food, in 41.8 % of males remains were found in the stomach, and in 89.6 % in the intestine, whereas food was found in the stomach in 40.4 % of females, and 76.9 % had food remains in the intestine

(Table S1). The dominant prey types of V. ammodytes were lizards (64.7 %, e.g. Podarcis muralis, Lacerta viridis, Anguis fragilis), followed by mammals (31.9 %). Other types of prey (birds, snakes (Natrix tessellata) and centipedes (Scolopendra cingulata)) were negligible in diet composition (3.4 %) (Table S1).

Comparison of the sexes and age categories

Approximately half of the dissected specimens (53.6 % and 46.4 % of males and females, respectively) had food remains in their digestive systems. The relative numbers of males and females with and without food remains were similar (χ^2 = 1.22, df = 1, p = 0.270, all age classes pooled). There were no differences between gravid and non-gravid females in presence of food items ($\chi^2 = 1.10$, df = 1, p = 0.298).

In the general analysis of diet composition, we found no differences between the sexes (χ^2 = 5.11, df = 4, p = 0.276). However, when we considered only the two most dominant types of prey (lizards vs. mammals), analyses of inter-sexual differences showed that males more frequently consumed lizards than mammals (χ^2 = 6.28, df = 1, p = 0.012), while in females both types of prey were present in a frequency not different from the null hypothesis (χ^2 = 1.46, df = 1, p = 0.227). Thus, we separated sexes in further analyses of two age categories. We found differences in the frequency of lizards and mammals as prey between immatures and adult males



Figure 2. Volumes of right testes of the nose-horned vipers (residual scores for regression of the right testes volumes (log10) against snout-vent length (log10 SVL)) vs. month of the activity period (only adult males).

(Yates corrected χ^2 = 3.95, df = 1, p = 0.047), but not in females of two age classes (Yates corrected $\chi^2 = 0.97$, df = 1. p = 0.324).

Seasonal differences in diet composition

Seasonal change in diet composition was statistically significant in males (χ^2 = 14.10, df = 6, p = 0.029), but not in females (χ^2 = 4.48, df = 6, p = 0.611). In the spring season, immature males fed almost exclusively on lizards and adults had a more diverse diet, while in summer both age categories fed on lizards and mammals. Opposite to males, immature and adult females had a diverse diet both in spring and in summer (Fig. 4). In February (males), as well as in August and September (females), V. ammodytes fed exclusively on lizards, while other prey items (birds, snakes and centipedes) were found in May and June, exclusively.

Fat reserves

Results of Mann-Whitney U tests showed that there were no inter-sexual differences in the amount of fat reserves in both subadults (Z = -1.48, p = 0.140) and adults (Z = -0.96, p = 0.336). Pregnant females had similar amounts of fat reserves as the non-pregnant ones (Z = -1.57, p = 0.117). Adult individuals had more fat reserves than subadults both in males (Z = 4.12, p < 0.001) and in females (Z =-2.51, p = 0.012). Kruskal-Wallis test showed that the amount of fat reserves was not different among months in adult males ($H_{(7, N = 52)} = 5.07$, p = 0.651), non-gravid females (H_(5, N = 34) = 2.81, p = 0.730) and gravid females $(H_{(5, N=21)} = 2.14, p = 0.830)$. The highest amount of fat bodies in adults was in early spring (February – March in males, March - April in females). The lowest amount in adult males was in May and remained relatively constant until the end of the season (Fig. 5a). Amount of body reserves varied more in non-gravid than in gravid females but was high in both groups until the end of August (Fig. 5b).



Figure 3. Volumes of developed eggs/embryos through months in adult females of nose-horned vipers.

DISCUSSION

Our study shows that anatomical dissections from museum specimens can provide fundamental information about size at maturity, reproductive output, diet composition and amount of body reserves for snakes. Information about life-history traits are essential for species conservation (Filippi & Luiselli, 2000; Pleguezuelos et al., 2007; Shine et al., 2014). Analyses of museum specimens have both advantages (e.g. inexpensive method, large samples from an important part of the species range) and limitations (e.g. preservation status of specimens precludes accurate measurements of some body parts, unequal representation of specimens from different parts of the species range, sex-related biases due to different activity patterns) (Crnobrnja-Isailović et al., 2007; Luiselli & Amori, 2016).

Reproductive characteristics

Although sexual dimorphism in nose-horned vipers was previously confirmed (Tomović et al., 2002) and adult males are generally larger than females (e.g. Beškov, 1977), results of our study showed that both sexes attain sexual maturity at approximately the same body size.

Males

Testes (relative size) were significantly larger in adult males than in subadults, but only the right side organs. In male snakes, there is a significant increase in testes size with the onset of reproductive maturation (White & Kolb, 1974). To the best of our knowledge, the only precise information regarding the size of testes at the onset of sexual maturation in vipers is available for Vipera latastei (Pleguezuelos et al., 2007, Fig 1A). However, this species attains a much smaller adult body size than V. ammodytes. Although we did find enlarged testes in individuals larger than 43 cm (SVL), our data did not show a clear difference in testes sizes between subadult and adult males. Therefore, we could not precisely set the threshold body size values for sexual maturity in males of the nose-horned viper.



Figure 4. Numbers of different prey types in males (M) and females (F) through months of all age groups of nose-horned vipers



Figure 5. Amount of fat reserves through months in a) adult males; b) adult gravid and non-gravid females of nosehorned vipers.

Our finding that right testes of V. ammodytes were significantly longer (and relatively more voluminous) than the left (like in *Thamnophis* sp. – White & Kolb, 1974; Shine et al., 2000), was most probably the consequence of the general asymmetry of internal organs in snakes (Shine, 1977; Shine et al., 2000; Gribbins & Rheubert, 2011). Also, our study found seasonal variation in testes volume in adult males: testicular growth began in March-April, peaked in May, dropped in summer (June-August), and then increased again in September (Fig. 2). It is generally known that testes size is variable during the reproductive cycle in vipers (e.g. Nilson, 1980; Saint Girons, 1992; Pleguezuelos et al., 2007), as well as in other snake species in temperate regions (Duvall et al., 1992; Pleguezuelos & Feriche, 1999; Sivan et al., 2016). In V. ammodytes, mating was recorded only in spring (Crnobrnja-Isailović et al., 2007; Tomović, 2015), which differs from V. latastei where copulation was observed both in spring and autumn, depending on the geographic region (Pleguezuelos et al., 2007). Our results indicate that in nose-horned vipers, perhaps both vernal and autumnal spermatogenesis occur (Fig. 2), but the small sample size precluded us from a definite conclusion. Further research is needed to understand if there is dissociated timing of male and female reproductive cycles in V. ammodytes, and whether short-term (in females) or long-term (in males) sperm storage (Saint Girons, 1992; Aldridge & Duvall, 2002; Pleguezuelos et al., 2007) is characteristic for this species across its entire distributional range.

Females

Results of this study enabled us to set the threshold body size value for sexual maturity in females of nosehorned vipers: the smallest female with developed eggs/ embryos had 43.8 cm SVL (49.0 cm TotL). The total body length of the smallest gravid female in our sample was lower than the values reported by some other authors (52.0 cm (Luiselli & Zuffi, 2002) and 59.5 cm (Beshkov, 1977)), probably due to large geographic variability in growth rates and adult body sizes, often resulting from environmental factors (Tomović et al., 2002; Zuffi et al., 2009), as well as because of larger sample sizes in our study.

Undeveloped follicles were found in most subadult and adult females in our sample, but only 38.1 % of adult females were gravid (had developed eggs/embryos). The latter stands for both the complete sample and for the subsample of adult females caught during the reproductive season (May, June, July and August). This result implies that V. ammodytes in the central and western parts of the Balkans reproduce on a biennial or even triennial basis (see Table 3), as was reported for some other parts of the species' range (Luiselli & Zuffi, 2002). Accordingly, V. ammodytes displays a capital breeding strategy (Bonnet et al., 1998), typical for other European vipers (e.g. Zuffi et al., 1999; Bonnet et al., 2002; Luiselli & Zuffi, 2002). Less-than-annual reproduction was shown to contribute to the species' vulnerability to extinction (Pleguezuelos et al., 2007; Tomović et al., 2015).

In our study, the average number of developed eggs/ embryos was 9.1, while the range was between 5 and 17. This average was much higher compared to literature data (5.6 – Luiselli & Zuffi, 2002; 7.3 – Beškov, 1977; 7.0 – Dushkov, 1978). Also, the observed range of numbers of developed eggs/embryos was wider than the published for the same species (4-8 - Luiselli & Zuffi, 2002; 4-12 - Beškov, 1977; 5-10 - Dushkov, 1978). This could be the consequence of the quantitative and qualitative differences in the sample structure. Previously published data were based on relatively good population samples from rather narrow locations/regions: 17 females (Luiselli & Zuffi, 2000) and six females (Beškov, 1977) from the same population, as well as 11 females from three close regions (Dushkov, 1978). Unfortunately, we do not have dense population samples, and the second drawback was that our sample covered a very wide distribution range (territory of Ex-Yugoslavia), with (implicitly) huge variation of abiotic and biotic conditions and factors. Possible explanations for the numbers of developed eggs or embryos smaller than expected (observed in three individuals not included in analyses) may be a reproductive failure (egg and embryo resorption as a compensatory mechanism for minimising nutrient losses during failed attempts at reproduction: Bonnet et al., 2008) or result from other constraints such as dehydration, protein deficiency or the presence of parasites (Lourdais et al., 2015; Dyugmedzhiev et al., 2018 and references therein).

As expected, we found a significant correlation between maternal body size (SVL) and numbers of developed eggs/embryos (Luiselli & Zuffi, 2002; Zuffi et al., 2009). It is known that the reproductive success of females is dependent on body size because larger females have more abdominal space (Shine, 1978, Madsen & Shine, 1994). Our result that more undeveloped follicles and developed eggs/embryos were found in right ovaries + oviducts, could also be the consequence of the general asymmetry of internal organs in snakes (Shine, 1977; Shine et al., 2000; Gribbins & Rheubert, 2011).

Diet

Approximately half of the nose-horned vipers we dissected had remains of food in their digestive systems. This suggests that V. ammodytes has low feeding frequency, which is also observed in other European vipers (Santos et al., 2007 and references therein). Nose-horned vipers almost exclusively consumed lizards (64.7 %) and mammals (31.9 %). Only a few individuals (3.4 %) ate birds, snakes and centipedes, which was already known from the literature (Clark, 1967; Beškov, 1977; Luiselli, 1996; Arsovski et al., 2014). A very high percentage of lizards in the diet of nose-horned vipers in our study is quite different from the only detailed analysis of the diet composition of this species in the literature (Beškov, 1977). In south-western Bulgaria, V. ammodytes individuals of all age categories fed predominantly on mammals (60.9 %), then on lizards (22.8 %), far less on centipedes (12.0 %) and the least on birds (3.3 %) (Beškov, 1977). Within European vipers, V. latastei and V. ammodytes are the most similar species in regards to general diet composition (Bea et al., 1992), with the smallest proportion of small mammals in the diet (Saint Girons, 1980; Bea et al., 1992). The reason could be the fact that both species inhabit predominantly Mediterranean and Submediterranean habitats with similar prey type availability. However, V. latastei shows significant variation in prey preferences along a latitudinal cline (Santos et al., 2008) with more mammals eaten in humid areas and a more diverse diet in drier and Mediterranean regions.

Comparison according to sex and age category

Our results showed no difference in the percentage of female and male V. ammodytes with and without food, although it has been reported that, generally, their activity patterns differ (Crnobrnja-Isailović et al., 2007). Our data were contradictory to the literature in that we found a higher proportion of individuals with food than those without food (Nilson, 1980; Madsen & Shine, 1993), suggesting that adult males still feed during the mating season. Also, there were no differences between gravid and non-gravid females regarding the presence of food items, contrary to published data which showed that reduced feeding in gravid females (at least in viviparous snakes) is a common phenomenon, probably due to limitation of abdominal space to accommodate food and embryos (Gregory et al., 1999; Lourdais et al., 2002). However, many studies show that female snakes occasionally feed during pregnancy (e.g. Barron et al., 2013; Claus & Bauwens, 2019).

In our analysis of complete diet composition, we found no difference between the sexes. However, when we considered only the two most dominant types of prey (lizards vs. mammals), analyses of inter-sexual differences showed that in the diet of males, lizards were significantly more prevalent than mammals, while in females both types of prey were equally present. These results are different from those obtained for V. latastei where no intersexual differences in diet composition were found (Brito, 2004; Santos et al., 2007). Sexual dimorphism could also be the cause of diet differences, as reported for other snake species (Camilleri & Shine, 1990; Vincent et al., 2004; Meik et al., 2012). In nosehorned vipers, sexual dimorphism was recorded for head size, with females having higher values of head width (Tomović et al., 2002) and thus, they may be able to feed on larger prey (i.e. mammals). Another possible reason for a greater percentage of mammals than lizards in the female diet may be due to the higher energetic demands of females since they must allocate more resources to reproduction, and mammals supply more energy per biomass than reptiles (Shine, 1989).

Results of our study may imply a subtle ontogenetic shift in diet (significant only in males), where towards adulthood the share of mammals increases at the expense of lizards. Similar results were obtained for other viper species (Saint Girons, 1980; Luiselli, 1996; Beškov, 1977; Bea et al., 1992; Santos et al., 2007, 2008). The ontogenetic shift in diet composition may be the consequence of the gape limitations of small snake individuals, which have a narrower range of prey types suitable for ingestion (e.g. Shine, 1991; Shine & Madsen, 1997).

Seasonal differences in diet composition

We found seasonal variation in the diet composition of nose-horned vipers. The consumption of lizards decreased from spring to summer in both sexes, while for mammals the trend was the opposite. However, this result should be taken as tentative given the limited sample of subadult individuals. Most probably, the prev spectrum depends on the availability of prey at a given moment, as previously shown for V. latastei (Santos et al., 2007, 2008). Our data needs to be taken cautiously given that result may include possible seasonal differences in the diet related to climatic heterogeneity across the wide distribution range of nose-horned vipers, as already recorded for some other viper species (e.g. Santos et al., 2008).

Fat reserves

The largest amount of fat reserves in adult individuals of both sexes was recorded in early spring (February -April) and it remained relatively constant until the end of the season (Fig. 5a,b). Gravid and non-gravid females did not differ in the amount of fat reserves. A possible explanation for similar amounts of fat reserves between gravid and non-gravid females may be feeding during gestation (Lourdais et al., 2002), that was confirmed in this study, i.e. there was no difference in the percentage of diet in the gut between gravid and non-gravid females. Results of our study showed that nose-horned vipers (like all other vipers) are capital breeders which are capable

of storing large fat reserves for reproduction (Bonnet et al., 1998). However, we also found pregnant females with prey, which means that this species may also be an income breeder and that capital and income attainment of energy may not be alternative, but additive strategies (Pleguezuelos et al., 2007). Feeding activity during and after gestation increases fitness and reproductive output; if food intake during gravidity is reduced or absent, females may not reproduce (have bi-annual or less frequent reproduction) (Zuffi et al., 1999; Luiselli & Zuffi, 2002; this study).

We did not find differences in the amount of fat bodies between the sexes, and this character is probably species-specific (Bonnet et al., 1998). Adults had a higher amount of fat reserves compared to subadults, probably because they have a bigger gape and greater range of prey types that can be ingested, or a higher feeding success (Shine, 1991; Shine & Madsen, 1997).

CONCLUSION

According to our knowledge, this is the most comprehensive analysis of reproductive characteristics, diet composition and amount of fat reserves in V. ammodytes. It represents the basis for future fieldoriented population (Capture-Mark-Recapture) studies that could reveal the influences of geographic/ecological variability on life-history characteristics of nose-horned vipers. The obtained data about reproductive traits (i.e. biennial or even triennial reproduction) and dietary specialisation demonstrate the vulnerability of this species (Tomović et al., 2015), generally regarded as "very common" and non-threatened (Tomović, 2015).

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Author Contributions:

MA and SN performed laboratory work. SN and MA analysed data and wrote the initial draft. All authors analysed and discussed the data. MA and LT wrote the final manuscript. All authors read and approved the final manuscript.

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FULL PAPER



New evidence for distinctiveness of the island-endemic Príncipe giant tree frog (Arthroleptidae: Leptopelis palmatus)

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The Principe giant tree frog Leptopelis palmatus is endemic to the small oceanic island of Principe in the Gulf of Guinea. For several decades, this charismatic but poorly known species was confused with another large tree frog species from continental Africa, L. rufus. Phylogenetic relationships within the African genus Leptopelis are poorly understood and consequently the evolutionary history of L. palmatus and its affinity to L. rufus remain unclear. In this study, we combined mitochondrial DNA (mtDNA), morphological, and acoustic data for L. palmatus and L. rufus to assess different axes of divergence between the species. Our mtDNA gene tree for the genus Leptopelis indicated that L. palmatus is not closely related to L. rufus or other large species of Leptopelis. Additionally, we found low mtDNA diversity in L. palmatus across its range on Príncipe. We found significant morphological differences between females of L. rufus and L. palmatus, but not between males. We characterised the advertisement call of L. palmatus for the first time, which is markedly distinct from L. rufus. Finally, we summarised our observations of L. palmatus habitats and additional notes on phenotypic variation and behaviour. Our study reinforces the distinctiveness of L. palmatus and provides information important for the conservation of this endangered species.

