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Front Cover: Yellow striped poison frog (*Dendrobates truncatus*) from Falan, Tolima, Colombia, see article on p. 41. Photographed by Taran Grant.

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### Alien chelonians in north-eastern Spain: new distributional data

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#### INTRODUCTION

A lien species pose a growing threat to native biodiversity because of their role as competitors, predators or as vectors of disease (Tompkins et al., 2003). In north-eastern Spain, aquatic ecosystems have been colonised by numerous alien species, including crustaceans, fishes, amphibians and reptiles (Clavero & García-Berthou, 2003; García-Berthou et al., 2007; Escoriza & Boix, 2012). These aliens may have an important effect on native biotic communities and although this impact has been widely studied in fish and amphibians, it is less well known in reptiles (Escoriza, 2018).

Catalonia in the north-east of Iberian Peninsula is relatively rich in reptile species (Salvador, 2014). In this region there are three species of native chelonians (*Emys orbicularis, Mauremys leprosa* and *Testudo hermanni*), with small and fragmented populations, that are sensitive to potential disturbances in habitat quality (Pleguezuelos et al., 2002; Couturier et al., 2014). Consequently, it is a conservation priority to assess the occurrence of alien species that may occupy similar habitats and to determine whether they have established successful breeding populations. Since the beginning of this century alien chelonian species have increased in number and in geographical range in the region but there has been no formal report drawing all the observations together.

The first records of alien turtles are from the mid-1990s. *Trachemys scripta* was the most widely distributed alien species, although restricted to the coastal regions of Barcelona and Girona (Llorente et al., 1995). Subsequently, other species of freshwater turtles were observed around Barcelona and its periphery: *Pseudemys concinna floridana*, *Chrysemis picta* and *Graptemys pseudogeographica* (Filella et al., 1999). Around the late 1990s, *Chelydra serpentina* was also observed in the locality of Castell d'Aro (Girona) (D. Boix, unpublished data). In 2010, three additional species were reported in Castellet (Barcelona): *Trachemys decussata*, *Mauremys reevesii* and *Pelodiscus sinensis* (Martínez-Silvestre et al., 2010).

In addition to the progressively increasing diversity of

aliens, some species have also shown an important and rapid expansion of their local range, especially *T. scripta* (Llorente et al., 1995; Filella et al., 1999). In the case of the terrestrial chelonians, most records refer to *Testudo graeca*, but there are also sporadic citations of other species: *Terrapene carolina*, *Testudo hermanni boettgeri*, *Testudo horsfieldii*, and *Testudo marginata* (Soler et al., 2010).

#### **MATERIAL & METHODS**

In the present study, we monitored the occurrence of alien turtles and tortoises in the provinces of Girona and Barcelona during the period 2000-2019. The habitats surveyed were mainly wetlands including rivers, lakes, reservoirs and artificial ponds located in natural reserves, river deltas and urban parks. The habitats were not chosen because they already had a record of the presence of an alien species. The surveys were undertaken using visual transects with binoculars and using baited funnel traps (Escoriza et al., in press). Terrestrial species were opportunistically located during these surveys. Taxonomy follows Uetz et al. (2019), and specimens were identified to species level following Bonin et al. (1996), Conant & Collins (1998) and Ernst & Lovich (2009).

Details of the chelonian records, on which this study is based, are presented in Supplementary Material on the British Herpetological Society website (see note at the end of this article).

#### RESULTS

Seventeen species of alien freshwater turtles and three species of terrestrial tortoise have been detected in the provinces of Girona and Barcelona. A preliminary assessment of the data obtained during the surveys showed a positive trend in both the richness of alien species and the number of sites occupied by them (Table 1 and 2, Fig. 1). *Trachemys scripta* is the most widely distributed species in our study area (Fig. 2) and it is also the only species in which reproduction has been confirmed (see Supplementary Material). The other species



**Figure 1.** Map showing previous and new chelonian records in the study area. On the left side is shown a compilation of two publications (Llorente et al., 1995; Filella et al., 1999), with the species richness of alien chelonians. On the right side is shown the number of alien chelonian species, based on the new data collected in this study.

Table 1. Alien chelonian species detected in Girona and Barcelona

Species	Period of observation	No. of sites
Apalone ferox	2013	1
Chelydra serpentina	2001-2016	5
Chrysemis picta	2011-2019	3
Graptemys ouachitensis	2014-2018	4
G. pseudogeographica	2007-2019	19
Kinixys belliana	2018	1
Macrochelys temminckii	2013	1
Mauremys mutica	2018-2019	1
M. reevesii	2016-2019	6
M. sinensis	2013-2019	12
Pelodiscus sinensis	2012-2019	2
Pseudemys concinna	2003-2019	19
P. nelsoni	2011-2019	20
P. rubriventris	2019	1
Sternotherus odoratus	2013-2018	2
Testudo graeca	2003-2006	2
T. horsfieldii	2016	1
Trachemys grayi emolli	2011-2019	6
T. ornata	2013	1
T. scripta	2001-2019	61

show a more restricted distributional range, mostly localised in urban or peri-urban areas, although their range also has increased in the last decade. This is particularly evident in *Graptemys pseudogeographica* and *Pseudemys nelsoni*. Both species were only occasionally recorded before 1999 (Filella et al., 1999) but presently appeared in 7 % and 9 % of the region (Fig. 2).