A rã gigante de Príncipe, Leptopelis palmatus, é endêmica da pequena ilha oceânica de Príncipe no Golfo da Guiné. Por várias décadas, esta espécie carismática mas pouco conhecida foi confundida com outra espécie grande de rã da África continental, L. rufus. As relações filogenéticas dentro do gênero africano Leptopelis são mal compreendidas e, conseqüentemente, a história evolutiva de L. palmatus e sua afinidade com L. rufus permanecem obscuras. Neste estudo, combinamos dados de DNA mitocondrial (mtDNA), morfológicos e acústicos de L. palmatus e L. rufus para avaliar diferentes eixos de divergência entre as espécies. Nossa árvore de genes de mtDNA para o gênero Leptopelis indicou que L. palmatus não está proximamente relacionada a L. rufus ou outras espécies grandes de Leptopelis, e encontramos baixa diversidade de mtDNA em L. palmatus em toda a sua distribuição em Príncipe. Encontramos diferenças morfológicas significativas entre as fêmeas de L. rufus e L. palmatus, mas não entre os machos. Caracterizamos o canto reprodutor de L. palmatus pela primeira vez, que é marcadamente distinto do de L. rufus. Finalmente, resumimos nossas observações dos habitats de L. palmatus e notas adicionais sobre variação fenotípica e comportamento. Nosso estudo fornece informações importantes para a conservação dessa espécie ameaçada de extinção

Keywords: bioacoustic, morphology, endemic, mitochondrial DNA, island, São Tomé and Príncipe

INTRODUCTION

The Príncipe giant tree frog *Leptopelis palmatus* (Peters, 1868) is endemic to the small (142 km²) oceanic island of Príncipe in the Gulf of Guinea. For several decades, this charismatic but poorly known species was confused with another large tree frog species from continental Africa, L. rufus Reichenow 1874 (Anderson, 1909; Parker, 1936; de Witte, 1941; Perret, 1962). Phylogenetic relationships within the African genus Leptopelis are poorly understood and consequently the evolutionary history of L. palmatus and its affinity to L. rufus remain unclear. Previous authors have hypothesised that *L. palmatus* is closely related to *L*.

rufus and other large-bodied species in West and Central Africa (L. macrotis, L. millsoni) based on a combination of mtDNA and morphological data (e.g., Idris, 2004). A more recent study estimating diversification across Afrobratrachia, which is composed of the families Arthroleptidae, Brevicipitidae, Hemisotidae, and Hyperoliidae, does not support this relationship (Portik et al., 2019); however, relationships among Leptopelis were not the primary focus of that study and the molecular data matrix was a combination of mtDNA and very sparse nuDNA loci. Here we include more comprehensive sampling of Leptopelis diversity (55 % of currently recognised species) and use a complete mtDNA data matrix with the sole aim of identifying the

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closest continental relatives of the island endemic. We also generate mtDNA sequence data from L. palmatus collected across its elevational and geographic range on Príncipe to assess genetic diversity and phylogeographic structure within the species.

Throughout nearly a century of taxonomic confusion between L. palmatus and L. rufus, L. palmatus was reported from several countries including Cameroon, Equatorial Guinea (including Bioko Island), Gabon, and Nigeria (Boulenger, 1882; Mocquard, 1902; Boulenger, 1906; Nieden, 1910; Ahl, 1931; Schiøtz, 1963; Mertens, 1965). After comparing a large series of male and female L. rufus with the sole female holotype of L. palmatus available for study, Perret resurrected L. rufus and clarified that L. palmatus was an insular species (Perret, 1973). Perret also remarked that L. rufus and L. palmatus differed in tympanum size and several additional morphological features, concluding that despite decades of taxonomic confusion the two species may not even be closely related (Perret, 1973). Loumont later collected a series of eight L. palmatus females, confirmed the results of Perret's morphological study and reported a snout-vent length of up to 110 mm, which remains the largest size record for the entire genus (Loumont, 1992; Channing & Rödel, 2019). Male specimens of *L. palmatus* were finally collected and formally described following an expedition to Príncipe in 2002 (Drewes & Stoelting, 2004). We have since collected additional male and female specimens of L. palmatus, providing the opportunity to compare large series of both sexes of L. palmatus and L. rufus to assess phenotypic variation within and between the species.

Very little is known about the natural history of Leptopelis palmatus, an endangered island endemic (IUCN SSC Amphibian Specialist Group, 2020). Although males lack vocal sacs (Drewes & Stoelting, 2004), they do produce advertisement calls and here we report the first recording and analysis of their call, which we compare to that of L. rufus. We also summarise our observations of L. palmatus habitats and additional notes on phenotypic variation and behaviour. In summary, our study combines mitochondrial DNA (mtDNA), morphological, and acoustic data for L. palmatus and L. rufus to assess different axes of divergence between the species. We couple our findings of distinctness of L. palmatus with additional notes on phenotypic variation and behaviour to provide important information for the conservation of this endangered species.

METHODS

Field sampling

We conducted six herpetological expeditions to Príncipe Island between 2001 and 2016, during which we searched for Leptopelis palmatus. Tissue samples (including liver or muscle) were preserved in 95 % ethanol or RNAlater and voucher specimens were fixed in formalin. The specimens were deposited at the California Academy of Sciences (CAS) and the U.S. National Museum of Natural History (USNM).

Phylogenetic and Population Genetic Analyses

We obtained 16S mtDNA sequences for 30 species in the genus Leptopelis (55 % of the currently recognised species) and two confamilial outgroups (Arthroleptis and Cardioglossa; Portik & Blackburn, 2015) from GenBank, only selecting sequences with associated voucher specimens (Table S1). We aligned the sequences with MAFFT using the automatic algorithm selection option (--auto) (Katoh et al., 2002; Katoh & Standley, 2013) and selected the HKY + I + G substitution model based on BIC in jModelTest (Darriba et al., 2012). To perform Bayesian phylogenetic analyses, we used BEAST v1.8 (Drummond et al., 2012) with a birth-death tree prior (Stadler, 2009), and a lognormal relaxed molecular clock. Two independent analyses were run for 10 million generations each with sampling every 1,000 generations, producing a total of 20,000 trees. We assessed convergence and the effective sample size of parameter estimates using Tracer v1.7 (Rambaut et al., 2018) and repeated simulations without sequence data to test the influence of priors on posterior distributions. We discarded a burn-in of 10 % prior to generating a maximum clade credibility tree from the remaining 18,000 trees.

To assess mtDNA structure across the entire known geographic and elevational range of *L. palmatus*, we extracted DNA from 35 tissue samples using a DNeasy Blood & Tissue kit (Qiagen Inc., Valencia, CA, USA), and polymerase chain reaction (PCR)-amplified and cycle sequenced a portion of the 16s mitochondrial gene using the primers 16SA and 16SB (Palumbi et al., 1991). Each reaction contained the following components: 1 µL of template DNA, 6.05 µL H₂O, 1.75 µL 10X Buffer, 0.3 µL of each primer, 0.25 µL dNTPs, 0.1 µL of Bovine serum albumin (BSA), 0.1 µL of MgCl, and 5 µL of goTAQ DNA Polymerase (Promega, Madison, WI, USA). We used a thermocycler protocol for amplification beginning with denaturation for 1 min 30 seconds at 94 °C, followed by 33 cycles, which consisted of 45s denaturation at 94 °C, 45s annealing at 55 °C, and 1 min 30s extension at 72 °C, followed by the final extension occurring at 72 °C for 5 min. We purified PCR products using ExoSAP-IT (USB Corp., Cleveland, OH, USA) and carried out sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI automated 3730xl Genetic Analyzer (Applied Biosystems). All sequences were edited using Geneious v.R8.0.04 (Biomatters Ltd.) and are available on GenBank (see Supplementary Materials). Due to the limited genetic diversity in our dataset, we estimated a mtDNA haplotype network using the TCS algorithm (Clement et al., 2000) implemented in PopArt (Leigh & Bryant, 2015).

Morphological data collection and analysis

To assess the maximum reported female body size of L. palmatus relative to other species included in the phylogeny of the genus we obtained measurements from the literature (Table S1) and plotted them alongside the phylogeny. To compare variation in diagnostic traits between L. rufus and L. palmatus (Perret, 1973; Schiøtz, 1999), we took morphological measurements of adult



Figure 1. (a) 16S mtDNA gene tree of 30 currently recognised species in the genus Leptopelis with maximum female (the larger sex) snout-vent length (SVL) in millimetres. Black circles on nodes indicate > 0.95 posterior probability, grey circles indicate > 0.85 posterior probability. All other nodes are poorly supported. (b) Geographic sampling of L. rufus and L. palmatus examined in this study. (c) Sampling map and mtDNA haplotype network for L. palmatus on Príncipe Island. Open circles indicate additional localities with vouchered L. palmatus specimens that are currently housed in the Museu Nacional de História Natural e da Ciência, Universidade de Lisboa, Portugal (Ceríaco, 2016; Ceríaco & Marques, 2018).

frogs to the nearest 0.1 mm using Mitutoyo Absolute Digimatic Callipers. The following measurements were taken by KEJ and RCB: snout-vent length (SVL); eye diameter, measured as the ocular aperture (EYE); maximum horizontal diameter of left tympanum, measured to outer margin of tympanic rim (TMP); and maximum diameter of disc toepad, measured from the fourth digit of the left hindfoot (DSC). The ratios TMP:EYE and DSC:TMP have been proposed as useful diagnostic comparisons, but without accounting for potential sexual variation (Schiøtz, 1999). Sex was determined by a) snout-vent length, b) the presence (or absence) of pectoral glands in preserved specimens, c) the presence of eggs, or d) field notes indicating calling behaviour. A total of 109 adult specimens were measured (39 L. palmatus and 70 L. rufus). Male and female measurements were analysed separately to account for sexual size dimorphism. We omitted 10 samples that could not be identified to sex. resulting in a dataset of 58 male (19 L. palmatus and 39 L. rufus) and 41 female specimens (17 L. palmatus and 24 L. rufus). To account for allometry across individuals, we corrected TMP measurements (Thorpe, 1975; Thorpe, 1983a; Thorpe, 1983b; Turan, 1999) using the allometric equation: Xadj = $X - \beta$ (SVL-SVLmean). In this equation, Xadj is the adjusted value of the morphometric variable measurement, X is the original value taken from the dial callipers, β is the coefficient of the linear regression of X against SVL in the dataset, SVL is the snout-vent length of the individual, and SVLmean is the overall mean snoutvent length in the dataset. To quantify divergence in

SVL, TMP, TMP:EYE, and DSC:TMP, we fit an ANOVA for each set of traits with measurements grouped by species and used a Tukey Honest Significant Differences test to calculate adjusted P values for group mean comparisons. All statistical analyses were performed in R v 4.0.2 (R Core Team 2020), and data were visualised using the ggplot2 package (Wickham, 2016).

Bioacoustic data collection and analysis

We collected an acoustic recording of a male L. palmatus on November 28, 2016 (25.9 °C) in situ at a presumed breeding site. The call was recorded using a Roland R-26 portable recorder paired with a Sennheiser ME-66 microphone at an approximate distance of 2 m from the male. We described the calling site and behavioural context, and prepared the male as a voucher specimen for genetic and morphological analysis (USNM 591754). The recording was archived in the Macaulay Library at the Cornell Lab of Ornithology (ML 206529). We analysed advertisement calls of *L. rufus* that were recorded in Cameroon (Amiet & Goutte, 2017). Audio spectrograms and oscillograms were made using Raven Pro 1.4 (Cornell Lab of Ornithology), and analysed with a Fast Fourier Transformation window of 512 points, a brightness of 70 points, and a contrast of 70 points following Gilbert and Bell (2018). The following parameters were measured for each call because they capture the primary axes of variation in Leptopelis advertisement calls (Amiet & Goutte, 2017): pulses per call, pulse duration, peak frequency (Hz), frequency range (Hz), and total number of notes recorded.



Figure 2. (a) Violin plots of adjusted Snout-Vent Length (SVL), Tympanum size (TMP), Tympanum-Eye ratio (TMP:EYE), and Disc-Tympanum ratio (DSC:TMP) in *L. palmatus* and *L. rufus*. Comparisons significant at an adjusted P < 0.05 with a Tukey Honest Significant Difference test are indicated with an asterisk. (b) Call parameter definitions and representative audio spectrograms of *L. palmatus* and *L. rufus* male advertisement calls. (c) colour variation in *L. palmatus* (from left to right: CAS 258958, CAS 258910, USNM 591753, USNM 591758) Photos A. Stanbridge and R. Bell.

RESULTS

Phylogenetic relationships within Leptopelis and genetic variation within L. palmatus

The 16S mtDNA gene tree of species-level relationships within the genus Leptopelis indicated strong support for the monophyly of some species groups including the largebodied West and Central African species L. rufus, L. macrotis, and L. millsoni, and the Ethiopian radiation L. vannutellii, L. yaldeni, L. gramineus and L. susanae (Fig. 1A). By contrast, the phylogenetic placement of most species including L. *palmatus* was poorly supported in the present dataset. However, our results indicate that L. palmatus does not appear to be closely related to L. rufus or to other, largerbodied species in the genus. We found very low diversity at 16S across the range of *L. palmatus* on Príncipe Island with one dominant haplotype, three minor haplotypes, and no pattern of phylogeographic structure (Fig. 1C).

Morphological divergence between L. rufus and L. palmatus

We found that morphological differences between L. rufus and L. palmatus were distinct in females versus males. SVL, adjusted TMP size, and TMP:EYE ratios were all significantly different between female specimens of L. palmatus and L. rufus, but not male specimens (Fig. 2). Overall, *L. palmatus* females exhibited larger body sizes and had larger TMP than *L. rufus* (P < 0.05; Fig. 2A). Likewise, the ratio of TMP:EYE was typically >1/2 in female *L. palmatus* and <1/2 in female *L. rufus* (P < 0.05; Fig. 2A). DSC:TMP ratios were not significantly different between L. palmatus and L. rufus in either males or females, and none of the remaining measurements were significantly different between males of the two species (Fig. 2A).

Table 1. Summary of call analysis for each species. Abbreviations are as follows: APF (Average Peak Frequency), APD (Average Pulse Duration)

Species	Location	Notes	Pulses	APF (Hz)	APD (s)	Recording Source
L. palmatus	Príncipe, trail from São Joaquim village to Pico Príncipe	14	1-8	1170 (937 – 1406)	0.04 (0.03 –0.05)	This study (ML 206529)
L. rufus 1	Cameroon, Ototomo	6	1	2857 (2756 – 3359)	0.24 (0.22 – 0.26)	Amiet & Goute, 2017
L. rufus 2	Cameroon, Kala Afomo	7	1	3248 (2842 – 3962)	0.23 (0.23 – 0.24)	Amiet & Goute, 2017

Calling sites, advertisement call, and colour variation in L. palmatus

We observed calling males on two occasions in 2015 (6 October 2015 and 7 October 2015) but were unable to obtain recordings. The first site was along the road between Gaspar and Sundi where we observed a calling male perched 2 m above ground on a vine at 1958h (CAS 258910). A gravid female was found nearby several minutes later (CAS 258911). The second site was along a small stream flowing through secondary forest in the vicinity of Nova Estrela where we found two calling males at 4 m (CAS 258916) and 2.5 m (CAS 258917) above ground at approximately 1800h. On 28 November 2016 we observed several calling males along the trail from São Joaquim village to Pico Príncipe, in a tributary of the Ribeira Banzú. Males were perched 2–3 m above ground on palm fronds, tree branches, and tree trunks in the vicinity of a medium stream (3 m across) and above a swampy ditch. We recorded one male (USNM 591754) at 1805h, which was perched approximately 2 m above the ground on a palm frond. The advertisement call consisted of a simple note with a dominant frequency of 1170 Hz, average pulse duration of 0.04 seconds, and a range of pulses from 1-8 (Fig. 2B, Table 1). In the period we recorded, several calling bouts progressed from notes with one pulse to two pulses to a series of eight pulses (Fig. 2; Table 1). The waveform and structure of the L. palmatus call is quite different from that of L. rufus, which has a much higher dominant frequency (2800+ Hz), longer average pulse duration (0.23 seconds), singlepulse notes, and numerous harmonics (Fig. 2B; Table 1). We documented extensive dorsal colour pattern variation in both male and female *L. palmatus* including dark green/black with or without spots, bright green, and bright yellow with dark mottling (Fig. 2C; Table S2).

DISCUSSION

Phylogenetic relationships within the genus Leptopelis are poorly understood; however, relationships among some species groups are strongly supported in our mtDNA gene tree and are consistent with phenotypically and/or geographically cohesive groups (Portillo & Greenbaum, 2014; Reyes-Velasco et al., 2018). Although the phylogenetic placement of L. palmatus remains unclear, our inference does not support a close affinity to L. rufus, as foreshadowed by Perret's morphological comparisons (Perret, 1973). More complete taxonomic sampling and a larger genetic dataset will be necessary to establish a robust phylogenetic inference for the genus Leptopelis and to inform the biogeographic history of L. *palmatus*. Our results indicate very low genetic diversity in L. palmatus and no evidence of phylogeographic structure across its range. Two other amphibian species are also endemic to Príncipe Island: the puddle frog Phrynobatrachus dispar (Peters, 1870) and the reed frog Hyperolius drewesi Bell 2016. Both are very abundant and based on our surveys, their distributions span even more of the geographic and elevational range of the island than does L. palmatus (Loumont, 1992; Drewes & Stoelting, 2004; Uyeda et al., 2007; Bell, 2016). Population genetic studies for both P. dispar and H. drewesi found very low genetic diversity and no patterns of genetic structure based on mtDNA-only (P. dispar, Uyeda et al., 2007) or a combination of mtDNA and genome-wide SNPs (H. drewesi, Bell et al., 2015). Consequently, there do not appear to be geographic barriers to gene flow across the ranges of the endemic amphibians of Príncipe.