Table 2.	Number of a	alien chelon	ian species	and sites	s, in Gi	rona	and
Barcelon	a during the	period 2001	L-2019				

Year	No. of alien species	No. of sites
2001	2	2
2002	1	1
2003	3	3
2004	2	4
2005	1	1
2006	2	2
2007	2	1
2008	2	3
2009	2	2
2010	3	3
2011	7	8
2012	8	15
2013	12	13
2014	8	14
2015	8	20
2016	10	21
2017	5	18
2018	12	25
2019	11	27

Semi-aquatic turtles have been found and these are mainly species of east Asian and North American origin (Table 1), from subtropical to temperate climates (Bonin et al., 1996; Ernst & Lovich, 2009), but also include one strictly tropical species (*Trachemys grayi emolli*; Table 1, Fig. 2 and Fig. 3). During the surveys we detected, for the first time in Europe, free-living *Mauremys mutica* (Table 1, Fig. 2 and Fig.



Figure 2. Map showing the records of the alien species. A. Trachemys; B. Pseudemys; C. Graptemys and Chrysemis; D. Sternotherus; E. Mauremys; F. Kinixys and Testudo; G. Macrochelys and Chelydra; H. Apalone and Pelodiscus



Figure 3. Examples of alien species of chelonians found in Girona and Barcelona. 1. Chelydra serpentina; 2. Pseudemys concinna; 3. Mauremys sinensis; 4 M. mutica; 5. Sternotherus odoratus; 6. Trachemys scripta; 7. Chrysemys picta; 8. Graptemys pseudogeographica; 9. P. nelsoni; 10. M. reevesii; 11. Pelodiscus sinensis; 12. Macrochelys temminckii; 13. Testudo horsfieldii; 14. T. graeca; 15. T. grayi emolli.

4), a species native to the subtropical regions of south-east Asia (Bonin et al., 1996). We marked the specimen and then recaptured it in two consecutive years, indicating that it is capable of withstanding the mild winter conditions of the region. We also detected two adult specimens of *Pseudemys rubriventris* (male and female) in the Lake Banyoles (Table 1, Fig. 2 and Fig. 4). This is only the second record of this species in the Iberian Peninsula (Arribas, 2017) and the first record outside an urban environment.

During the surveys we also observed some isolated specimens of three alien species of land tortoise T. graeca, T. horsfieldii, and K. belliana (Table 1, Fig. 2 and Fig. 3); there was no evidence that these species were reproducing. Testudo graeca was found in several peri-urban locations and possibly these specimens had survived for long periods in the region. Testudo horsfieldii is native to the continental steppes of south-western Asia and appears very frequently in the pet trade (Türkozan et al., 2008). During our surveys it was observed only on a single occasion, in an agricultural field near to the city of Puigcerdà (Fig. 3, and see Supplementary Material). Also a single specimen of K. belliana was observed in an urban park in Barcelona (Fig. 3, and see Supplementary Material); this is a tropical species and it is unlikely that it could survive for extended periods outdoors in a Mediterranean climate.

#### DISCUSSION

Overall our results showed that the coastal regions of Catalonia are highly vulnerable to the establishment of alien reptile populations, due to the mild climate and the importance of the pet trade. This is supported by the presence of several alien turtles, but also by other reptiles, such as monitor lizards (Soler & Martínez-Silvestre, 2013) and snakes (*Pantherophis guttatus* in Girona and Barcelona; D. Escoriza & G. Pascual, unpublished data). These alien turtles presumably have their origin in the pet trade because



**Figure 4.** Detail of diagnostic traits of some species of turtles with a problematic identification. Left column: head shape; Middle column: bridges; Right column: plastron. Upper line: *P. rubriventris*; Middle line: *M. mutica*; Bottom line: senile individuals of *T. scripta* superficially similar to *T. decussata*.



**Figure 5.** Example of a senile individual of *T. scripta elegans* (right side) similar in appearance to *T. decussata*, to the left side a normal-coloured *T. s. elegans* for comparison

there are no turtle breeding farms in this region, unlike other countries (Shi & Parham, 2001). These animals are usually isolated specimens and may have been released by their owners or otherwise have escaped captivity.

In other regions of the world, such as Florida, similar factors have favoured the establishment of numerous alien species with negative effects on native fauna (Dove et al., 2011). In our study we have not evaluated the effect of these species on native fauna, but these could be: (i) competition with native turtle species, because they have a similar trophic range and thermoregulation requirements (Pérez-Santigosa et al., 2011); (ii) hybridisation between congeneric species, e.g. *M. sinensis* and *M. mutica* with the native species *M*.

*leprosa*, as has been observed in other contact areas of the world, involving introduced *M. mutica* and the native *M. japonica* (Suzuki et al., 2013); (iii) predation on native aquatic fauna (Polo-Cavia et al., 2010), including vulnerable species of amphibians such as *Triturus marmoratus* or *Pelobates cultripes*; and (iv) the transmission pathogens (Meyer et al., 2015).

Previous publications (e.g. Filella et al., 1999; Martínez-Silvestre et al., 2010) also reported the presence of the Cuban Slider (Trachemys decussata) in the study region. However, we did not find this species, but we found senile specimens of T. scripta with a similar external appearance (Fig. 4 and Fig. 5). However, these senile T. scripta individuals always retained a faded plastral spotting (Fig. 4), differing from that expected in T. decussata (an almost imperceptible spotting; Bonin et al., 1996). Therefore, we consider the presence of T. decussata in Catalonia at least doubtful, and that previous records could be assigned to senile T. scripta. The fact that all cited T. decussata were full-grown specimens also supported this statement. However, genetic analyses will be required to state with certainty the absence of T. decussata in the region, because there is the possibility of hybridisation with *T. scripta* (Parry, 2009).

We consider that it would be advisable to limit the trade of these invasive species, as was suggested by Maceda-Veiga et al. (2019). However, following the initial ban on the trade in one species (T. scripta) other species have filled the gap in the market with the result that they have been released (i.e. Graptemys pseudogeographica, Mauremys sinensis, Pseudemys concinna and P. nelsoni). For this reason we believe that the application of more efficient bans and regulations on the pet trade are necessary in order to end further turtle releases in urban ponds; furthermore naturalised individuals need to be removed. Maceda-Veiga et al. (2019) concluded that the release rates of invasive species have remained unchanged since the 2011 ban on their trade. However, the time period analysed in their study starts only after the ban which is too short a period to allow any inference, specially taking into account the long lifespan of turtles. Given the current situation, further studies are needed to evaluate the effectiveness of restrictions on the pet trade.

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## Long-term comparison of relocated and resident box turtles, Terrapene carolina carolina

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**ABSTRACT** - The eastern box turtle, *Terrapene carolina carolina*, is a long-lived turtle species that is declining across much of its range. A mark-recapture study of this species was carried out for over thirty years at the Mason Neck National Wildlife Refuge in Virginia. During this time, box turtles were relocated to the reserve and became part of the study. Several individuals were recaptured more than ten years after relocation including one that was recaptured after almost 28 years. Overall, however, turtles relocated to the study area appeared not to fare as well as resident turtles. Significantly fewer relocated turtles were recaptured after their first winter at the new site (33.3 %) than resident turtles (51.5 %) though the survival rates for relocated and resident turtles were similar for subsequent years. Relocation can work as a rescue strategy for some individuals but it can also negatively impact relocated individuals. This study is the first to show long-term residency of relocated box turtles.

#### INTRODUCTION

The eastern box turtle, *Terrapene carolina carolina*, is declining across its range (Ernst & Lovich, 2009) and effective management techniques are required to protect populations that are in danger. In the case of habitat loss, one such technique is the relocation of individuals to reserves. However, studies highlighted below have shown that relocation programmes may not be effective rescue strategies for *Terrapene* spp.

The principles of population ecology tell us that a particular habitat can only support a given number of turtles and for this reason relocated turtles may have to disperse away from where they are released. Most adults have a homing tendency when displaced (Nichols, 1939) so these relocated box turtles may simply try to return to their home range, expending valuable time and energy in the process (Dodd, 2001). Belzer (1997) found that few relocated turtles established home ranges in the new site into which they were introduced in Pennsylvania. In New York, Cook (1996) found that many relocated T. carolina did establish home ranges but survivorship was lower for relocated individuals than for individuals that had not been relocated. Relocated T. carolina in North Carolina had larger home ranges, moved a greater average daily distance and were more likely to experience mortality or disappearance than resident box turtles (Hester et al., 2008).

Since 1980, a long-term, mark-recapture study of the eastern box turtle has been undertaken at the Mason Neck National Wildlife Refuge. Besides the established population, other eastern box turtles were relocated to this reserve during the 1980s and 1990s and the present study provided an opportunity to measure the long-term residency rates of these relocated turtles. Survivorship and recapture rates of relocated turtles are compared with those of resident turtles.

#### MATERIALS AND METHODS

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The study area was a 30 ha tract of wildlife reserve (Elizabeth Hartwell Mason Neck National Wildlife Refuge, 38°67' N, 77°10' W, ~25-35 m asl) in Fairfax County, Virginia. Located within the Washington DC suburbs, the Mason Neck peninsula contrasts sharply with most of the metropolitan area in remaining largely undeveloped. The reserve contains an 83 ha freshwater tidal wetland marsh (the "Great Marsh"). A variety of habitats can be found on the reserve but it is generally a well-drained mixed deciduous upland forest.

The study site is an abandoned farm located at the end of a gravel road that bisects the reserve and located adjacent to the Great Marsh and the Potomac River. This area of the reserve is accessible only by permit except during the annual deer hunt. Since being ceded to the Federal Government in the 1960s, the farmland has undergone succession and is now mostly wooded. At the time of this study a few open areas remained including a small grass parking lot, a shooting range and a 3 ha (ca.) field that was mowed at three-year intervals.

The work done in this study conformed to the British Herpetological Society's Ethical Policy and Guidelines (British Herpetological Society, 2017) and met all legal requirements. Between 1984 and 1993, seventy-eight *T. carolina* were relocated, primarily for mitigation reasons, to the Mason Neck National Wildlife Refuge study site from other locations (by authors CE and TB). Most of the relocated individuals had been found on or adjacent to roads and paths in the northern Virginia area, while others were relocated from George Mason University's Fairfax campus which was then undergoing various construction and expansion projects. Some of these turtles were subsequently recaptured during the study.