Cryptic morphological variation has eluded species relationships in the genus Leptopelis, including nearly a century of taxonomic confusion between L. palmatus and L. rufus. Our morphological assessments of overall body size and tympanum measurements indicate significant differences between L. palmatus and L. rufus in females but not in males. In particular, the ratio of tympanum size to eye size, which has been proposed as a diagnostic character among large-bodied Leptopelis (L. macrotis, L. millsoni, L. palmatus, L. rufus; Schiøtz, 1999) is adequate for differentiating among L. rufus and L. palmatus females, but not males. Although the ratio of disc to tympanum width has also been proposed as diagnostic (Schiøtz, 1999), toe disc dimensions may change with preservation and be a less reliable feature to measure than tympanum or eye diameter. Sexual dimorphism in tympanum size is known in other anurans and may have functional consequences for acoustic signalling and sensitivity (Fox, 1995). Consequently, estimating acoustic sensitivity in male and female L. palmatus relative to male and female L. rufus would be an interesting extension of the pattern we identified here. Our results support that exceptional body size in *L. palmatus* is due to females reaching very large sizes whereas male body size appears to be within the range of male *L. rufus*. Despite large adult body sizes, post-metamorphic individuals of *L. palmatus* are quite small (10-11 mm; Drewes & Stoelting, 2004) and it is unknown how long individuals take to reach

reproductive maturity or how old very large females are. It is unclear what selective pressures (or lack thereof) on Príncipe have resulted in the apparent island gigantism of this species.

The advertisement call of L. palmatus is rather quiet and our observations thus far indicate that males form small breeding aggregations. The call of L. palmatus is quite distinct from that of L. rufus, further suggesting that these species are not closely related. We observed the other two island endemic anurans P. dispar and H. drewesi at breeding sites with L. palmatus. The calls of the three species are different in waveform and frequency with P. dispar producing long trills at higher frequencies (4500-5000 Hz; Loumont, 1992), H. drewesi producing single to multi pulsed notes at intermediate frequencies (2520-3020 Hz; Gilbert & Bell, 2018), and L. palmatus producing single to multi pulsed notes at lower frequencies (937-1406 Hz; this study). Consequently, there is very little acoustic overlap between the species and passive acoustic recording devices could be an effective strategy to gather more extensive data on geographic occupancy and seasonal activity in the three species (e.g., Sugai et al., 2019).

Previous authors have reported extensive dorsal colour pattern variation in L. palmatus ranging from dark green/black with or without small white spots to light brown and bright green (Manaças, 1958; Loumont, 1992; Drewes & Stoelting, 2004). We have observed all of these colour variants as well as vibrant yellow coloration with dark mottling. From our observations to date, it is not clear if colour variation differs systematically between the sexes or across ontogeny. Colour and pattern variation is known in other species of Leptopelis in which there are green phases and brown phases that may vary across ontogeny in some species (e.g., L. bocagii, L. boulengeri, L. cynnamomeus, L. mossambicus, L. natalensis, L. notatus, L. occidentalis, L. ragazzii, L. susanae, L. vermiculatus, L. uluguruensis, L. valdeni; Schiøtz, 1999; Amiet, 2012). The genetic basis and ecological relevance of this colour pattern variation have not yet been characterised and coloration in *L. palmatus* seems to be among the most variable of all species in the genus.

Extensive surveys indicate the Príncipe giant tree frog, L. palmatus, is primarily found in forested habitats from sea level to over 600 m elevation (Loumont, 1992; Drewes & Stoelting, 2004; this study). Consistent with previous survey efforts, we found both males and females perched one meter or higher off the ground on branches or leaves, especially near small, flowing streams at night (Loumont, 1992; Drewes & Stoelting, 2004). Previous reports indicated that large females may be encountered on or near the ground (Drewes & Stoelting, 2004); correspondingly, we encountered one large female (CAS 258958 SVL = 100.0 mm) on the ground near a waterfall at 1245h, a second large female (USNM 591753 SVL = 76.6 mm) on a thin branch approximately 20 cm above ground in the forest at 1735h, a third large female (CAS 261010 SVL = 97.7 mm) on a tree root approximately 10 cm above ground near a wide, muddy stream at 1817h, and a fourth large female (USNM 591798 SVL = 90.5

mm) in a puddle in the road at 1943h. Unfortunately, the reproductive biology of *L. palmatus* is entirely unknown and we have been unsuccessful in locating eggs/larvae or observing amplexus in this species whereas we have observed egg deposition sites and collected larvae of the other two anuran species on Príncipe (RCD, RCB, per. obs.). Documenting this species' reproductive mode and specific breeding habitat will be critical to understanding the habitats L. palmatus relies upon throughout its lifecycle.

Our study confirms the distinctiveness of L. palmatus relative to other large-bodied tree frogs in the genus. Our mtDNA gene tree for the genus Leptopelis indicated that continental species of large tree frogs (L. rufus, L. macrotis, L. millsoni) form a distinct clade, and that L. palmatus is not closely related to this species group, reinforcing that gigantism has evolved multiple times in the genus. We found low mtDNA diversity and high colour variation within L. palmatus, suggesting no mtDNA genetic structure associated with phenotypic variation across the island, although more studies are needed to characterise the genetic basis and ecological relevance of colour variation. Morphological differences between L. palmatus and L. rufus are most apparent in females, which could be in part why taxonomic confusion persisted for decades. Finally, we demonstrated that the advertisement call of L. palmatus is markedly distinct from L. rufus and further supports L. palmatus being distinct from L. rufus. Future studies that describe the reproductive strategy of *L. palmatus* will provide essential information to guide conservation of this unique and endangered species.

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Description of the tadpole of *Cruziohyla calcarifer* (Boulenger, 1902) (Amphibia, Anura, Phyllomedusidae)

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Specimens belonging to the genus Cruziohyla from Panama, Costa Rica and Honduras, collected by the scientific community as Cruziohyla calcarifer are now known to represent a different species, Cruziohyla sylviae. Similarly, the tadpole previously described for C. calcarifer also now represents that of C. sylviae. Here we describe the tadpole of the true C. calcarifer for the first time, including information on ontogenetic changes during larval development. The tadpole of *C. calcarifer* is characterised in having distinctive morphology, mouthpart features and markings.

Keywords: larvae, splendid leaf frog, reproduction, development, morphology

INTRODUCTION

he tadpole of *Cruziohyla calcarifer* (Boulenger, 1902), named Agalychnis calcarifer until 2005 (Faivovich et al., 2005), was previously described from specimens collected at La Selva Biological Research station, 2.6 km SE of Puerto Viejo de Sarapiqui, in the north of Costa Rica (Donnelly et al., 1987), which is approximately 1200 km from the type locality for that species. However, following a review of the genus in 2018, when the closely related species Cruziohyla sylviae Gray, 2018, was identified and described, it was confirmed that the only species of Cruziohyla known occurring at La Selva is C. sylviae. No specimens matching the description of the true C. calcarifer have ever been recorded at La Selva and the last specimens of C. calcarifer to be recorded in Costa Rica, and representing that species' northernmost point of distribution, are an adult male (UCR6480 [UCR = Universidad de Costa Rica]) and female (UCR6285) collected near the Panamanian border in 1996, and a juvenile collected just south of the port of Limon in 1997 (UCR7199).

By comparison, C. sylviae has a geographic range known to extend from Panama northwards through Costa Rica to Nicaragua and Honduras (Gray, 2018). As such, La Selva Biological Research Station is central to the known geographical range of C. sylviae, and this is the only species in the genus recorded there or any further north. La Selva is only 70 km from the Type locality of C. sylviae, being Guayacan, Costa Rica, and all specimens previously recorded as C. calcarifer at La Selva, and northward to Honduras, are confirmed as C. sylviae

(Gray, 2018). Subsequently, the only Cruziohyla tadpole found at La Selva and further north belongs to that of *C. sylviae*, and those found agree with the description provided by Donnelly et al. (1987) (Sub: A. calcarifer) (e.g. CRE 6697 [CRE = Natural History Museum of Los Angeles, USA] from Puerto Viejo de Sarapiqui, Costa Rica, and SMF 79425 [SMF = Forschungsinstitut und Natur-Museum Senckenberg, Germany] from Guasimo, Olancho, Honduras).

Cruziohyla calcarifer was described in 1902 (Boulenger, 1902), with the type specimen originating from Ecuador. To date, the species has remained extremely rare with almost nothing known of its breeding biology and the tadpole has not been described. Herein we describe the tadpole of *C. calcarifer*, a species confirmed as having a distribution from north-western Ecuador to only the very south-eastern part of Costa Rica. The description presented herein is based on wildcollected tadpoles and those produced by wild collected specimens from Ecuador, which have had their identity confirmed by 16s mitochondrial DNA (Gray, 2018).

METHODS

16 live juvenile specimens of C. calcarifer were obtained from Alto Tambo, Esmeraldas, Ecuador, a locality within the species' recognised range where specimens are considered representative of those from the type locality (Faivovich et al., 2010). The morphological characteristics of the specimens obtained fully match those for C. calcarifer, as defined by Boulenger (1902) and Gray (2018). Following exportation to Europe, a representative

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specimen (e.g. MM1021 [MM = Manchester Museum, UK]) was genetically confirmed as a true C. calcarifer (Gray, 2018). The live specimens developed to adulthood and were subsequently housed at Manchester Museum, England, and also within the private herpetological collection of Konstantin Taupp in Germany.

Two pairs of adult C. calcarifer maintained in Germany produced fertile clutches of eggs on 25 April 2020 and 11 May 2020. The egg clutches consisted of 19 (15 fertile) and 11 (6 fertile) eggs that were laid on open leaves, from which five and four tadpoles hatched on 7 May 2020 and 23 May 2020, respectively. The tadpoles were maintained in an aquarium at 20.5 °C (+/- 0.5 °C), and grew slowly but consistently throughout their development. Tadpoles were staged according to Gosner (1960). Measurements of single representative tadpoles at given stages of development were taken directly with digital calipers to the nearest 0.1 mm, using a digital microscope, and also from accurately scaled digital images (Table 1). Of the nine tadpoles studied, two specimens were euthanised using MS222 at stages 26 and 37 and preserved in formalin so as to afford scientific description and the best direct morphological comparisons of congeneric species (Grosjean, 2005): C. sylviae (Donnelly et al., 1987: sub: A. calcarifer) and C. craspedopus (Hoogmoed & Cadle, 1991). The remaining seven tadpoles completed metamorphoses successfully, taking between 91-101 days from hatching to emergence.

The main description of the tadpole of C. calcarifer, its external morphology, is based on a well-developed specimen at Stage 37 (NHMUK/BMNH.2021.6359), and is further supported by a specimen at Stage 26 (NHMUK/ BMNH.2021.6360). These specimens are housed with the type specimen of C. calcarifer (Boulenger, 1902) at the Natural History Museum, London (NHMUK). Other specimens examined or cited as part of the work are housed at: Manchester Museum (MMUK), Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ), Natural History Museum of Los Angeles, USA (CRE/LACM), Forschungsinstitut und Natur-Museum Senckenberg, Germany (SMF), Coleccion de Herpetología, Escuela de Biología, Universidad de Costa Rica (UCR). Institutional abbreviations used follow Frost (2020).

Terminology of the tadpole description is that of Altig and McDiarmid (1999) and Grosjean (2001). Acronyms used for tadpole measurements are as follows: TL (total length = direct line distance from tip of snout to posterior tip of tail), BL (body length = direct line distance from tip of snout to body terminus), TAL (tail length = direct line distance from body terminus to absolute tip of tail), BW (body width = greatest transversal distance of body), BH (body height = widest vertical point from ventral-dorsal surface), TMH (tail muscle height = vertical distance from the ventral margin of the tail muscle to dorsal margin of tail musculature at midpoint), UFH (upper fin height = maximum vertical distance from tail musculature to dorsal fin margin), LFH (lower fin height = maximum vertical distance from tail musculature to ventral fin margin), MTH (maximum tail height = vertical distance from dorsal to ventral fin margins at mid-point), ED (eye diameter = distance from anterior to posterior corner of eye), IOD (interorbital distance = shortest distance between the centre of the orbits), NW (nostril width), IND (internarial distance = shortest distance between the inner margins of the nostrils), RN (rostro-narial distance = straight line from anterior corner of nostril to tip of labium), RP (rostro-pupilar distance = straight line from anterior corner of eye to tip of snout), NP (nostrilpupilar distance = straight line from anterior corner of eye to posterior margin of nostril), ODW (oral disc width = greatest transversal distance from oral disc margins, LTRF (labial tooth row formula).

RESULTS

Body shape: Ovoid in lateral view, elliptical in dorsal view, depressed (body width = 9.5mm; body height = 8.2 mm), highest and widest at about midpoint of the body. Body length: Approximately 30 % of total length (BL = 16.2 mm; TAL = 53.4 mm). Snout shape: In dorsal and lateral profiles the overall snout shape is rounded. The rounded profile of the snout extends anteriorly, medial to distinct nares which are minimally raised and situated on a shallow fold either side of the mouth: The shallow fold slopes anteroventrally, from nostril to outer margin of oral disc. Nares: Small (0.4 mm) yet well-defined, positioned dorsolaterally, directed anteriorly, located on the same lateral plane as the centre of the pupil. Distance of nostrils from upper labia (RN = 1.5 mm), from the eye (NP = 4.2 mm), apart (IND = 3.6 mm).

Eyes: Dorsolateral, directed laterally, (ED = 2.0 mm), interorbital distance over twice that of internarial distance (IOD = 8.7 mm). Spiracle: Ventral, sinistral to the midline, short (1.0 mm), oblique spiracular opening (1.3 mm wide), situated approximately midway and at the posterior edge of the body, equal distance from eye and mouth (5.0 mm). Tail: Ventral and dorsal tail fins curve outward distally, narrowing toward the terminus; tail highest at the midpoint (MTH = 13.0 mm). Caudal musculature is moderately high and gradually tapers, not quite extending to the tip of the tail. Dorsal fin emerges at the junction of body and tail musculature, ventral fin does not extend onto the body. Height of dorsal fin and caudal musculature at the midpoint of the tail are approximately equal (4.2 mm), ventral fin slightly higher (4.6 mm). Anal tube: Short (1.5 mm), dextral to the caudal fin.

Mouth: Moderately small (ODW = 3.8 mm wide), anteroventral, directed anteriorly. Oral disc not emarginate, labia completely bordered by papillae. Anteriorly a single row of marginal papillae joined by very short length (n = 4 papillae) forming a second papillar row at the mid-dorsal margin. Additional papillae are present submarginally, diminishing to double row ventrally, having a short tertiary row and convex-shaped cluster of papillae mid-ventrally (Fig. 1c). Upper jaw sheath with fine serrations, medially convex, forming a smooth broad arch with long slender lateral processes extending distally. Lower jaw sheath V-shaped, with distinct sharply pointed serrations. Labial teeth present in two anterior (upper) and three posterior (lower) rows. LTRF: 2 (2) / 3 (1). Anterior (upper) rows are long,



Figure 1. Tadpole of Cruziohyla calcarifer (NHMUK/BMNH 2021.6359) Stage 37. (A) Dorsal view; (B) Lateral view, scale bar = 5mm; (C) oral disc, scale bar = 1mm.

extending laterally nearly to the submarginal papillae, first row forms distinct dip medially, second upper row narrowly interrupted medially. Posterior rows becoming progressively shorter posteriorly, upper posterior labial teeth row narrowly interrupted medially, lower two rows are complete.

Coloration: In life, background colour of body at Stage 37 is tan brown, having fine pale vellow marbling on the dorsal and ventral surfaces of the body, the spiracle, and anterior part of tail. Tail musculature pale tan, pale grey reticulations to both dorsal and ventral fins extending three-fourths the length of the tail. A series of fine goldcoloured speckles on the body surface represent clearly visible lateral line configurations (Fig. 2). The Internal organs are not clearly visible through the ventral surface of the body. Iris is silver-grey, its periphery having gold reticulation. In preservative, coloration of body pale yellow having grey markings throughout, area anterior to the eyes pale yellow, dorsally a dark grey band extends interorbitally. Pancreas and duodenum seen through ventral surface of the body as light and dark areas respectively. Tail musculature predominantly pale yellow with fine grey mottling anteriorly. Dorsally the paleyellow colour of the tail musculature extends onto body, symmetrically either side of a dark grey mediodorsal line. Spiracle, dorsal and ventral fins translucent. Iris is black. Oral disc and papillae remain translucent with fine dark speckling, oral surfaces of upper and lower jaw sheaths are black.

Ontogenetic changes:

Changes in colour

Stage 24: Body and tail very dark brown, tail musculature

lighter brown. Iris brown with golden inner edge. Some internal organ coloration is visible through the ventral surface of the body, primarily the intestinal tube which is turquoise blue. Stages 25-26: The overall body coloration lightens considerably, the tail musculature is pink and redness of the heart muscle is visible through the ventral surface of the body. Externally the orientation of neuromasts forming the lateral line system are clearly visible as fine gold-coloured speckles on the body surface and thus able to be detailed (Fig. 2). Stage 25-30: Body tan-brown with fine yellow marbling to lateral body surfaces and anterior of tail. Pupil round, iris silver with reticulations, periphery has gold reticulation. Internally, the intestinal tube loses its blue coloration to become dark grey, externally a pink hind limb bud develops (Fig. 3). Stages 30–39: Spiracle is evident but transparent, brown/ yellow marbling coloration on the body and at junction of caudal musculature becomes progressively more extensive and more contrasted in the later stages as the background body coloration becomes a darker brown. In later stages, undersides of toes develop dark brown pigmentation, tubercles and toe-pads pale yellow. Stages **40–43:** Dorsal median, upper labium and tail is tan brown. Dorsal surfaces of arms, legs, flanks and eve-snout light grey-brown, concealed surfaces yellow. Distinct dark brown markings present on ventral thigh region. Stage 43: In contrast to the brown tail musculature the dorsal and lateral fins turn distinctly black as tadpole emerges and the tail atrophies.

Changes in morphology

Notable morphological changes include a distinct change in body shape: in the early stages (stages 25-26) the body is higher than wide, becoming dorsally depressed (wider than high) from Stage 26 (Table 1). This phenomenon is known in both other members of the genus (Donnelly et al., 1987; Hoogmoed & Cadle, 1991). At stages 25–26 the tail musculature and both dorsal and ventral fins at the midpoint are of equal width, whereas from Stage 26 the ventral fin is marginally higher than the caudal musculature and dorsal fin. At Stage 30 the bilateral myotonic muscle masses in the tail musculature are more clearly defined, the nostrils become more prominent either side of snout. At Stage 37 the pupil has developed an elliptical shape, the spiracle is still evident, and the dorsal lateral lines remain. Between stages 37–41 a notable increase in the growth of the tail length is seen compared with that of the body length, the hind limbs become well-developed, and at later stages further widening of the body is evident, attributable to the developing forearms that have not yet emerged. Between stages 39–40 the distance between the anterior labium and medial aspect of the snout reduces as the snout becomes truncate in lateral plane. From Stage 41, with the onset of metamorphosis, the tail begins to shrink, viewed from above the snout becomes more pointed, and the nostrils are further defined. A small notch also appears at the bottom of the pupil in respect of initiating the pupils' development to becoming vertical in shape. The outward facing calcar on the heel becomes apparent in some specimens.



Figure 2. Tadpole of Cruziohyla calcarifer (NHMUK/BMNH 2021.6360) at Stage 25, showing lateral line organs. (A) Lateral view: (B) Dorsal view: (C) In life.

Day from	Day 1	Day 7	Day 14	Day 21	Day 28	Day 42	Day 54	Day 61	Day 70	Day 77	Day 82	Day 89
Stage	24	25	25	26	26	30	36	37	38	39	40	41
Total length	19.0	26.1	32.0	37.3	47.3	50.1	52.9	53.4	58.6	64.0	67.0	66.0
Body length	4.4	7.1	8.8	10.3	15.3	15.8	16.0	16.2	18.0	20.0	20.0	20.0
Tail length	14.6	19.0	23.2	27.0	32.0	34.3	36.9	37.2	40.6	44.0	47.0	46.0
Body Height	3.5	5.1	5.8	6.3	7.8	8.0	8.2	8.2	10.5	10.8	10.8	9.4
Body width	-	-	-	-	8.8	9.2	9.4	9.5	11.8	12.0	12.1	13.0
Tail muscle height	0.8	2.0	2.6	3.0	3.0	3.7	4.0	4.2	4.4	5.4	5.5	5.0
Dorsal fin height	1.2	2.0	2.6	2.9	3.0	3.2	4.0	4.2	4.4	4.4	4.8	4.0
Ventral fin height	1.7	2.0	2.6	3.0	3.2	3.6	4.2	4.6	4.8	5.5	5.5	4.5
Eye diameter	0.9	1.1	1.1	1.2	1.4	1.6	1.8	2.0	2.0	2.0	2.0	2.0
Eye to nostril	0.6	1.6	1.8	2.0	3.4	3.6	4.0	4.2	4.4	4.5	4.3	4.0

DISCUSSION

Reproductive behaviour, egg deposition and clutch size

Initial observations of the breeding behaviour of the true C. calcarifer, both in captivity and in the wild, indicate the species has reproductive traits which differ from both other members of the genus: C. calcarifer deposit their egg clutches on the lower central section of leaves overhanging small ponds or open water bodies, whereas C. craspedopus and C. sylviae lay egg clutches above flooded hollows or water cavities between buttresses of fallen trees (Marquis et al., 1986, sub: A. calcarifer; Donnelly et al., 1987: sub: A. calcarifer; Hoogmoed & Cadle, 1991; Caldwell, 1994: sub: A. calcarifer; Duellman, 2001, sub: A. calcarifer; Savage, 2002, sub: A. calcarifer; Kubicki, 2004, sub: A. calcarifer). The courtship and breeding behaviour witnessed for C. calcarifer appears most similar to that of Agalychnis spurrelli (Boulenger, 1913), where adult C. calcarifer congregate 'en masse' and lay their eggs on leaves in and around open ponds (personal communication with Miguel Solano in reference to observations in the mid-nineties in the Fila Carbon, Costa Rica). In the Changuinola drainage, Panama, groups of calling *C. calcarifer* males with females were also commonly found together in the understory above a headwater streamlet (Myers & Duellman, 1982),

Table 1. Morphometric measurements (in mm) of single representative tadpoles of Cruziohyla calcarifer during ontogeny

and in Alto Tambo, Ecuador, 17 eggs of *C. calcarifer* were found deposited in the centre of an open leaf (Fig. 3a) with the species' tadpoles found in pools by the side of a road (e.g. QCAZ37723), together with those of A. spurrelli.

Egg clutch sizes of *C. calcarifer* produced in captivity (n = 11–19) are consistent with those found in the wild (n = 17) at Alto Tambo, Ecuador (Fig. 4a). These small clutch sizes are comparable with those produced by its sister species, C. craspedopus, which laid between 9-22 eggs (mean 14, n = 13) in captivity (personal observation of 3rd author). The numbers are consistent with clutch sizes for *C. craspedopus* in the wild: 14–21 eggs (mean 17, n = 10), Hoogmoed & Cadle, 1991 and 2–16 eggs (mean 10.8, n = 5), Block et al. (2003). However, in contrast to both C. calcarifer and C. craspedopus, egg clutch sizes for C. *sylviae* are repeatedly reported to be significantly larger: 30-40 eggs, Marquis et al., 1986 (sub: A. calcarifer); 13-27 eggs, Donnelly et al., 1987 (sub: A. calcarifer); 20-28 eggs, Caldwell, 1994 (sub: A. calcarifer); 10-54 eggs, Savage, 2002 (sub: A. calcarifer). More recent findings through committed long-term monitoring of wild C. sylviae in Costa Rica confirm that egg clutches of 12-38 eggs (mean 29, n = 22) are indeed representative for that species (personal communication, Pepo Marsant, 2020).



Figure 3. Tadpole of Cruziohyla calcarifer at various stages of development: (A) Stage 24; (B) Early Stage 25; (C) Stage 29; (D) Stage 34; (E) Stage 36. Scale bar = 5mm.

Tadpole development and characteristics

Development times in Cruziohyla tadpoles are highly variable between and within species, presumably due to the differing conditions, including water temperature, volume, tadpole density and food supply. The development of wild collected C. calcarifer tadpoles from Durango, Ecuador, took approximately 50 days from egg to metamorphosis (e.g. QCAZ 37745), whereas the development of captive C. calcarifer tadpoles raised during this work took approximately twice as long (Mean: 96 days). Similarly, C. sylviae tadpoles raised in captivity have taken between 66–78 days to metamorphose (mean 71 days; Gray, 2002, sub: A. calcarifer), compared with those raised by Donnelly at al., 1987 (sub: A. calcarifer) which took 6 months to complete metamorphosis. Allowing for varying developmental timescales, some growth differences between the species during ontogeny are reported. For example, in this study some increases in growth were seen in the tail length of *C. calcarifer* tadpoles between stages 37–41, similar to that reported for C. craspedopus tadpoles by Hoogmoed & Cadle, 1991, whereas in the tadpoles of C. sylviae reported on by Donnelly et al. (1987) (sub: A. calcarifer) tail lengths at the same stages showed little if any growth.

The mouthparts of tadpoles belonging to each *Cruziohyla* species share some common features as well as individual defining characteristics: Having a complete row of marginal papillae on the anterior labium is a characteristic seen in tadpoles of all members of the genus from Stage 27. The anterior (upper) top tooth row of *C. calcarifer* shows a distinct median dip, which it shares only with C. craspedopus (Hoogmoed & Cadle, 1991) and not C. sylviae (Donnelly et al., 1987: sub: A. calcarifer); Tadpoles of C. calcarifer also possess a short double row of papillae to the medial-upper labium which is a unique feature among the tadpoles in the genus. Apart from overall visual differentiation (Fig. 5), reproductive traits and larval characteristics can be combined when summarising the diagnostic comparisons one can make between the tadpoles of species in the genus Cruziohyla (Table 2).



Figure 4. Tadpole of Cruziohyla calcarifer, in early and late stages of development: (A) Prior to hatching from small egg clutch laid on open leaf, Alto Tambo, Esmeraldas, Ecuador; (B) Showing dark markings on thighs, a unique characteristic of the tadpole.

Table 2. Diagnostic comparison of egg deposition and tadpoles in the genus Cruziohyla

	C. calcarifer	C. sylviae	C. craspedopus
Body: Widest point of body, flatness of dorsal surface, hind-limb shape	Body widest at midpoint. Back is not flattened. No indentation formed in tarsal fold	Widest point of body posteriorly. Back is not flattened. No indentation formed in tarsal fold	Body widest just behind eye. Back is distinctly flattened. Pronounced indentation in tarsal fold
Coloration: Overall body and tail colouration in later stages of development	Tan brown. No conspicuous spotting to body or tail	Dark olive-green to brown. No conspicuous spotting to body or tail	Dark olive-green to grey-black. Having conspicuous blue-grey spotting
Mouthparts: Papillae and tooth rows	Short double row of papillae to medial-anterior labium. Anterior top tooth row possesses distinct medial dip	Single row of papillae to medial- anterior labium. No top tooth row medial dip	Single row of papillae to medial- anterior labium. Anterior top tooth row possesses distinct medial dip
Markings: Dark ventral thigh markings present in later stages of development	Yes	No	No
Lateral lines: Neuromasts form defined 'M'- shape on dorsal body surface	Yes	No	No
Ecology: Site of egg deposition and water-body type	Deposits eggs on centre/tip of a leaf, over open water-bodies such as small roadside pools, ditches, ponds	Deposits eggs on roots, vines, leaf stems over flooded hollows or water cavities between buttresses of fallen trees	Deposits eggs on roots, vines, leaf stems over flooded hollows or water cavities between buttresses of fallen trees
Reproduction: Egg clutch size	< 25	10 – 55 >	< 25

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Figure 5. Tadpoles of the genus Cruziohyla (Stage 41): (A) Cruziohyla calcarifer; (B) Cruziohyla sylviae; (C) Cruziohyla craspedopus.

The morphology and overall coloration of captivebred C. calcarifer tadpoles originating from Ecuadorian C. calcarifer agree fully with wild specimens and those collected and raised in-country (e.g. QCAZ 37745). The dark brown markings on the ventral surfaces of the thigh region seen later in development (stages 40>) in the tadpole of *C. calcarifer* is a feature unique to this species (Fig. 4b).

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Author Contributions:

We can confirm that the above authors all played a high level of contribution to this research and that without each the work would not have been possible: The corresponding author instigated and led the project, conducted fieldwork in Ecuador, facilitated the acquisition of the key specimens, heavily researched comparative material, and wrote up the major part of the description. The second author was also involved in specimen acquisition, captive breeding and rearing the specimens concerned, measurements, and writing. The third author was likewise involved, breeding and rearing congener species to provide key data, specimen acquisition, and manuscript contributions. The fourth author contributed to writing and was also responsible for conducting the invaluable genetic work that clarified the identity of the specimens involved. The fifth author contributed greatly by illustrating the detailed morphology shown in the figures, and with manuscript contribution.

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A new species of Bent-toed gecko (Squamata: Gekkonidae: Cyrtodactylus Gray, 1827) from the Garo Hills, Meghalaya State, north-east India, and discussion of morphological variation for C. urbanus

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Recent taxonomic and systematic research on Cyrtodactylus khasiensis has found that this nomen comprises a large number of superficially similar but deeply divergent species-level taxa from throughout north-east India and surrounding countries. In this study we focus on the taxonomic status of recently surveyed populations from the East Garo Hills and West Garo Hills districts and a single specimen collected from Ri Bhoi District in Meghalaya State, north-east India. Based on a combination of morphological and molecular data we found that the Ri Bhoi specimen is conspecific with the recently described C. urbanus, and that the Garo Hills populations represent a new species of Cyrtodactylus described herein. Molecular analyses (using the NADH dehydrogenase 2, nd2 and adjoining tRNA genes) demonstrate that the new species is nested within the khasiensis group of the Indo-Burma radiation of Cyrtodactylus, and is well-supported as sister to a clade that comprises C. septentrionalis and C. guwahatiensis. We morphologically compare the new C. urbanus specimen with the original description of the species, identify a number of errors and ambiguities in the original description, and notably expand the known morphological variation for the species based on 23 characters. The discovery of an endemic new species of lizards from the Garo Hills further highlights the region as an overlooked centre of biodiversity importance. We discuss several misidentifications in the literature of other reptile species from the region emphasising the need for further attention by taxonomists to review the herpetofauna of the Garo Hills.

Keywords: Biodiversity, taxonomy, lizard, reptile, endemic

INTRODUCTION

he Bent-toed geckos of the genus Cyrtodactylus Gray, 1827 represent the most species-rich radiation of squamates with more than 300 species known to date (Grismer et al., 2021). Cyrtodactylus species range from Pakistan, throughout south and south-east Asia, the Indo-Australian Archipelago to northern Australia (e.g., Annandale, 1913; Darevsky et al., 1998; Mahony & Reza, 2008; Mahony et al., 2009a, 2009b; Wood et al., 2012; Das et al., 2016; Agarwal et al., 2018b; Grismer et al., 2018b; Rivanto et al., 2018; Davis et al., 2019; Wang et al., 2020). Until recently, north-east India had only two reported species of Cyrtodactylus, C. gubernatoris (Annandale, 1913), known from only two specimens collected in the Darjeeling hills of northern West Bengal State, and C. khasiensis (Jerdon, 1870), a species long considered to be widespread throughout north-east India and neighbouring regions of southern China, Bangladesh and northern/western Myanmar (Smith, 1935; Ahsan,

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1998; Li, 2007). Mahony (2009) and Mahony et al. (2009a) reviewed the taxonomic status of specimens referred to C. khasiensis from Myanmar and Bangladesh respectively, concluding that these populations represent other superficially similar species. Agarwal et al. (2014) was the first to conduct molecular phylogenetic analyses on populations of C. khasiensis from north-east India, demonstrating that most represent deeply divergent lineages. Agarwal et al. (2018b, 2018c) reviewed the known valid and synonymised taxa from northeast India, formally described eight new species, and elevated a synonym and a subspecies to species level. Purkayastha et al. (2020a) described a new species, C. urbanus Purkayastha, Das, Bohra, Bauer and Agarwal, 2020a, from the suburbs of Assam's state capital city, Guwahati, and expanded the known morphological variation for the other Cyrtodactylus species reported from the city, C. quwahatiensis Agarwal, Mahony, Giri, Chaitanya and Bauer, 2018, further emphasising the high species-level diversity in the region. Most recently, Mirza

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et al. (2021) described C. arunachalensis Mirza, Bhosale, Ansari, Phansalkar, Sawant, Gowande and Patel, 2021, a widely distributed species from Arunachal Pradesh State. Thirteen, mostly range restricted species are now known to occur in north-east India (Jerdon, 1870; Annandale, 1906, 1913; Agarwal et al., 2018b, 2018c; Purkayastha et al., 2020a; Mirza et al., 2021), and all bar one, C. tripuraensis Agarwal, Mahony, Giri, Chaitanya and Bauer, 2018, are considered endemic to India (Al-Razi et al., 2018). In this paper we provide a taxonomic study on Cyrtodactylus populations from the East Garo Hills, West Garo Hills and Ri Bhoi districts of Meghalaya, resulting in the discovery of a new species of Cyrtodactylus from forested habitat in the Garo Hills (Fig. 1) and an additional population of C. urbanus from Ri Bhoi. Our study increases the known diversity of Cyrtodactylus in Meghalaya State to four species, and permits an expanded description of morphological variation for C. urbanus.