The tract was searched with special emphasis given to

areas where turtle captures were abundant during previous research. The study ran for three years (2011-2013) with the field season beginning in April and ending before the annual deer hunt in November. Since the annual activity cycle for this turtle had already been established (Ernst & Lovich, 2009), field work was concentrated in the spring when, historically, most captures occurred (Boucher, unpublished data). At this time of year, the turtles, passing though and flattening the invasive Japanese stiltgrass (*Microstegium vimineum*), could be tracked easily. The hottest part of summer (late June to the end of August), when few turtles are found (Boucher, 1999), was avoided.

For individual identification we used a method modified from Ernst et al. (1974), in which turtles were marked with notches on various marginal scutes. These were assigned numbers (Fig. 1) and the sum of those filed was the individual turtle number.



**Figure 1**. System for marking box turtles by filing marginal scutes. The turtle's unique identifying number is determined by finding the sum of the numbers assigned to the marked scutes.

Sex was determined by visual inspection of the turtle using several sexually dimorphic traits, e.g. carapace/plastron shape, tail size, hind leg claws, eye colour (Ernst & Lovich, 2009). The IBM SPSS Statistics 19 package was used for all statistical tests and differences were treated as statistically significant when the probability of them occurring by chance was 5 % or less (p≤0.05).

#### RESULTS

The adult sex ratio favoured males in both the relocated and resident turtles (Table 1) and there was no significant difference in adult sex ratio between the two samples (proportion test, z = -0.03, p = 0.976). At the time juveniles were introduced to the reserve they made up 15.4 % (12 of 78) of the relocated population and 14.5 % (62 of 428) of the resident population caught during the same period (Table 1) with no significant difference between the two samples (proportion test, z = -0.21, p = 0.834).

**Table 1.** Comparison of resident turtles and relocated turtles,adult (10 years+) sex ratio, and juveniles (<10 years) proportion of</td>population, 1984-1993

	Adult sex ration (M:F)	% Juveniles
Resident	1.35:1 (n=366)	14.5 (n=62)
Relocated	1.36:1 (n=66)	15.4 (n=12)

Relocated turtles had a 43.6 % (34 of 78) recapture rate. By comparison, resident turtles that were originally captured during the same period had a 55.0 % recapture rate. The difference in recapture rates for relocated and resident turtles is not significant (z = -1.86, p = 0.063).

Resident turtles were also recaptured more frequently and over a greater period than relocated turtles. Resident turtles were recaptured a mean of 2.1 times (range = 0 - 38) compared to 1.6 times (range = 0 - 21) for relocated turtles. For turtles that were recaptured, mean difference between first and last capture for resident turtles was 3034.5 days (range = 1 - 10,586; n = 237) while for relocated turtles it was 2512.8 days (range = 7 - 10,217; n = 34).

The survivorship at the study site, based on raw recapture rates, through the first ten years is shown in Figure 2. The only year with a significant difference in survival was the first year (proportion test, z = 2.96, p = 0.003). Survival through each subsequent year was similar for resident and relocated individuals (Table 2). Once a relocated *T. carolina* persisted through the first winter, its likelihood of being recaptured at the study site was similar to that of resident turtles.



Figure 2. Survivorship of resident and relocated turtles

**Table 2.** Survivorship through each winter following year of originalcapture

Winter	1*	2	3	4	5	6	7	8	9	10
Resident % persistence	51.5	84.2	90.9	93.5	92.5	91.2	89.6	96.7	88.8	90.3
Relocated % persistence	33.3	88.5	78.3	100	88.9	81.3	100	92.3	91.7	81.8

\*Significant difference between resident and relocated turtles (proportion test)

**Table 3.** Recapture rate, mean number of recaptures, and mean number of days between first and last capture for resident and relocated turtles

	Recapture rate	Mean no. of recaptures*	Mean period (days) between first and last capture
Resident			
Juveniles	37.8% (14/37)	0.49	1757.7
Adult females	58.1% (100/172)	2.42	3221.9
Adult males	55.9% (123/220)	2.06	3027.4
Relocated			
Juveniles	30.8% (4/13)	0.46	3393.8
Adult females	39.3% (11/28)	2.25	2261.0
Adult males	51.4% (19/37)	1.49	2065.3

\* The mean value for the number of times each individual was recaptured

**Table 4.** Relocated turtles with survivorship of more than ten winters at the study site

Number	Sex	Date of release	Last date of recapture	Winters survived
239	м	7 June 1984	28 May 2012	28
74	М	17 May 1988	16 Sept. 2012	24
402	F	6 June 1984	14 June 2003	19
157	М	7 Sept. 1992	21 May 2011	19
260	М	22 June 1984	7 Sept. 2002	18
296	F	11 June 1985	22 Sept. 2001	16
398	М	20 Sept. 1987	23 May 2003	16
331	F	30 July 1985	17 May 1997	12
1034	М	7 August 1992	26 April 2003	11

Among relocated turtles, we recaptured more males than either females or juveniles (Table 3) but for resident turtles, females recapture rates were higher and occurred over a longer period. Relocated juveniles had a lower recapture rate and lower mean number of recaptures than either males or females but were recaptured over the longest mean time interval. Some relocated turtles were found at the study area long after relocation (Table 4), up to almost 28 years later in one case.

Some relocated turtles had injuries. If the recapture rate is recalculated separately for uninjured and injured turtles, then the recapture rate is lower for uninjured, relocated turtles (39.6 %, n=53) than for injured, relocated turtles (52.0 %, n=25). Uninjured, relocated turtles were recaptured a mean number of 1.9 times while injured, relocated turtles were recaptured a mean number of 1.0 times. The difference was not significant (two-sample t-test, p = 0.17).