MATERIALS & METHODS

Fieldwork and curation. Fieldwork was carried out by R.G. Kamei (RGK) at Daribokgre Village (East Garo Hills District), and Jangrapara Village, Tura and Rangsaggre Village (West Garo Hills District) between 4 and 28 June 2014, and at Saiden Village, Nongpoh (Ri Bhoi District) on 3 July 2014 (Fig. 1). GPS coordinates and elevation for collection localities were determined using a Garmin GPSMAP 62s. Collected specimens were humanely euthanised by injecting ca. 2 % lidocaine solution into the body cavity, fixed in 5 % aqueous formalin in the field, and subsequently washed for 24 hours in water before being transferred to 70 % ethanol for long-term preservation. Prior to fixation, a muscle tissue sample from the pectoral region was excised and stored in PCR grade absolute EtOH at -4 °C for molecular analyses. After morphological examination, specimens were deposited at the Bombay Natural History Society, Mumbai, India (BNHS 2752-2756; BNHS 2852). Abbreviations used for other museum collections are as follows: Bernice Pauahi Bishop Museum, Honolulu, Hawaii, USA (BPBM); Brigham Young University, Provo, Utah, USA (BYU); California Academy of Sciences, San Francisco, California, USA (CAS); Centre for Ecological Sciences, Bangalore, India (CES); Chulalongkorn University Museum of Zoology, Bangkok, Thailand (CUMZ); Departmental Museum of Zoology, Mizoram University, Aizawl, Mizoram, India (MZMU); Field Museum of Natural History, Chicago, Illinois, USA (FMNH); La Sierra University Herpetological Collection, La Sierra University, Riverside, California, USA (LSUHC); Museum of Vertebrate Zoology, Berkeley, California, USA (MVZ); National Museum of Natural History, Smithsonian, Washington, USA (USNM); Natural History Museum, London, UK (NHMUK; previously British Museum [Natural History], BMNH); Pakistan Museum of Natural History, Islamabad, Islamabad Capital Territory (PMNH); North East Regional Centre, Zoological Survey India, Shillong, Meghalaya, India (NERC/ZSI; previously the Eastern Regional Station, Zoological Survey India, ERS/ZSI); Zoological Survey of India, Kolkata, West Bengal, India (ZSIK).

Molecular data. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit[®] (Qiagen) following manufacturer's instructions. Partial sequences for nd2 were amplified due to the extensive availability of homologous congeneric sequences available on GenBank (Benson et al., 2017) for phylogenetic reconstruction. Sequences were generated using either the primer pair L4437b and H5540 (Macey et al., 1997, 2000; ca. 1,010 nucleotides) or ND2-METF1 and ND2-COIR1 (Macey et al., 1997; 1,256 nucleotides). The PCR was performed in a 25 µl reaction mix comprising of 1.5 µl extracted DNA (~10 ng/µl), 10 µl PCR grade H₂O, 12.5 µl MyTaq[™] Mix (Bioline) and 0.5 μ l each of forward and reverse primers (10 ng/ μ l). The PCR reaction protocol for the primers L4437b and H5540 was as follows: initial denaturation at 95 °C for two minutes, then 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 30 seconds, and extension at 72 °C for one minute. The final extension was at 72 °C for five minutes. The PCR reaction protocol for the primers ND2-METF1 and ND2-COIR1 was as follows: initial denaturation at 95 °C for three minutes, then 35 cycles of denaturation at 95 °C for 35 seconds, annealing at 50 °C for 35 seconds, and extension at 72 °C for 35 seconds. The final extension was at 72 °C for 10 minutes. PCR product purification and sequencing were outsourced to either Medauxin (Bangalore, India) or Barcode Biosciences (Bangalore, India). Sequence chromatograms were quality checked, edited and assembled into contigs using Geneious V.8.1.9 (Kearse et al., 2012). A BLAST (Altschul et al., 1990) search was performed on the NCBI BLAST website (http://blast.ncbi. nlm.nih.gov) against GenBank sequences to verify their approximate identity. Newly generated sequences are available on GenBank (MW367435.1-MW367439.1; see Table 1). Comparative sequences (Table 1) comprising all members of the Indo-Burma radiation of Cyrtodactylus along with several outgroup taxa were obtained from GenBank, generated in the following studies: Siler et al. (2010); Johnson et al. (2012); Wood et al. (2012); Bauer et al. (2013); Agarwal et al. (2014, 2018c); Grismer et al. (2018a, 2018b, 2019a, 2019b); Muansanga et al. (2020); Purkayastha et al. (2020a). A sequence for C. myaleiktaung Grismer, Wood, Thura, Win, Grismer, Trueblood and Quah, 2018 was obtained directly from the original authors (Grismer pers. comm., 2020) as it was not available on GenBank at the time of writing. Sequence identities of some species were corrected following Mahony & Kamei (in review). A sequence alignment was generated using MUSCLE (Edgar, 2004) on MEGA7 (Tamura & Nei, 1993; Kumar et al., 2016) using default parameter settings. The alignment was converted from DNA to amino acid sequences to check for open reading frames and premature stop codons in the *nd2* sequences. Non-coding regions of the alignment were checked manually and adjusted where necessary. The total alignment length comprised 1,309 bp including the complete nd2 + tRNA-trp + tRNA-ala + partial tRNAasn genes. Phylogenetic relationships were estimated with RAxML-HPC2 (Stamatakis, 2014) on XSEDE (CIPRES platform: Miller et al., 2010) for maximum likelihood, on an unpartitioned alignment dataset using the GTR CAT

Table 1. List of *Cyrtodactylus nd2* sequences used in this study (newly generated sequences in **bold**). Museum abbreviations are defined and original citations for sequences given in the "Materials & Methods" section. Dist. District; Subdist. Subdistrict; Div. Division; Reg. Region; Prov. Province; N.P. National Park; W.S. Wildlife Sanctuary; Mts. mountains. * specimen number and collection locality were recently corrected on GenBank by the original authors (Wood, Bauer & Jackman pers. comm., 21/12/2020).

Species	Museum No.	Locality	GenBank Accession No.
C. annandalei Bauer, 2003	CAS 215722	Myanmar, Sagaing Div., Alaungdaw Kathapa N.P.	JX440524.1
<i>C. arunachalensis</i> Mirza, Bhosale, Ansari, Phansalkar, Sawant, Gowande & Patel, 2021	CES13/1465	India, Arunachal Pradesh State, Changlang Dist., Glow Lake.	KM255193.1
<i>C. aunglini</i> Grismer, Wood, Thura, Win, Grismer, Trueblood & Quah, 2018c	LSUHC 13948	Myanmar, Mandalay Reg., Pyin Oo Lwin Dist., Kyauk Nagar Cave.	MH764589.1
*C. ayeyarwadyensis Bauer, 2003	CAS 212459 [previously as "CAS 216459"]	Myanmar, Ayeyarwady Div., vicinity of Mwe Hauk Village [previously as "Myanmar, Rakhine State, Than Dawe Dist., Gwa Township"].	JX440526.1
C. cf. ayeyarwadyensis	CAS 216446	Myanmar, Rakhine State, vicinity of Kantheya Beach.	GU550715.1
C. battalensis Khan, 1993	PMNH 2301	Pakistan, North-West Frontier Prov., Battagram City.	KC151983.1
C. bapme sp. nov.	BNHS 2752	India, Meghalaya State, East Garo Hills Dist., Daribokgre Hamlet.	MW367437.1
C. bapme sp. nov.	BNHS 2754	India, Meghalaya State, West Garo Hills Dist., Jangrapara Village.	MW367438.1
C. bapme sp. nov.	BNHS 2755	India, Meghalaya State, West Garo Hills Dist., Tura Peak.	MW367436.1
C. bapme sp. nov.	BNHS 2756	India, Meghalaya State, West Garo Hills Dist., Jangrapara Village.	MW367435.1
C. bhupathyi Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018b	BNHS 2255	India, West Bengal State, Kalimpong Dist., near Lower Mongpong.	KM255204.1
C. brevidactylus Bauer, 2002	CAS 214105	Myanmar, Mandalay Reg., Popa Mountain Park.	GU550714.1
C. chamba Agarwal, Khandekar & Bauer, 2018c	CES11/1291	India, Himachal Pradesh State, Chamba Dist., near Chamba.	KM255191.1
C. chanhomeae Bauer, Sumontha & Pauwels, 2003	CUMZ 2003.62	Thailand, Saraburi Prov., Phraputthabata Dist., Khun Khlon Sub- dist., Thep Nimit Cave.	JX440529.1
C. chrysopylos Bauer, 2003	LSUHC 13937	Myanmar, Shan State, Taunggyi Dist., Ywnagan Township.	MH764604.1
C. fasciolatus (Blyth, 1861)	CES11/1337	India, Himachal Pradesh State, Shimla Dist., near Subathu.	KM255184.1
C. cf. fasciolatus 1	CES11/1345	India, Uttarakhand State, Almora Dist., Almora.	KM255169.1
C. cf. fasciolatus 2	CES09/1196	India, Uttarakhand State, Dehradun Dist., Mussoorie-Kempty Road.	KM255172.1
C. gansi Bauer, 2003	CAS 222412	Myanmar, Chin State, Min Dat Township.	JX440537.1
C. gubernatoris (Annandale, 1913)	BNHS 2207	India, Sikkim State, East Sikkim Dist., Singtam Town.	KM255181.1
C. guwahatiensis Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018c	BNHS 2146	India, Assam State, Guwahati Dist., Guwahati.	KM255194.1
C. himalayanus Duda & Sahi, 1978	CES11/1317	India, Jammu and Kashmir State, Kishtwar Dist., near Kishtwar.	KM255187.1
C. jaintiaensis Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018c	BNHS 2248	India, Meghalaya State, Jaintia Hills Dist., near Jowai.	KM255195.1
C. kazirangaensis Agarwal, Mahony, Giri, Chait- anya & Bauer, 2018c	BNHS 2147	India, Assam State, Golaghat Dist., Kohora.	KM255170.1
C. khasiensis (Jerdon, 1870)	BNHS 2249	India, Meghalaya State, East Khasi Hills Dist., Cherrapunjee Resort.	KM255188.1
C. lawderanus (Stoliczka, 1871)	CES11/1343	India, Uttarakhand State, Almora Dist., Almora.	KM255189.1
C. meersi Grismer, Wood, Quah, Murdoch, Grismer, Herr, Espinoza, Brown & Lin, 2018b	LSUHC 13455	Myanmar, Bago Reg., Yangon (north) Dist., Taikkyi Township.	MH624104.1
C. mombergi Grismer, Wood, Quah, Thura, Herr & Lin, 2019b	LSUHC 14734	Myanmar, Kachin State, Mohnyin Township, Indawgyi W.S.	MN059875.1
C. cf. mombergi	LSUHC 14591	Myanmar, Kachin State, Mohnyin Township, Indawgyi W.S.	MN059869.1
C. montanus Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018c	BNHS 2231	India, Tripura State, North Tripura Dist., Phuldungsei Village.	KM255200.1
<i>C. montanus</i> Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018c	MZMU 1630	India, Mizoram State, Dampa Tiger Reserve, Pathlawi Lunglen Hang.	MT250544.1
<i>C. myaleiktaung</i> Grismer, Wood, Thura, Win, Grismer, Trueblood & Quah, 2018c	LSUHC13965	Myanmar, Mandalay Reg., Mandalay Dist., Mya Leik Taung.	Grismer pers. comm., 17/10/2020
<i>C. myintkyawthurai</i> Grismer, Wood, Quah, Mur- doch, Grismer, Herr, Espinoza, Brown & Lin, 2018b	CAS 245200	Myanmar, Bago Reg., Central Bago Yoma.	MH624107.1
C. myintkyawthurai (erroneously as C. "feae" on GenBank)	USNM 559805	Myanmar, Mandalay Reg., Popa Mountain Park.	JX440536.1
<i>C. nagalandensis</i> Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018c	BNHS 2253	India, Nagaland State, Kohima Dist., Khonoma.	KM255199.1
C. novaeguineae (Schlegel, 1837)	BPBM 23316	Papua New Guinea, West Sepik Prov., Parkop, Toricelli Mts.	JX440547.1
C. nyinyikyawi Grismer, Wood, Thura, Win & Quah, 2019a	CAS 226139	Myanmar, Magwe Reg., Min Bu Township, Shwe Settaw W.S.	MH624118.1
C. peguensis (Boulenger, 1893)	LSUHC 13454	Myanmar, Bago Reg., Myin Mo Shwe Taung Pagoda.	MH756190.1
C. philippinicus (Steindachner, 1867)	FMNH 236073	Philippines, Romblon Island.	JX440550.1
C. pyadalinensis Grismer, Wood, Thura, Win & Quah. 2019a	LSUHC 13932	Myanmar, Shan State, Ywangan Township, Panluang-Pyadalin Cave W.S.	MK488057.1

Species	Museum No.	Locality	GenBank Accession No.
C. pyinyaungensis Grismer, Wood, Thura, Zin, Quah, Murdoch, Grismer, Lin, Kyaw & Lwin, 2018d	BYU 52234	Myanmar, Mandalay Reg., 5.3 km north of Pyinyaung Village at the Apache Cement Factory mining site.	MF872307.1
C. russelli Bauer, 2003	CAS 226137	Myanmar, Sagaing Div., Htamanthi W.S.	JX440555.1
C. septentrionalis Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018c	BNHS 1989	India, Assam State, Bongaigaon Dist., near Abhayapuri.	MH971164.1
C. slowinskii Bauer, 2002	CAS 210205	Myanmar, Sagaing Div., Alaungdaw Kathapa N.P.	JX440559.1
C. tibetanus (Boulenger, 1905)	MVZ 233251	China, Tibet Autonomous Region, Lhasa, 3 km from of Potala Palace.	JX440561.1
C. tripuraensis Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018b	BNHS 2238	India, Tripura State, Sepahijala Dist., Sepahijala W.S.	KM255183.1
C. cf. tripuraensis	BNHS 2230	India, Tripura State, North Tripura Dist., Rowa W.S.	KM255201.1
C. urbanus Purkayastha, Das, Bohra, Bauer & Agarwal, 2020a	VR/ERS/ZSI/688	India, Assam State, Kamrup Metropolitan Dist., Guwahati, Basishta Temple.	MN911174.1
C. urbanus	BNHS 2852	India, Meghalaya State, Ri Bhoi Dist., Saiden.	MW367439.1
C. sp. Changlang1	CES11/1349	India, Arunachal Pradesh State, Changlang Dist., Miao.	KM255179.1
C. sp. Changlang2	CES13/1459	India, Arunachal Pradesh State, Changlang Dist., Miao.	KM255192.1
C. sp. Khellong	CES13/1464	India, Arunachal Pradesh State, West Kameng Dist., Khellong.	KM255196.1
C. sp. Magway	LSUHC 226139	Myanmar, Magway Reg., Min Bu Township, Shwesettaw W.S.	MH624118.1
C. sp. Mizoram	CES13/1455	India, Mizoram State, near Aizawl Town.	KM255197.1

model under default settings for 1,000 rapid bootstrap (bs.) replicates. The resulting phylogenetic tree was visualised in FigTree (Rambaut, 2009), and rooted with Cyrtodactylus tibetanus (Boulenger, 1905) which has previously been resolved as the sister taxon to remaining Cyrtodactylus species (e.g., Wood et al., 2012; Agarwal et al., 2014).

Morphology. Measurements were made using a Mitutoyo[™] digital calliper and rounded to the nearest 0.1 mm. Measurements and scale counts were made on the right side of the specimens unless otherwise stated. Abbreviations and terminology used are as follows: SVL, snout to vent length; TRL, trunk length; BW, maximum body width; TL, tail length; TW, tail width taken on the first tail segment; TD, tail depth taken on the first tail segment; HL, head length from the snout tip to the retroarticular process of the jaw; HW, maximum head width; JW, jaw width taken ventrally at the retroarticular process of the jaw; HD, maximum head depth; FL, forearm length; CL, crus length; OD, orbit diameter taken horizontally between the bony orbital borders (not equivalent to eyeball diameter); NO, distance between the posterior edge of the nostril and the anterior orbital border; SO, distance between the snout tip and the anterior orbital border; OE, distance between the posterior orbital border and the anterior border of the ear; EL, maximum ear length/diameter; ES, anterior border of the ear to the snout tip; IN, internarial distance; IO, minimum interorbital distance between the left and right supraciliary rows; FW, minimum frontal width; RL, maximum rostral length; RW, maximum rostral width; ML, maximum mental length; MW, maximum mental width; PMIL, maximum length of the inner postmental; PMIL, maximum length of the outer postmental; FIVL, length of digit IV of the manus, from the proximal apex with digit III to the tip (excluding the claw); TIVL, length of digit IV of the pes, from the proximal apex with digit III to the tip (excluding the claw); PcP, precloacal pores, a continuous series of pore-bearing scales on the precloacal region that does not extend onto the thighs; PcFP, precloacofemoral pores, a continuous series of pore-bearing scales that extends from the precloacal region onto the thighs; FP, femoral pores, a series of pore-bearing scales on the thighs that are separated from a PcP/PcFP series by a diastema of non-pore-bearing scales; PCS, post cloacal spurs; MVSR, mid-ventral scale rows, counted between the ventrolateral folds; PVT1, paravertebral tubercles, counted from the most anterior tubercle on the occiput to the mid-sacrum; PVT2, paravertebral tubercles on the trunk only, counted between the level of the axilla and the level of the groin; DTR, dorsal tubercle rows, counted transversely across the trunk at its widest point; SL, total supralabials; IL, total infralabials. Two separate series for subdigital lamellae were counted on all digits of the right manus and right pes, a basal series, that includes scales of a width at least twice the diameter of the palmar scales up to and including a single large scale at the digital inflection, and an apical series, including lamellae distal to the digital inflection and not including the ventral claw sheath or nonlamellar scales between the basal and apical lamellae series (counted separately), abbreviated as follows: on manus, FILam, FIILam, FIILam, FIVLam, FVLam; on pes, TILam, TIILam, TIIILam, TIVLam, TVLam. The morphological variation sections are based on the comparison of each paratype against the full original description of the holotype to determine variation within the species. The new species is compared morphologically with the following taxa that comprise all known species from the khasiensis clade (Lowland clade of Agarwal et al., 2014) of the khasiensis group (sensu Grismer et al., 2021) and other species from north-east India and surrounding countries that have not yet been included in a molecular phylogenetic study and so their systematic placement is currently unknown (marked with *): C. ayeyarwadyensis Bauer, 2003; *C. cayuensis Li, 2007; C. guwahatiensis; *C. himalayicus (Annandale, 1906); C. kazirangaensis Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018; C. khasiensis; *C. mandalayensis Mahony, 2009; *C.



Figure 1. Topographic map of the Garo and Khasi Hills of Meghalaya State and bordering areas of Assam State, north-east India showing the distribution of Cyrtodactylus bapme sp. nov. and verified localities of C. urbanus: triangles represent the type localities; spots represent additional localities; cyan for C. urbanus; pink for Cyrtodactylus bapme sp. nov. Inset: map of north-east India with purple box representing the area depicted in the main map. Country borders in black; Indian state borders in brown; major rivers in blue; N Nepal; Bd Bangladesh; Bt Bhutan; C China; M Myanmar; As Assam; AP Arunachal Pradesh; Ma Manipur; Me Meghalaya; Mi Mizoram; N Nagaland; S Sikkim; T Tripura; WB West Bengal.

markuscombaii (Darevsky, Helfenberger, Orlov & Shah, 1998); *C. martinstollii (Darevsky, Helfenberger, Orlov & Shah, 1998); C. septentrionalis Agarwal, Mahony, Giri, Chaitanya & Bauer, 2018; *C. tamaiensis (Smith, 1940); C. tripuraensis, and C. urbanus. Morphological characters cited for congeners in the comparisons sections are taken directly from either their original descriptions (i.e., Darevsky et al., 1998; Bauer, 2003; Li, 2007; Mahony, 2009; Agarwal et al., 2018b, 2018c; Purkayastha et al., 2020a), literature that reviewed the species based on their type specimens for C. khasiensis and C. himalayicus (Agarwal et al., 2018b), C. guwahatiensis (Purkayastha et al., 2020a) and C. tamaiensis (Mahony, 2009), and/or direct examination of specimens (Appendix I).

The new species is known only from female specimens and thus does not have the often-useful diagnostic character of PcP (otherwise observed on male *Cyrtodactylus* from the region). On the females, these scales instead possess a shallow pit (in lieu of a true pore). The number of pitted scales on females when present is either the same as or less (but never higher) than found on males of other related species for which both males and females are known (Agarwal et al., 2018b, 2018c), thus the total number of pitted scales in the precloacal series in females of the new species are considered indicative of the minimum number of PcP that would be expected on males of this species (see Bauer, 2003:488, 492, for similar discussion on the use of female PcP counts in morphological comparisons with males of other species). The comparison section of the species account therefore includes a comparison of female pitted precloacal scale number for the new species with PcP counts of males of related taxa for which information is not available in the original description whether females possess pitted scales, e.g., both Bauer (2003: for C. ayeyarwadyensis)

and Darevsky et al. (1998: for C. markuscombaii & C. martinstollii) state only that pores are absent on females, Purkayastha et al. (2020a: for C. guwahatiensis) does not mention whether pores or pits are present on females. Measurements and meristics for the juvenile paratype (BNHS 2756) were not taken.

Map. GPS coordinates of localities depicted on the distribution map were converted to Decimal Degrees (DD) format using GPS Visualizer (https://www.gpsvisualizer. com/calculators); coordinate system used throughout is DD format. The topographic map was made using Quantum GIS (QGIS v.2.14.3-Essen) using the 250 m spatial resolution Shuttle Radar Topography Mission (SRTM) layer available from DIVA-GIS (http://www. diva-gis.org), and other basic layers from the Natural Earth Quick Start Kit (http://www.naturalearthdata. com). The type locality for *C. urbanus* was plotted based on the revised GPS coordinates (see discussion in the "Cyrtodactylus urbanus distribution and morphological variation" section).

Additional notes. Within the comparison section, the elevational range of C. himalayicus was assessed based on data given in Agarwal et al. (2018b) for the type locality and the only known referred specimen from Gopaldhara Tea Estate (ZSIK 19546). The official Gopaldhara Tea Estate website (accessed 24/06/2020) gives the elevation range of the estate as 5,500-7,000 feet (converted to metres herein).

RESULTS & DISCUSSION

The overall topology within the Indo-Burma radiation of Cyrtodactylus on the phylogenetic tree agrees well with those published elsewhere (e.g., Purkayastha et al., 2020a), with the exception of the placement of the clade



Figure 2. Maximum likelihood phylogeny based on the nd2 + tRNA-trp + tRNA-ala + partial tRNA-asn genes for the Indo-Burma radiation of Cyrtodactylus and outgroup taxa, showing the systematic position of the new Garo Hills species (blue) and newly discovered population of C. urbanus (red). GenBank numbers in parentheses (* specimen number). Bootstrap support values \geq 95 are represented by a green spot, values <95 are given next to relative nodes.

comprising C. russelli and C. slowinskii that is usually resolved as sister to a clade containing all Indo-Burma radiation taxa bar C. fasciolatus. However, the position of the *russelli-slowinskii* clade is often poorly resolved given similar taxonomic sampling (e.g., Purkayastha et al., 2020a). All newly generated sequences from populations sampled in the Garo Hills of Meghalaya formed a distinct clade. This Garo Hills clade is sister to a clade comprising C. septentrionalis and C. guwahatiensis with high support (bs. 100) (Fig. 2), which is nested within the khasiensis clade (Lowland clade of Agarwal et al., 2014) of the khasiensis group (sensu Grismer et al., 2021) in the Indo-Burma radiation of Cyrtodactylus. The sequence for the Saiden specimen was sister to a paratype (VR/ERS/ ZSI/688: Purkayastha et al., 2020a) of *C. urbanus* with sufficiently low divergence to be considered conspecific (Fig. 2; sequences 99 % identical).

The morphological examination of the genetically distinct Garo Hills lineage demonstrated that it is diagnosable from other known species in the *khasiensis* clade, thus based on the combined morphological and molecular evidences, we consider the Garo Hills lineage to represent a distinct new species (described below).

Systematics

Cyrtodactylus bapme sp. nov.

{urn:lsid:zoobank.org:pub:B1B43276-4E4D-46C1-BF43-7AB52FD5D137}

Figs. 3-5; Table 2.

Holotype. Adult (gravid) female, BNHS 2752 (field number RGK 0435; Figs. 3E, F & 4), from the banks of a stream below Daribokgre Wildlife Inspection Bungalow (25.494975, 90.323572, 1,015 m above sea level [asl.]), Daribokgre Hamlet, East Garo Hills District, Nokrek Ridge National Park in Nokrek Biosphere Reserve, Meghalaya State, north-east India, collected on 26 June 2014 by RGK.

Paratypes. Adult (gravid) female, BNHS 2753 (field number RGK 0486; Figs. 3D & 5A), from Chibonga chiring (25.494721, 90.329413, 1,015 m asl.), Daribokgre Hamlet, East Garo Hills District, Nokrek Ridge National Park in Nokrek Biosphere Reserve, Meghalava State, north-east India, collected on 28 June 2014 by RGK; an adult female,

Table 2. Morphometric and meristic data for adult *Cvrtodactylus bapme* sp. nov. and referred specimen of *C. urbanus*. L/R data taken on left/right side; + incomplete tail; - data not taken. Refer to the "Materials & Methods" section for explanation of abbreviations.

		Cyrtodactyl	us papme sp. nov.		
Specimen no.	BNHS 2752	BNHS 2753	BNHS 2754	BNHS 2755	BNHS 2852
Status	Holotype	Paratype	Paratype	Paratype	Referred specimen
Village	Daribokgre	Daribokgre	Jangrapara	Tura	Saiden
Sex (maturity)	Female (adult)	Female (adult)	Female (adult)	Female (adult)	Male (subadult)
SVL	77.0	71.9	69.9	71.8	47.9
TRL	33.1	33.8	33.0	30.6	20.7
BW	16.2	15.7	11.4	16.9	10.6
TL	88.2	+	+	+	55.4
TW	7.1	5.4	5.9	5.8	3.9
TD	6.6	5.2	5.2	5.1	3.3
HL	21.5	19.6	18.6	19.3	13.2
HW	15.8	13.8	12.5	14.0	9.2
WL	14.0	11.5	11.8	12.5	8.6
HD	8.5	7.9	7.1	7.6	5.3
ES	19.5	18.1	17.1	18.1	12.4
SO	8.6	7.6	7.4	7.5	5.1
OE	6.3	5.6	5.1	5.6	3.3
NO	6.6	5.9	5.9	5.7	4.0
OD	6.2	6.0	5.7	6.0	4.1
EL	1.6	1.9	1.2	-	1.2
IN	2.7	2.6	2.3	2.6	1.6
FW	3.2	3.0	3.1	3.0	2.0
10	7.5	6.2	6.1	6.2	4.6
RL	2.2	2.1	1.8	2.0	1.1
RW	3.3	3.2	3.1	3.4	2.2
MI	2.4	1.9	1.9	2.2	1.4
MW	3.2	3.0	3.0	3.0	23
PMII	2.3	2.2	2.2	2.2	13
DMIII	1.5	1.2	1.0	1.4	0.8
EI	12.0	11.5	9.4	0.0	6.9
	12.0	11.2	5.4 11 E	5.5 11 6	0.5
	7.0	13:0	II.5 E 4	11.0 E 7	0.7
	7.0	0.0	5.4	5.7	4.0
	8.4	7.3	6.9	6.9	5.8
РСР	0 [10 pitted]	0 [10 pitted]	0 [0 pitted]	0 [13 pitted]	13
FP	0	0	0	0	0
PCS(L/R)	4/4	3/3	3/3	4/4	3/–(damaged)
MVSR	30	36	37–38	39	~39
DTR	23	23	21	24	20
PVT1	~47	-	49–51	~50	-
PVT2	~32	~32	~37	~35	~30
SL(L/R)	9/8	10/9	11/10	12/11	12/11
IL(L/R)	9/8	8/9	8/10	9/9	11/10
FILam	3(3)6 [L]	4(2)5	5(3)4	5(3)6	5(3)6
FIILam	4(4)7	5(0)9	4(3)6	5(3)7	5(3)7
FIIILam	5(3)11	5(1)11	5(4)7	5(3)8	5(2)10
FIVLam	6(5)8	6(0)13	5(4)7	6(4)7	6(4)9
FVLam	5(2)9	4(1)9	4(3)6	6(4)6	5(3)8
TILam	4(4)7	4(3)6	4(4)5	4(3)6	4(4)6
TIILam	5(3)9	6(1)9	5(3)7	5(4)7	5(2)8
TIIILam	5(0)14	5(0)12	5(4)8	6(3)11	5(2)10
TIVLam	8(0)14	6(0)13	7(5)8	7(4)9	6(3)11
TVLam	6(1)12	5(1)10	6(3)9	6(3)11	4(3)11
Paired dark dorsal spots	~9	7	~9	~9	~9
Dark tail bands	10	-	_	_	13



Figure 3. Cyrtodactylus bapme sp. nov. in life from Meghalaya State, north-east India, showing variation in colour and pattern: A. adult female paratype (BNHS 2754) from Jangrapara, West Garo Hills District; B. adult female paratype (BNHS 2755) from Tura Peak Reserved Forest, West Garo Hills District; C. juvenile paratype (BNHS 2756) from Jangrapara, West Garo Hills District; D. adult female paratype (BNHS 2753) from Daribokgre, East Garo Hills District; E. and F. adult female holotype (BNHS 2752) from Daribokgre, East Garo Hills District. Images taken ex-situ.

BNHS 2754 (field number RGK 0254; Figs. 3A & 5C) and an unsexed juvenile, BNHS 2756 (field number RGK 0253; Fig. 3C), from a betel nut plantation close to the Public Works Department Inspection Bungalow (25.845801, 90.286975, 90 m asl.), Jangrapara Village, West Garo Hills District, Meghalaya State, north-east India, collected on 08 June 2014 by RGK; adult (gravid) female, BNHS 2755 (field number RGK 0413; Figs. 3B & 5B), from the banks of Nengsandra dare Stream in Nikrang A'ding (25.504235, 90.231431, 750 m asl.), near a trail towards Tura Peak summit, Tura Peak Reserved Forest, Tura Hill Range, West Garo Hills District, Meghalaya State, north-east India, collected on 25 June 2014 by RGK.

Morphological character summary. Cyrtodactylus bapme sp. nov. is a moderate-sized gecko (SVL 69.9-77.0 mm); 8–12 supralabials; 8–10 infralabials; dorsal tubercles are smooth to weakly keeled, bluntly conical; 21-24 dorsal tubercle rows; ca. 32-37 paravertebral tubercles between the level of the axilla and the level of the groin; 30–39 midventral scale rows; 0–13 pit-bearing (without true pore) precloacal scales in a continuous series in females, condition in males unknown; 12-19 subdigital lamellae on finger IV (excluding non-lamellar scales between the proximal and apical lamellae series); 15-22 subdigital lamellae on toe IV (excluding nonlamellar scales between the proximal and apical lamellae series); no single row of transversely enlarged subcaudal scales; 7–9 paired dark brown dorsal blotches on the dorsum; 10–12 alternating dark and light bands on the dorsum of the tail.

Holotype description (Figs. 3E, F & 4). Holotype in generally good preservation condition, except for a portion of skin and muscle tissue removed from the right pectoral region as a tissue voucher.

Adult female (SVL 77.0 mm), habitus robust (BW/ SVL 0.21; TRL/SVL 0.43), dorsoventrally depressed. Head moderately long (HL/SVL 0.28), dorsoventrally depressed (HD/HW0.54), distinct from neck; occipital region enlarged relative to the jaw width (JW/HW 0.89); loreal region convex; interorbital area flat; canthus rostralis rounded; narrow longitudinal furrow medially on the snout is absent; snout short (SO/HL 0.40), longer than the orbit

diameter (OD/SO 0.72); scales on the dorsal surfaces of the head, evelids and snout are primarily homogeneous in size and shape, granular, longitudinally oval to circular, juxtaposed, those on the snout largest; granular scales on the occipital region and the rear of the head intermixed with sparse larger, rounded, bluntly pointed tubercles, smallest and sparse dorsally on the occipital region, increasing in size laterally over the occipital region and posteriorly onto the nape where they are three times larger than adjacent granules; enlarged tubercles are absent on the parietal and frontal regions of the head; orbits are moderately large (OD/HL 0.29); pupils vertical with crenulated margin; supraciliaries large, mucronate, largest bluntly pointed, smaller posterior supraciliaries sharply pointed and protruding laterally, decreasing in size towards the posterior and anterior end of the orbits, largest about one-third the way from the anterior edge of the orbits; a row of three slightly enlarged blunt tubercles parallel to the supraciliaries on the anterior outer upper evelids; ear openings oval, obliquely orientated, small (EL/HL 0.07); orbit to ear distance is almost equal to the orbit diameter (OE/OD 0.98); rostral is wider than deep, divided dorsally to ca. half of the rostral depth by a weak rostral groove; a single enlarged supranasal on either side, separated by two small granular internasals; rostral is in contact with first supralabials, nasals, supranasals and two internasals; nostrils oval, openings directed postero-laterally, posterior half of the nasal is covered by the nasal pad, each nasal in broad contact with the rostral and surrounded by the supranasal, first supralabial, and four/five (left/right side) small postnasals; mental is wider than long, triangular; two well-developed postmentals on either side, inner pair in broad contact behind the mental, less than twice the size of and separating the outer pair from each other; inner postmentals are bordered by the mental, first infralabials, outer postmentals and three slightly enlarged gular scales; outer postmentals are bordered by the inner postmental, first and second infralabials, three slightly enlarged and two much enlarged gular scales, the largest positioned laterally; supralabials (to the midorbital position), eight on each side; nine/eight (left/right) supralabials to the angle of the jaw; nine/ eight (left/right) infralabials, second to fifth infralabials are bordered by one or two rows of enlarged scales that increase in size anteriorly; gular scales are mostly small, granular, smooth, juxtaposed, homogeneous in size except those mentioned above and where they increase in size posteriorly on the throat becoming more imbricate.

Dorsal scales on the body are heterogeneous, mostly small rounded granular scales, intermixed with irregularly arranged, enlarged bluntly conical tubercles which are smooth to weakly keeled dorsally, becoming more conical and slightly smaller laterally towards the lower flanks; dorsal tubercles extend from the nape onto the tail base; tubercles on the nape are smaller than those of the dorsum, largest on the posterior dorsolateral region where they are ca. five times the size of adjacent granular scales; ca. 23 dorsal tubercles across the mid-dorsum; ca. 32 paravertebral tubercles between

the axilla and the groin, ca. 47 from the nape to the mid sacral region; slightly enlarged tubercles separated by small scales on the ventrolateral folds, tubercles rounded (not pointed), ventrolateral folds weak; ventral scales smooth, cycloid, imbricate to subimbricate, much larger than dorsal scales; ca. 30 ventral scales between ventrolateral folds at mid-trunk; ten pit-bearing (without true pore) precloacal scales in a continuous shallow "V"shaped series, pit-bearing scales slightly larger than the anteriorly contacting ventral scales; one row of slightly enlarged scales between the precloacal pit-bearing scale series and the cloacal opening, largest at the apex of the "V"-shaped pit-bearing scale series and ca. 1.5 times larger than the adjacent pit-bearing scales; precloacal groove absent; two large well developed eggs are visible through the abdominal skin.