#### DISCUSSION

Survivorship is difficult to measure in an open *T. carolina* population. If an individual turtle is never recaptured it could be that: a) the turtle had died, b) the turtle had emigrated, or c) it had remained in the area but had avoided detection. It was impossible in this study to distinguish between death and emigration unless a dead turtle was found. Much more likely than finding a dead turtle was the possibility that a turtle

was released and simply never seen again and consequently 'survival' estimates in this study are conservative. An alternative approach to estimate survival, not employed in the present study, is to use CMR models (Dodd, 2016; McDiarmid et al., 2012).

Cook (2004) found that all *T. carolina* that established home ranges at the release site did so within three years of being released. In this study, only 23.1 % of relocated turtles persisted at least three winters after being released as compared to 39.4 % for resident turtles. Some relocated turtles, however, survived and were found at the study area long after relocation. Based on these individuals, assuming they are a true representation, then *T. carolina* relocation seems to be of limited value as a conservation measure as only a third survived the first winter and less than a quarter persisted long enough to establish home ranges. Nevertheless, this is preferable to a 0 % survival rate for turtles that are not relocated.

An examination of factors that determine whether a turtle will establish a home range at its release site could be a useful conservation tool. Females might be more sedentary or less likely to disperse from a release site than males even though studies have shown that female home ranges are larger (Ernst & Lovich, 2009). Lower recapture rates and mean number of recaptures for juveniles (Table 3) can be explained by lower juvenile survivorship and the fact that small turtles are more difficult to find and recapture. Younger juveniles at Mason Neck often remain concealed in the leaf litter (C. Ernst personal observation). That juveniles had the longest mean time interval between release and last recapture (Table 3) is expected because juveniles have more possible years of survival ahead of them. Juveniles may also be less likely to disperse far from the release site because of their small size. They are slower than adults and have a shorter stride (Marvin & Lutterschmidt, 1997). Terrapene carolina juveniles also have smaller home ranges than adults (Ernst & Lovich 2009).

One of the goals of relocating turtles is to establish or strengthen breeding populations. Little information has been reported on reproduction by relocated *T. carolina* though they have been observed laying eggs (Cook, 2004). Some of the relocated turtles in this study may have reproduced after their release at the Mason Neck National Wildlife Refuge and their genes may now contribute to the Mason Neck population. One relocated male and one relocated female were found courting resident turtles, six years after relocation in the case of the male and two years after relocation for the female.

Complicating the issue of lower persistence by relocated turtles compared to resident turtles at our research site, is that relocated turtles were more likely to have been originally found with injuries than residents. It is possible that this influenced the lower recapture rate for relocated turtles, especially if injured individuals later died from their injuries. This does not, however, appear to be the case. Injured turtles appear to be more subject to recapture. It could be that their injuries limit their ability to disperse. Only 67.9 % of relocated turtles (53 of 78) were found without injury compared to the 90.2 % reported for the Mason Neck population (Boucher & Ernst, 2004). The nature of the injuries was also often

more serious for relocated turtles. Some appeared to have injuries from vehicle encounters which was not surprising since many were found along roads. Vehicle injuries are rare on the wildlife reserve which has limited vehicular access though one resident reserve turtle was injured when struck by a mower. It seems likely that *T. carolina* on the Mason Neck National Wildlife Refuge are less susceptible to injury than *T. carolina* from most of the rest of northern Virginia because the reserve and surrounding land on the Mason Neck peninsula form a large area of undeveloped protected land.

Terrapene carolina relocation may work as a rescue measure for some individual turtles. This study is the first to provide evidence of long-term residency of relocated box turtles, including one individual that was recaptured almost 28 years after relocation. Relocation should not, however, be treated as a mitigation or conservation measure that has no negative impact on relocated animals. Only a third of relocated animals were recaptured after their first winter at the new location. Managers involved in relocation decisions should question whether the effort of relocation is justified when only a third of the relocated animals survive their first winter at the new site. Releases may also have negative impacts on the resident population if the population density is increased to the point that resident and released turtles are in competition for limited resources, or if the released turtles introduce diseases. Relocating animals into large, semi-natural enclosures at the new location could help them to acclimatise, particularly if the enclosures provide habitat for successful hibernation and overwintering such as deep leaf litter, soil that is well drained and easily burrowed into, and a protective canopy.

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## Clinical and naturalistic substrates differ in bacterial communities and in their effects on skin microbiota in captive fire salamanders (Salamandra salamandra)

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**ABSTRACT** - The fire salamander (Salamandra salamandra and its relatives) is of increasing priority for ex situ conservation due to the spread of the fungal pathogen Batrachochytrium salamandrivorans in Europe. In captivity, the species may be maintained on a clinical paper-based or a naturalistic substrate, either of which has its own advantages and disadvantages. However, the impact of these two substrates on bacterial microbiotas within an enclosure and on the salamanders themselves is unknown. To investigate this, we maintained captive fire salamanders on either paper towels or a naturalistic substrate and quantified the culturable microbiotas of both substrates across the one-week lifespan of a paper towel and of the salamanders themselves over a six-month period. We found significant differences in the bacterial communities associated with the two substrates. Over a week-long period, there were major fluctuations in the community composition and abundance on paper towels while on the naturalistic substrate bacterial communities were relatively stable. The skin microbiota of salamanders were indistinguishable at the beginning of the study but after six months differed significantly between the two treatments, although the bacterial morphotypes present remained relatively similar compared with changes between substrates. These data show that husbandry protocols may have a strong influence on the culturable bacterial communities to which captive amphibians are exposed. Nevertheless, the animals were apparently able to maintain their own microbiota to a considerable degree. These findings should be borne in mind when determining husbandry protocols. Given the relative benefits of both types of enclosure, it is possible that a hybrid approach could be used whereby a small amount of naturalistic substrate is provided in a container within an otherwise clinical enclosure, to act as a bacterial reservoir.