Fore and hind limbs slender (FL/SVL 0.16, CL/SVL 0.17); digits strongly inflected at each joint, all bearing a robust recurved claw; enlarged subdigital lamellae not scansorial, proximal(granular)distal lamellae formula for the right manus unless stated otherwise as follows: FILam 3(3)6 (taken on the left side as FILam on the right side is incomplete), FIILam 4(4)7, FIIILam 5(3)11, FIVLam 6(5)8, FVLam 5(2)9, proximal(granular)distal lamellae formula for the right pes as follows: TILam 4(4)7, TIILam 5(3)9, TIIILam 5(0)14, TIVLam 8(0)14, TVLam 6(1)12; interdigital webbing is absent on the manus and pes; relative length of the digits: I < V < II < IV < III on the right manus, I < II < III < V < IV on the right pes; scales on the palms and soles smooth, raised, juxtaposed to subimbricate; scales on the forelimbs are heterogeneous in size and shape, the dorsal and ventral surfaces of the upper arms and the ventral surface of the forearms are covered with small granular, subimbricate to juxtaposed scales, scales on the dorsum of the forearms are more heterogeneous in size and intermixed with a few slightly enlarged blunt, scattered tubercles, grading to smooth, flat, imbricate scales on the lower dorsum of the forearms; scales on the hind limbs are heterogeneous in size and shape, dorsal part of the thighs and shanks with small, blunt granular scales intermixed with scattered, enlarged, bluntly conical, smooth tubercles, equally dense on the shanks and the thighs; preaxial portion of the thighs and ventral aspect of the hind limbs with enlarged, smooth, flat, imbricate scales approximately equal in size to the ventral scales, small, granular scales on the postaxial surface of the thighs with a clear border where granular and subimbricate scales transition; femoral pores and enlarged femoral scale series are absent.

Tail original, complete, slightly dorsoventrally depressed (TD/TW 0.93), slender, tapering; caudal segments are barely distinct proximally, indistinct distally; a few enlarged, flat and distinctly keeled tubercles are randomly scattered on the first three segments of the tail, remaining dorsal caudal scales smooth, flat, heterogeneous in size and shape, subimbricate, smallest middorsally, becoming larger on the lateral aspect; subcaudal scales smooth, subimbricate and heterogeneous in size, distinctly larger than dorsal scales, no enlarged transverse plates or distinct series of enlarged paired subcaudal scales; the second tail



Figure 4. Cyrtodactylus bapme sp. nov. adult female holotype (BNHS 2752) in preservation: A. dorsal view: B. ventral view; C. details of dorsal pholidosis; D. dorsal view of head; E. ventral view of head; F. lateral view of head. A and B to scale; D-F to scale. Scale bar is 10 mm.



Figure 5. Cyrtodactylus bapme sp. nov. adult female paratypes in preservation from Meghalaya State, north-east India, showing variation of colour and pattern: A. BNHS 2753 from Daribokgre, East Garo Hills District; B. BNHS 2755 from Tura Peak Reserved Forest, West Garo Hills District; C. BNHS 2754 from Jangrapara, West Garo Hills District. Scale bar is 10 mm.

segment with eight rows of scales dorsally reduced to four rows ventrally; four small, smooth post cloacal spurs on each side of the tail base.

Coloration in life (Fig. 3E, F): Dorsal and lateral surfaces of the head primarily light brown, densely mottled with dark brown, with pale greyish-brown supralabials and a dark brown rostral; anterior supraciliaries yellowishbrown becoming dark brown posteriorly; iris light grey with a brown venous pattern, pupil with a light grey border; dorsal and lateral surfaces of the neck and trunk are primarily light grey with nine pairs of dark brown blotches that are parallel anteriorly, becoming phaseshifted posteriorly; flanks with dark brown mottling; dorsal and lateral surfaces of the forelimbs and hind limbs mottled light grey and brown; tail, dorsally and laterally with ten contrasting dark brown and light grey transverse bands, light bands approximately half as wide as dark bands; entire ventral surface of the head, body and limbs white; ventral surfaces of the manus and pes are light greyish-brown to yellowish-brown; ventral surface of the tail primarily whitish proximally with brown mottling, becoming darker distally where the dark and light bands are distinct. Coloration in preservative (Fig. 4): generally

similar to the coloration in life though less vibrant; ventrally the white in life darkened to pale brown, darker laterally on the abdomen and on the ventral surfaces of the manus and pes; iris dark grey; yellow tones on the supraciliaries faded to light brown.

Variation. Refer to Table 2 for morphometric and basic pholidosis variation within the type series of Cyrtodactylus bapme sp. nov., comprising four adult females. Additional details are given here for one unsexed juvenile paratype (BNHS 2756; Fig. 3C). The paratypes morphologically correspond with the holotype description, but with the following exceptions:

Head: 8-12 supralabials and 8-10 infralabials (Table 2); number of small gular scales posteriorly bordering the inner postmentals varies by individual: one or two granular scales on BNHS 2753 and BNHS 2755, and four on BNHS 2754; four or five small to large gular scales posteriorly border the outer postmentals on the paratypes; a narrow weakly developed longitudinal furrow is present medially on the snout of BNHS 2753, BNHS 2754 and BNHS 2755; internasals vary by number and size: one large internasal on BNHS 2753 and BNHS 2755, and three small granular internasals on BNHS 2754; rostral suture is ca. 60 % of the

rostral depth on BNHS 2754.

Tubercles: unlike the holotype, three paratypes (BNHS 2753, BNHS 2754, BNHS 2755) possess small densely packed tubercles on the parietal region; dorsal tubercle rows range 21–24, and the number of paravertebral tubercles range ca. 32–37 between the level of the axilla and the level of the groin (Table 2); tubercles on BNHS 2754 are mostly conical on the body and limbs, with some weakly keeled on the posterior dorsum of the body; on BNHS 2755, tubercles are mostly conical on the anterior body and shanks, those on the mid-body and thighs have a weak median keel, those on the sacral region are more strongly keeled; some shank and thigh tubercles have a weak keel on BNHS 2753; largest dorsal tubercles are up to six times larger than the adjacent granular scale size on BNHS 2754; tubercles in the ventrolateral fold are bluntly spinose on BNHS 2754; dorsum of the forearms of BNHS 2754 with more dense tubercles than on the holotype; a row of slightly enlarged blunt tubercles run parallel to the supraciliaries on the anterior outer upper eyelids, varying as follows: the row absent on BNHS 2753, four or five tubercles in a row on BNHS 2754, and three or four tubercles in a row on BNHS 2755; post cloacal tubercle number varies as follows: three left and three right on BNHS 2753 and BNHS 2754, and four left and four right on BNHS 2755 (as on the holotype).

Body: ventrolateral folds are weakly defined on all specimens except on BNHS 2753 where the folds appear to be absent (possibly due to being heavily gravid); number of pit-bearing scales in the precloacal series varies from none to 13 (Table 2); the largest scale between the precloacal pit-bearing scale series and the cloacal opening varies as follows: ca. 1.3 times larger than adjacent pit-bearing scale size on BNHS 2753, ca. 1.5 times larger on BNHS 2755, and two times larger on BNHS 2754; midventral scale rows vary from 30 to 39 (Table 2); numbers of subdigital lamellae and presence/absence of non-lamellar scales between the proximal and distal portion of the digits varies considerably by individual (see Table 2).

Tail: second tail segment with nine transverse scale rows dorsally reduced to four ventrally on BNHS 2754; tubercles also extend to the third caudal segment on BNHS 2754 (as on the holotype); original tail is absent on BNHS 2753 and BNHS 2755.

Coloration and markings (Figs. 3–5): BNHS 2754, BNHS 2755 and BNHS 2756 have approximately nine pairs of dark brown dorsal blotches (as on the holotype), whereas BNHS 2753 has seven pairs; dark brown dorsal blotches are not phase-shifted on either side of the vertebral line on BNHS 2753, BNHS 2754, BNHS 2755 and BNHS 2756; complete original tail of BNHS 2756 has 12 dark brown blotches/bands, with alternating light and dark blotches/ bands less distinct proximally, light bands significantly narrower than dark bands proximally, but almost equal in width distally; regenerated tail portion of BNHS 2754, BNHS 2755 and BNHS 2753 is dark greyish-brown; in life, iris coloration varies from grey to greenish-grey; pupil with an orange border in BNHS 2754 in life; see Figures 3–5 for additional variation in colour and pattern between the holotype and paratypes.

Comparisons. *Cyrtodactylus bapme* sp. nov. is here compared with all known members of the *khasiensis* clade within the *khasiensis* group of *Cyrtodactylus*, including species not yet assigned to any clade due to the absence of available molecular data, i.e., *C. cayuensis*, *C. martinstollii*, *C. markuscombaii*, *C. himalayicus*, *C. tamaiensis* (see discussion in Mahony & Kamei, in review).

Cyrtodactylus bapme sp. nov. can be diagnosed from C. aveyarwadyensis by having dark dorsal spots not bordered posteriorly by white punctuations or a narrow white band, N=5 (vs. "usually" bordered posteriorly by white punctuations or a narrow white band: Bauer, 2003), four subcaudal scale rows per segment on the original tail, N=4 (vs. six on the holotype—variation not given by Bauer, 2003); from C. cayuensis by possessing up to 13 pit-bearing scales in the precloacal scale series in females indicating the likely minimum number of precloacal pores on males (vs. 6-9 PcPs in males, N=9: Li, 2007), the largest scale at the apex of the precloacal pit-bearing scale series is between 1.3 and two times larger than the pit-bearing scales, N=4 (vs. ca. 3 times larger than the pore-bearing scales on the holotype: determined from Li, 2007: fig. 3); from C. guwahatiensis by having a larger maximum adult body size, SVL 77.0 mm, N=4 (vs. max. 70.5 mm, N=8: Agarwal et al., 2018c; Purkayastha et al., 2020a), dark dorsal spots not bordered posteriorly by white punctuations or a narrow white band, N=5 (vs. bordered posteriorly by white punctuations on the holotype and other referred specimens: Agarwal et al., 2018c; Purkayastha et al., 2020a; Purkayastha pers. comm., 2020); from C. himalayicus by having a larger maximum adult body size, SVL 77.0 mm, N=4 (vs. 64.5 mm, N=2: Agarwal et al., 2018b; examined specimens), weak ventrolateral folds (vs. absent: Agarwal et al., 2018b; examined specimens), the largest scale at the apex of the precloacal pit-bearing scale series is between 1.3 and two times larger than the pit-bearing scales, N=4 (vs. ca. 3 times larger than the pore-bearing scales, N=2: Agarwal et al., 2018b; examined specimens), elevational distribution limit of 90-1,015 m asl. (vs. ca. 1,525-2,130 m asl.: Agarwal et al., 2018b; Gopaldhara Tea Estate website, accessed 24/06/2020); from C. kazirangaensis by having tubercles extending to the third segment of the tail, N=3 (vs. tubercles not extending beyond the first segment of the tail, N=2: Agarwal et al., 2018c), dorsal body pattern with 7–9 pairs of dark brown blotches, N=5 (vs. dorsal pattern composed of six or seven irregular light and dark crossbars, N=3: Agarwal et al., 2018c), largest dorsal tubercles ca. 4-6 times larger than adjacent granular scales, N=4 (vs. approximately three times larger than adjacent granular scales on the holotype: determined from Agarwal et al., 2018c: fig. 7c-variation for paratypes not given); from C. khasiensis by having distinctly enlarged scales between the pit-bearing precloacal scale series and the cloaca, N=4 (vs. not enlarged relative to pore-bearing scales, N=6: examined specimens), a relatively wider mental scale, ML/MW ratio 0.62-0.74, N=4 (vs. 0.77-0.96, N=4: examined specimens); from C. mandalayensis by having a greater number of dorsal tubercle rows,

21-24 DTR, N=4 (vs. 18, N=1: Mahony, 2009; examined specimen), by possessing up to 13 pit-bearing scales in the precloacal scale series in females indicating the likely minimum number of precloacal pores on males, N=4 (vs. minimum 5+1 [possibly up to eight] pores in the PcP series, N=1: Mahony, 2009; examined specimen); from C. markuscombaii by possessing up to 13 pit-bearing scales in the precloacal scale series in females indicating the likely minimum number of precloacal pores on males, N=4 (vs. seven pore-bearing scales on the only known male, N=1, condition in females unknown: Darevsky et al., 1998), dorsal body pattern with 7–9 pairs of dark brown blotches, N=4 (vs. irregular transverse dark brown bands, N=2: Darevsky et al., 1998), elevational distribution limit of 90-1015 m asl. (vs. elevation 1200-1300 m asl.: Darevsky et al., 1998); from C. martinstollii by possessing up to 13 pit-bearing scales in the precloacal scale series in females indicating the likely minimum number of precloacal pores on males, N=4 (vs. 0-8 PcP on males, N=18, condition in females unknown: Darevsky et al., 1998), dorsal body pattern with 7-9 pairs of dark brown blotches, N=4 (vs. densely mottled, ca. 10-11 paired blotches when distinguishable, N=4: determined from Darevsky et al., 1998: Fig. 1), elevational distribution limit of 90-1,015 m asl. (vs. 1200–1300 m asl.: Darevsky et al., 1998); from C. septentrionalis by having a larger maximum adult body size, SVL 77.0 mm, N=4 (vs. max. 65.2 mm, N=2: Agarwal et al., 2018c), fewer paravertebral tubercles between the level of the axilla and the level of the groin, 32-37 PVT2, N=4 (vs. 38–42 PVT2, N=2: Agarwal et al., 2018c); from C. tamaiensis by having a smaller maximum adult body size, SVL 77.0 mm, N=4 (vs. 90.0 mm, N=1: Mahony, 2009; examined specimen), dorsal tubercles extend posteriorly onto the third segment of the tail, N=3 (vs. to the fourth segment, N=1: Mahony, 2009; examined specimen); from C. tripuraensis by having a larger maximum adult body size, SVL 77.0 mm, N=4 (vs. max. 70.7 mm, N=11: Agarwal et al., 2018b), pit-bearing precloacal scale series in females comprises 0-13 scales, N=4 (vs. 19-29 pitbearing scales or PcFP in the precloacal scale series of females, N=4: Agarwal et al., 2018c), typically higher number of dorsal tubercle rows, 21–24, N=4 (vs. 19–21, N=11: Agarwal et al., 2018c); from C. urbanus by having fewer apical subdigital lamellae on first finger, 4-6 (vs. 7–9, N=5: Purkayastha pers. comm., 2020).

Distribution and Natural History. This species is currently known from multiple low to mid elevation (90-1015 m asl) localities in the East and West Garo Hills districts (Fig. 1), of which the upper limits extend the maximum known elevation of the khasiensis clade from 900 m asl. for C. khasiensis (Agarwal et al., 2018b). Comparing with what is known of other north-east Indian Cyrtodactylus species, besides C. tripuraensis which has been reported from several localities in Tripura State (Agarwal et al., 2018b) and adjacent Bangladesh (Al-Razi et al., 2018), and C. arunachalensis with a known distribution extending ca. 350 km along the Brahmaputra Valley (Mirza et al., 2021), Cyrtodactylus bapme sp. nov. is the third most widespread species of this genus in northeast India. All other north-east Indian Cyrtodactylus species are currently confirmed from only one or two

localities (Agarwal et al., 2018b, 2018c; Purkayastha et al., 2020a, 2020c; herein).

BNHS 2755 was collected from a boulder on the bank of the Nengsandra dare (ca. 20:30 hours), an ephemeral stream in Nikrang A'ding near a trail towards Tura Peak (Tura Peak Reserved Forest). During other years surveyed (between 2008 and 2011), this stream becomes torrential in June which is the peak of south-west summer monsoon in north-east India. However, in 2014 it was relatively dry exposing more of the large boulders along the stream bank. The stream is surrounded on both sides by secondary forests with fairly dense undergrowth vegetation. BNHS 2754 and BNHS 2756 were collected from an indigenous agroforestry system called paan jhum (a practice of cultivating betel vine, Piper betle L.: e.g., Rahman et al., 2009; Nandy & Das, 2013) at around 20:00 hours on a rainless night. BNHS 2754 was spotted by eyeshine from the trunk of an areca nut tree (or betel palm tree, Areca catechu L.) at approximately two metres above ground level. The jhum had broad-leaved trees including jackfruit tree (Artocarpus heterophyllus Lam.) which is extensively grown in the West Garo Hills District (Phaomei & Mathew, 2019), and had a few fallen tree trunks and sparse herbaceous undergrowth. BNHS 2752, BNHS 2753 and BNHS 2755 each contain two large well developed eggs visible through the abdominal skin (e.g., Fig. 3D, F) indicating that egg-laying occurs during the monsoon season.

Etymology. The specific epithet "bapme" is derived from the word for "gecko" in the Garo language of the A'chik Mande ("Garo people"), the tribe indigenous to the region where the species occurs. The name is treated as a noun in apposition.

Suggested Common name. Garo Hills bent-toed gecko.

Cyrtodactylus urbanus distribution and morphological variation.