#### INTRODUCTION

he establishment of ex situ populations of amphibians has become a core part of the strategy to counter global amphibian declines (Wren et al., 2015). Consequently, the number of amphibian species and individuals in captivity has greatly increased for conservation breeding (Tapley et al., 2015a), the investigation of disease dynamics (Burggren & Warburton, 2007), and the development of captive-care protocols (Antwis et al., 2014a; b; Michaels et al., 2014; 2015; Tapley et al., 2015b). In captivity, amphibian enclosures are arranged with a 'clinical' approach to husbandry in order to standardise methods, to improve biosecurity, and to ease maintenance workload. Such enclosures often have a paper towel substrate that is changed at regular intervals (~1-10 day) when they become soiled with amphibian waste and heavily contaminated with bacteria and fungi (Bishop et al., 2009; Garner et al., 2009; 2011; Retallick & Miera, 2007; Weinstein, 2009; Carver et al., 2010; Gahl et al., 2012; Ogilvy et al., 202; Ohmer et al., 2015; Venesky et al., 2015).

It has been shown previously that husbandry conditions significantly influence traits associated with amphibian health and fitness, in particular the composition and diversity of the skin microbiota (Antwis et al., 2014; Loudon et al., 2014; Michaels et al., 2014). Likewise environmental reservoirs are important in determining the microbiota of amphibian populations both in the wild (Fitzpatrick & Allison, 2014; Walker et al., 2014) and in captivity (Loudon et al., 2014). In nature, however, terrestrial amphibians live on a variety of complex mixtures of organic and inorganic substrates. Captive conditions should aim not only to promote health, welfare and ease of care for the animals being maintained but also to produce animals as similar as possible to wild individuals. This will ensure that experimental results are applicable to wild conditions and will generate a captive stock that has the best chances of survival after translocation to the field. Clinical substrates may act differently from natural substrates as reservoirs of bacteria (Loudon et al., 2014; Michaels et al., 2014), and this may have implications for the microbiota not only in the enclosure environment, but also on the skins of the animals maintained therein.

The fire salamander (*Salamandra salamandra*) is a widespread amphibian, distributed throughout most of continental Europe, and with close relatives in Europe (*S. atra, S. lanzai, S. corsica*) and in the Near East and North Africa (*Salamandra infraimmaculata* and *Salamandra algira* respectively). *Salamandra salamandra* is a complex of regionally restricted forms with radically differing morphology and ecology (Seidel & Gerhardt, 2017), and its conservation status is potentially better understood in this light. The recent discovery (Martel et al., 2013) and spread (Sabino-Pinto et al., 2015; Feldmeier et al., 2016) of the lethal

pathogen *Batrachochytrium salamandrivorans* (Bsal) in fire salamanders is a growing threat for the taxon, and plans for the creation of ex situ rescue populations of local forms of this species have been proposed and, in the case of the initial outbreak site for Bsal, enacted (Spitzen van der Sluijs et al., 2018). There are many approaches to maintaining fire salamanders in captivity (Seidel & Gerhardt, 2017), including the use of clinical paper-based substrates. These have been demonstrated to work well in terms of the clinical health of animals, and also offer the advantages of standardisation and biosecurity. However, their impacts on the skin microbiota of salamanders are currently unknown.

Here we quantify clinical and naturalistic substrates as environmental reservoirs of bacteria, and compare the effects of these husbandry conditions on a captive population of Spanish fire salamanders (*S. salamandra gallaica*) in order to inform husbandry practice for this species.

#### MATERIALS AND METHODS

#### **Ethics Statement**

All methods used in this study were non-invasive and did not require a UK Home Office Licence as they fell within best practice husbandry for this species. The University of Manchester Ethics Committee approved this study prior to commencement. Care was taken to ensure animals were not harmed during data collection, and individuals were monitored daily for signs of distress or injury, of which none were observed. Animals were rehomed after the completion of the study and no animals were destroyed as part of it.