Cyrtodactylus urbanus was recently described based on a type series comprising seven specimens collected from "Basistha, Guwahati, Assam State, India (26.106301°N, 91.787199°E), 106 m elevation above sea level" (Purkayastha et al., 2020a). The authors stated in the distribution section that the type series was collected "from around Basistha Temple", however, the GPS coordinates plotted in Google Maps (accessed 17/01/2021) place the type locality within the relatively more developed suburb of Basistha, ca. 1.3 km north of Basistha Temple. The authors did not provide the district name in the type locality, although it was mentioned elsewhere in the table of sequences as "Kamrup (M) district" (Purkayastha et al., 2020a: table 1). We have subsequently confirmed that the holotype was collected from the Basistha Temple grounds (Purkayastha pers. comm., 27/03/2021) so we suggest the following revision of the type locality of C. urbanus: "Basistha Temple, Guwahati city, Kamrup Metropolitan District, Assam State, India (26.094846, 91.784545), 106 m elevation above sea level". Basistha Temple is situated on the border of Garbhanga Reserve Forest where the presence of C. urbanus is expected in contiguous habitat. Figure 6 (C, D) shows an additional example of a live (uncollected) individual likely conspecific with C. *urbanus* from nearby Basistha Temple. This individual was observed at ca. 22:00 hours on a vertical path-side embankment adjacent a small seep and surrounded by dense foliage. Purkayastha et al. (2020a) mentioned that they possessed "photographic vouchers" of C. urbanus from "Deeporbeel, Garbhanga Reserve Forest, Gotanagar and Rani Reserve Forest" in the vicinity of Guwahati but they neither published the photo vouchers nor cited the digital repository/registration numbers for the photo vouchers. We encourage the original authors to provide verifiable evidence for the presence of C. urbanus from these localities; but for now, we recommend that these reserve forests are not included in the confirmed distribution of the species.

Purkayastha et al. (2020c) reported a range extension for C. urbanus into Meghalaya State, at Nongpoh ("25.9081°N, 91.8543°E"), Nongpoh Subdivision of Ri Bhoi District, less than 25 km straight distance from the type locality; the map in Purkayastha et al. (2020c: Fig. 2) erroneously depicted this locality a further ca. 30 km south of the coordinates they provided (see Fig. 1 for corrected distribution). The elevation (not mentioned in Purkayastha et al., 2020c) at this GPS location is ca. 680-700 m asl. (assessed on Google Maps), revising the confirmed elevational range of the species to 100–700 m asl. The authors identified the single female specimen based on a comparison of its nd2 gene sequence with congeners (p-distance 0.7–0.9% from C. urbanus from the type locality), but provided no morphological details. We herein report the third confirmed locality for *C. urbanus* based on a single subadult male specimen (BNHS 2852, field number RGK 0554) collected from Saiden Village (25.880219, 91.887277, 565 m asl.), Nongpoh Subdivision in Ri Bhoi District, Meghalaya State. This third locality lies ca. five kilometres south-east of Purkayastha et al.'s (2020c) Nongpoh locality, and ca. 26 km south-east of the type locality (Basistha Temple). The Ri Bhoi localities are adjacent to the Nongkhyllem Wildlife Sanctuary (29 km²) within the Nongkhyllem Reserve Forest (125.91 km²) and Umsaw Reserve Forest (0.44 km²); both are provided state-level protection by the Meghalaya Forest Department (online, accessed 24/11/2020: http://www. megforest.gov.in/). Mathew (1995) listed two specimens (specimen numbers not given) identified as C. khasiensis in the collection of NERC/ZSI from "Umtham" Village and "Barapani" (~15-20 km south of Saiden) in Ri Bhoi District (localities erroneously reported to be in the East Khasi Hills District), Meghalaya. These localities are ca. 80 m higher in elevation than the Nongpoh population and are situated in contiguous habitat so may represent additional distribution records for C. urbanus. We recommend that these two specimens be re-examined to determine their species-level identifications.

Besides providing standard measurements and basic meristics for the type series of C. urbanus, Purkayastha et al. (2020a) did not provide any morphological variation section to accompany the description of the holotype. In the absence of a detailed morphological variation section for a given species, it is not possible to determine how

much of a holotype description represents taxonomically relevant morphological characters for that species; holotypes are often arbitrarily selected specimens that do not necessarily show "typical" or representative morphological characters for the species. A detailed comparison of the newly collected specimen (BNHS 2852) with the holotype description of Purkayastha et al. (2020a), and characters mentioned elsewhere in the paper (or visible in figures) for the type series demonstrates that the newly collected specimen expands the known morphological variation of the species, as follows: 1) granular scales are intermixed with a few small tubercles on the parietal region of the head, increasing in size posteriorly and laterally (vs. "scales on interorbital and occipital regions heterogeneous, without distinct tubercles" on the holotype); 2) nostrils oval (vs. "nostrils semicircular" on the holotype); 3) inner postmental pair less than twice the size (1.3 mm) of the outer postmental pair (0.8 mm) (vs. "inner [postmental] pair more than twice the size (2.6 mm) of and separating outer [postmental] pair (1.0 mm)" on the holotype); 4) few distinctly pointed tubercles on the ventrolateral folds (vs. "enlarged, smooth tubercles" on the holotype); 5) dorsal tubercles mostly conical to bluntly pointed on the body and limbs, without a median keel (vs. "bluntly conical and feebly keeled throughout" on the holotype); 6) original tail is dorsoventrally depressed (vs. "Tail original, ... rounded in cross-section" on the holotype); 7) subcaudal scales enlarged and heterogeneous in size, do not form a distinct midventral series of paired scales (vs. "subcaudal scales ... with midventral series of paired scales" on the holotype); 8) no distinct light-coloured middorsal stripe (vs. "a mid-dorsal cream coloured line divides the trunk and extends posteriorly to the level of the cloaca" on the holotype); 9) 11 supralabials on the right side, 12 on the left side (vs. 9–11 according to the species' "Diagnosis" section, however, this contrasts with counts provided elsewhere in the paper, i.e., 8-11 in the type series, N=7, according to table 4); 10) ten infralabials on the right side, 11 on the left side (vs. 8–10 in the type series, N=7); 11) DTR 20 (vs. 21-24 according to the species' "Diagnosis" section, however, this contrasts with counts provided elsewhere in the paper, i.e., 22-24 in the type series, N=7, according to tables 4 & 5); 12) MVSR ~39 (vs. 30-34 in the type series, N=7); 13) PcP 13 (vs. 9-12 on males in the type series, N=4); 14) scale row posteriorly bordering the PcP row is slightly enlarged relative to the pore-bearing scales (vs. "no enlarged scales between precloacal pores and vent" on the holotype, however, their fig. 4B depicting the PcP region of the holotype shows that the scale row posteriorly bordering the PcP row is distinctly enlarged relative to pore bearing scales and conflicting information was given in the species' "Diagnosis" stating "a row of enlarged scales above and below precloacal pore-bearing scales, slightly larger than pore-bearing scales"); 15) 13 dark brown transverse bands on the complete original tail (vs. "approximately 14 dark bands" stated in the species' comparison section, however, their figures 5 & 8 show that the type series have 13 or 14 dark bands on the original tails, N=5; however, the specimen reported in

Purkayastha et al. [2020c: fig. 1] from Nongpoh has 11 dark bands on a complete original tail, so the known range for the species is now 11-14 dark bands on a complete original tail); 16) in life, iris dark green with brown reticulations and orange pupil border on the Saiden specimen, and emerald green with brown reticulations and light brown pupil border on the Basistha uncollected individual (Fig. 6) (vs. "Iris in life silver-grey with brown reticulations"); 17) total TIVLam 17 (vs. 19-21 on the type series, N=7 according to table 4 and the "comparisons" section, however, conflicting information is given in the holotype description that stated 18 TIVLam [7 basal + 11 distal lamellae], and the species' "Diagnosis" section gave the ranges 6-7 basal and 10-12 distal lamellae, thus a maximum of 19 TIVLam in the species); 18) total FIVLam 15 (vs. 16–19 on the type series, N=7 according to table 4, however, conflicting information is given in the holotype description that stated 15 FIVLam [6 basal + 9 distal lamellae], and the species' "Diagnosis" section gave the ranges 5-6 basal and 8-10 distal lamellae, thus a maximum of 16 FIVLam in the species). The "Materials & Methods" section stated that the basal and apical lamellae series were counted separately, but did not mention whether non-lamellar scales between the basal and apical series were counted as apical lamellae in total counts, and the authors only provided the separate (basal and apical) counts for the holotype. We suggest that the authors of Purkayastha et al. (2020a) publish a table giving separate basal and apical lamellae counts, and intervening non-lamellar scale counts for all specimens in the type series to resolve this confusion; 19) PVT2 ca. 30 counted between the level of the axilla and the level of the groin (vs. PVT count for the holotype was reported in the holotype description as "45", but in table 4 as "38". Three different PVT ranges were given for



Figure 6. Cyrtodactylus urbanus in life: A-B. subadult male (BNHS 2852) from Saiden, Ri Bhoi District, Meghalaya State, north-east India; C-D. gravid adult female (not collected) from the type locality near Basistha Temple, Guwahati Metropolitan District, Assam State, north-east India. Images A–B taken ex-situ, C–D taken in-situ.

the species, i.e., "38-45" in the species' "Definition" section, "38–40" in the comparison section with C. khasiensis, and then "37-40" in tables 4 and 5). Purkayastha et al. (2020a) stated in the "Materials & Methods" section that PVT counts were made as follows: "PVT, para vertebral tubercles (PVT, counted from the most anterior tubercle on the occiput to mid-sacrum)", however, the close-up figure of the trunk of the holotype of C. urbanus (Purkayastha et al., 2020a: fig. 5C) has sufficient resolution to count ca. 38 PVT between the level of the forelimb and hind limb insertions (a common alternative method for counting PVT in some studies: see Discussion in Mahony & Kamei, in review). The authors are encouraged to clarify which numbers are correct for the PVT counts and accurately describe how the count was taken; 20) postcloacal tubercles, three on the left side, the region where spurs are generally found is damaged on the right side (vs. the presence and number of postcloacal spurs is not mentioned in Purkayastha et al., 2020). Purkayastha et al. (2020a: figs. 5 & 8) show three postcloacal spurs are visible on the left and right sides of the holotype, and in their figure 8, three are visible on the right side of paratypes VR/ERS/ZSI 685 and VR/ERS/ZSI 687, and three are visible on the left side of paratype VR/ERS/ZSI 686, demonstrating that males of this species typically possess at least three spurs on each side. Postcloacal spurs are not visible/discernible on the three female paratypes in their figure 8 (VR/ERS/ZSI/684, VR/ERS/ZSI/688, VR/ERS/ZSI/689), however, unpublished images provided to us (Purkayastha pers. comm., 2020) show three spurs of considerably reduced size (compared to males) are present on the three female specimens; 21) The presence or absence of PcP on females of C. urbanus is not clear from Purkayastha et al. (2020a) as it is not explicitly mentioned in the text. In the character table

(table 4), a hyphen is given to represent PcP number for the three paratypes, without an explanation of what the hyphen represents (e.g., it may mean "0", or "data not assessed"). In the absence of a PcP series in females of some Cyrtodactylus species, often a series of pitted scales is present in its place. This information is important to describe since it can be very useful for comparing females of Cyrtodactylus species that are otherwise morphologically very similar. Unpublished images provided to us (Purkayastha pers. comm., 2020) of the cloacal region of the three female paratypes of C. urbanus clearly show that pitted scales are present (at least 11 on two individuals), but it cannot be determined with certainty whether additional peripheral scales possess pits/depressions, or whether any of the medial scales of the series with clearly deeper pits are fully perforated, representing true pores. We recommend that these specimens be re-examined to confirm the number of pits/pores; 22) nine transverse pairs of dark brown blotches on the dorsum counted from the nape to the sacrum on the Saiden specimen (BNHS 2852), 9-10 on the uncollected individual from Basistha (Fig. 6C, D) (vs. not described in Purkayastha et al., 2020a). The number of dark blotches on the dorsum has been used in comparisons between species of Cyrtodactylus and can be a useful character. Purkayastha et al. (2020a) described the dorsal coloration as follows "Dorsal pattern of 6-7 longitudinal rows of two indistinct transverse pale buff blotches outlined by light edges" in the species' "Diagnosis" section, which based on their figures (figs. 5 & 8) the authors appear to be describing transverse pale patches between the dark brown blotches, though it is also not clear whether these counts were made on the trunk only, or from the nape to the sacrum. Purkayastha et al. (2020a: table 5) compared "Transverse series of blotches on the dorsum" between related species, stating "6–8" for *C. urbanus* but again it is not clear whether they were describing the dark brown blotches, or aforementioned "pale buff blotches", or how the counts were made. Based on Purkayastha et al. (2020a: figs. 5 & 8; 2020c: fig. 1) we determine that within the type series and their Nongpoh referred specimen, 8–10 transverse rows of paired dark brown blotches counted from the nape to the sacrum are present (N=8); 23) Tail segments indistinct, the second tail segment has eight scale rows dorsally, reduced to five rows ventrally (vs. not mentioned in Purkayastha et al., 2020a)

The previous section has highlighted a considerable number of errors, inconsistencies and ambiguities in the description of morphological characters between the "Diagnosis and comparison with regional congeners" section, the holotype description, and meristic counts given in tables in Purkayastha et al. (2020a). These issues urgently require clarification by the original authors of Purkayastha et al. (2020a) in the form of a published erratum to enable the correct diagnosis of further new species awaiting description in north-east India, and to prevent the potential misidentification of additional populations of C. urbanus for which molecular data are not available.

DISCUSSION

With regards to herpetofaunal diversity, Meghalaya State is historically the most well surveyed of the hilly states in north-east India. The past two decades has seen a growing number of new species of reptiles and amphibians being described from the state (e.g., Das et al., 2010; Mahony et al., 2011, 2013, 2018, 2020; Purkayastha & Matsui, 2012; Datta-Roy et al., 2013; Kamei et al., 2013; Agarwal et al., 2018c) and the taxonomic status of other poorly known species being revised based on newly collected material (e.g., Mahony, 2008; Biju et al., 2016; Kamei & Biju, 2016; Agarwal et al., 2018b; Mahony et al., 2018, 2020; Giri et al., 2019a, 2019b). The Garo Hills, however, are still relatively poorly surveyed. Few studies highlighting the region's herpetofaunal diversity have been published (e.g., Pillai & Chanda, 1981; Mathew, 2010; Sen & Mathew, 2010; Mathew & Meetei, 2013; Sen et al., 2013; Sangma & Saikia, 2014a, 2014b, 2015); however, several of these papers must be dealt with critically as they report several species misidentifications-e.g., Sangma and Saikia (2014a) reported Hemidactylus flaviviridis Rüppell, 1835, for the first time from Meghalaya giving figures (Sangma & Saikia, 2014a: fig. 4) of a specimen clearly identifiable as H. cf. platyurus (Schneider, 1792), and H. "garnooti" (sic.-H. garnotii Duméril & Bibron, 1836) for the first time from the Garo Hills giving a figure (Sangma & Saikia, 2014a: fig. 5) of specimens clearly identifiable as H. frenatus Duméril and Bibron, 1836; Sangma and Saikia (2014b) reported Oligodon nikhili Whitaker and Dattatri, 1982 (otherwise known only from the type locality in south India) and O. kheriensis Acharji and Ray, 1936, based on photographed specimens without giving diagnostic characters that allow an accurate identification of either species; figured specimens (Sangma & Saikia, 2014b: fig. 1) appear superficially similar to O. cyclurus (Cantor, 1839) and O. cinereus (Günther, 1864) (respectively), both of which are known from neighbouring regions.

Until now, published reports of *Cyrtodactylus* species from the Garo Hills include the following: C. "khasiensis" ("VR/ERS/ZSI/71"—specimen not examined in this study) from Baghmara Reserve Forest, South Garo Hills District (Mathew, 2010); C. "khasiensis" (no reported specimen voucher) from Balphakram National Park, South Garo Hills District (Pawar & Birand, 2001); C. "khasiensis" (one specimen presumably in NERC/ZSI, voucher number not given) from Songsak, Williamnagar, East Garo Hills District (Mathew, 1995). At the time of those reports, the large-scale hidden diversity within the north-east Indian Cyrtodactylus was not known (Agarwal et al., 2014) and all populations superficially similar to C. khasiensis were lumped under this catch-all name. We suggest that these specimens be re-examined and compared with Cyrtodactylus bapme sp. nov., since current molecular sampling suggests that C. khasiensis sensu stricto should be considered as a Khasi Hills endemic species (e.g., Agarwal et al., 2014, 2018b, 2018c).

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APPENDIX I

Comparative specimens examined.

- Cyrtodactylus himalayicus: holotype: male (ZSIK 15716), from "Kurseong, Darjeeling Dist. (5,000 ft)" [=Kurseong Subdivision (ca. 1524 m asl.), Darjeeling District, West Bengal State, India]; referred material: adult female (ZSIK 19546), from "Gopaldhara, Darjeling Dist." [=Gopaldhara Tea Estate, near Mirik Town, Darjeeling District, West Bengal State, India].
- *Cyrtodactylus khasiensis*: lectotype: adult female (BMNH 1906.8.10.4) from Khasi Hills, Meghalaya, India; paralectotypes: adult female (ZSIK 6199), adult male (ZSIK 6197), collected along with lectotype; referred material: adult males (ZSIK 5831, ZSIK 5832), adult female (ZSIK 5828), from "Cherrapunjee" [now Sohra Town, East Khasi Hills District, Meghalaya State, India].
- *Cyrtodactylus mandalayensis*: holotype: subadult male (BMNH 1900.9.20.1), from Mogok, Pyin Oo Lwin District, Mandalay Region, Myanmar.
- Cyrtodactylus tamaiensis: holotype: adult male (BMNH 1946.823.22), from "Pangnamdim, Nam Tamai Valley, Burma" [Pangnamdim, Kachin State, Myanmar].

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