#### Experimental design and animal husbandry

All experimental work was conducted in 2013 at the University of Manchester. Captive-bred (F2) S. salamandra gallaica were obtained as recently metamorphosed juveniles from a private breeder. Of eighteen salamanders used in test, the grandparents of eight were collected in the Sierra de Grandola, Portugal and ten from an unknown site in northern Spain. Animals from each site were allocated equally to each treatment by alternately selecting animals at random from each group. Animals were maintained individually in plastic containers (28 x 16.5 x 10 cm; Monkfield Nutrition, UK) with well-ventilated lids and access to a small water dish filled with aged tap water (GH <20mg/L, pH c. 6.5) changed weekly or when soiled. Refuges were provided in the form of overturned plastic plant saucers with doorways cut in the rim. 'Clinical' enclosures had a substrate of paper towels (blue paper towel hygiene rolls, Essential Supply Products, UK) dampened to saturation but not super-saturated with aged tap water. The towelling was spot cleaned daily for faecal material and then replaced weekly. 'Naturalistic' enclosures had a substrate consisting of coir coco-fibre (Wiggly Wigglers, UK), peat compost (B&Q, UK), rinsed silver sand (B&Q, UK), fine orchid bark (Monkfields Nutrition, UK) and crushed beech leaves in a 10:10:2:2:1 ratio. In nature, S. salamandra is found in woodland habitats, especially beech forest (Kuzmin et al., 2009) and this substrate was designed to mimic the leaf-mould substrate often found in this environment. All components of this mix were sterilised by autoclave prior to setting up enclosures but unlike the towelling it was not

changed throughout the study, but was still subject to the manual removal of faecal material as it was produced.

Enclosures were arranged alternately in a climate controlled growth cabinet (Percival Scientific, Iowa, USA) with a 12:12 photoperiod, diurnal surface temperature of 17 °C and nocturnal surface temperature of 13 °C. All animals were fed every third day with black crickets (*Gryllus bimaculatus*) of an instar appropriate to the size of the salamander (cricket length approximately equal to the distance between the eyes), cricket guts were loaded for at least 24 hours on fresh fruit and vegetables and dusted externally with Nutrobal (Vetark, Winchester, UK) vitamin and mineral supplement immediately prior to being offered. Animals were also offered chopped earthworms (*Lumbricus terrestris*, Worms Direct, Maldon, UK) every fourth feed. All salamanders received the same prey species at any one feed and uneaten food items were removed after 24 hours.

#### Salamander growth rates

The mass of salamanders was measured at the beginning and end of the study (6 months) using Ascher AS2001 balances accurate to two decimal points.

#### Bacterial community culturing

Six months after the start of the study, bacterial communities associated with the clinical and natural substrates were characterised. A wet weight of 1 g of each substrate was collected using sterile tools, both immediately after fresh paper towels were placed in enclosures (day 1) and from soiled towels one week later (day 7). Substrate was placed in 10 ml of 1M NaCl, and vortexed vigorously for one minute. Substrate was left to settle for 30 seconds and then 1 ml of liquid pipetted off and used to construct serial dilutions to a concentration of 10-3 under sterile conditions. Bacterial communities from the salamanders were collected and cultured one week after animals were placed in experimental set-ups ('month 1'), and again six months later ('month 6'). The ventral region of the body was rinsed with sterile water and swabbed ~20 times (Michaels et al., 2014). Swabs were placed in 1 ml of 1M NaCl, to facilitate subsequent culturing methods, which were conducted under sterile conditions. Tubes containing swabs were vortexed to dissociate bacteria and then diluted ten-fold with 1M NaCl<sub>2</sub>. For both substrate and salamander samples, dilutions of 100 and 10-1 were plated out on R2A agar media (Lab M Ltd., United Kingdom) and incubated at the same temperatures at which the salamanders were maintained. Bacterial colonies were grouped according to morphology and counted seven days after plating, after which negligible new colony growth was observed. Genetic sequencing was beyond the scope of the project, and so bacterial identification was not possible.

#### Data conversion and statistical analyses

Salamander body mass data were compared between treatment groups using repeated measures ANOVA in RStudio.

Bacterial counts were multiplied by the necessary dilution factors and averaged across the two dilutions for a given sample. All statistical analyses were conducted in RStudio. Differences in the total abundance of colony forming units (CFUs) isolated from the two different substrates on day 1 and day 7 were analysed using a generalised linear model with 'tank' included as a random factor to account for the repeated sampling at two different time points. Differences in microbiota composition of the environmental substrates at days 1 and 7 were also analysed using an Adonis analysis with Bray-Curtis distance using raw count data and including 'tank' as a random factor, and these data were then visualised using nonmetric multidimensional scaling (NMDS).

The relative abundance of each bacterial morphotype in the overall community was calculated by dividing the number of colony forming units (CFUs) of a given bacterial morphotype by the total number of CFUs for each sample. In order to account for differences in bacterial abundance between the two treatment groups, and a high proportion of rare morphotypes in some samples, additional Adonis analyses were performed with this relative abundance data using Euclidean (as a distance measure) and Morisita–Horn (as a dissimilarity index) distances to test for differences between treatment groups at the two sampling points, with 'tank' included as a random factor.

Differences in overall bacterial community composition of salamanders were analysed separately at month 1 and month 6 using an Adonis analysis with Bray-Curtis distance using raw count data, and visualised using NMDS. Additionally, the relative abundance of each bacterial morphotype in the community was calculated for each individual at each sampling point and Adonis analyses with Euclidean and Morisita–Horn distances were used to test for differences between treatment groups. The microbiotas associated with salamanders six months after the start of the study were compared to bacterial communities associated with the substrate (using data from day 7) using an Adonis analysis with Bray-Curtis distance using raw count data and visualised using NMDS, and analysed using Adonis with Morisita–Horn and Euclidean distances for relative abundance data.

#### RESULTS

Mean body mass at the start of the study across treatment groups was 1.02 ( $\pm$  0.41) g and mean mass at the end of the study (i.e. at 6 months) was 12.89 ( $\pm$ 3.23) g. There was no significant difference between experimental groups of salamanders in the change in body mass according to treatment ( $F_{1.85}$  = 1.191, p = 0.278).

Total abundance of bacterial communities isolates from the substrates was significantly affected by sampling time (X<sup>2</sup> = 25.680, d.f. = 1, p < 0.001), treatment group (X<sup>2</sup> = 19.963, d.f. = 1, p < 0.001), and their interaction (X<sup>2</sup> = 23.343, d.f. = 1, p < 0.001). Post hoc contrast analyses showed that the abundance of cultured bacteria was significantly higher for the clinical substrate at day 7 (p < 0.001 in all cases; Fig. 1). Bacterial community composition of the two substrates, based on raw bacterial abundance counts, were significantly different according to sampling time (F<sub>1,32</sub> = 24.788, p = 0.001), treatment group (F<sub>1,32</sub> = 26.415, p = 0.001) and their interaction (F<sub>1,32</sub> = 20.516, p = 0.001). The NMDS figure shows that culturable bacterial communities associated with naturalistic substrates remained stable over a one week period (Fig. 2; black shapes), but that communities associated with the clinical substrate were initially similar yet differentiated from those of the naturalistic substrate (Fig. 2; grey squares), and one week later these were significantly differentiated from naturalistic samples (Fig. 2; grey triangles).

The Adonis analyses of relative abundance data showed that for both distance measures there was a significant effect of sampling time (Morisita–Horn:  $F_{1,32} = 14.762$ , p = 0.002; Euclidean:  $F_{1,32} = 10.111$ , p = 0.001), treatment (Morisita–Horn:  $F_{1,32} = 6.860$ , p = 0.014; Euclidean:  $F_{1,32} = 7.531$ , p = 0.003), and their interaction (Morisita–Horn:  $F_{1,32} = 38.166$ , p = 0.001; Euclidean:  $F_{1,32} = 19.902$ , p = 0.001). Community composition and relative abundance of bacteria were stable over the one-week sampling period for the naturalistic substrate, but fluctuated massively for the clinical substrate, with one bacterial morphotype dominating the substrate by day 7 (Fig. 3A).

At month 1, the culturable bacterial community



**Figure 1.** Total abundance of bacteria associated with clinical (paper towel) and naturalistic (organic material) substrates at day 1 and day 7. The \* indicates a significantly (p < 0.001) different result to all others.



**Figure 2.** Non-metric multidimensional scaling figure depicting the bacterial community composition of salamander environments; naturalistic (black) and clinical (grey) environments at day 1 (squares) and day 7 (triangles), stress value = 0.04.



**Figure 3.** Relative abundance of culturable bacteria: **A.** Isolated from naturalistic and clinical substrates on which salamanders were maintained, **B.** Isolated from the skin of salamanders maintained in naturalistic and clinical environments 6 months after the beginning of the study. Different colours/shades represent different bacterial morphotypes, and colour/shade coding is conserved between the two figures.

composition of salamanders on the two different substrates were not significantly different based on total abundance counts ( $F_{1.16}$  = 1.390, p = 0.186; Fig. 4A) or relative abundance data (Morisita–Horn:  $F_{1.16}$  = 3.077, p = 0.055; Euclidean:  $F_{1.16}$ = 1.755, p = 0.129). After six months on the two different substrates, overall community composition of salamander microbiotas were significantly differentiated based on raw abundance data ( $F_{1,16}$  = 3.102, p = 0.021; Fig. 4B) and relative abundance data (Morisita–Horn:  $F_{1.16}$  = 9.779, p = 0.002; Euclidean:  $F_{1.16}$  = 10.030, p = 0.003). Salamanders maintained on the naturalistic substrate had only one dominant morphotype, with two intermediate morphotypes and a number of low abundance morphotypes (Fig. 3B), despite the naturalistic substrate exhibiting a relatively even community composition across bacterial morphotypes (Fig. 3A). Conversely, salamanders maintained on the fluctuating clinical substrate had a number of bacterial morphotypes with intermediate to high relative abundances, despite the substrate shifting from a relatively even representation of morphotypes on day 1, to the dominance of one bacterial morphotype by day 7.

Six months after the start of the study there were

significant differences in the microbiotas associated with salamanders and substrates ( $F_{132}$  = 10.285, r<sup>2</sup> = 0.150, p = 0.001), between the two treatment groups ( $F_{132} = 13.965$ ,  $r^2 =$ 0.203, p = 0.001), and a significant interaction between these two parameters ( $F_{132}$  = 12.482,  $r^2$  = 0.182, p = 0.001; Fig. 5) based on raw abundance counts of bacteria, which was also supported by the Adonis analyses of relative abundance data (p < 0.001 for all parameters and interactions using both)Morisita-Horn and Euclidean distance measures). On the whole, bacterial communities associated with the skin of salamanders maintained on the naturalistic substrate (black circles; Fig. 5) closely resembled the bacterial community associated with the naturalistic substrate (black diamonds; Fig. 5). Bacterial communities associated with the skin of salamanders on the clinical substrate (grey circles; Fig. 5) were similar but slightly differentiated from those of the naturalistic substrate or salamanders maintained on the naturalistic substrate, whereas the bacterial community associated with the substrate in the clinical environment was vastly different from all others (grey diamonds; Fig. 5).





Figure 4. Non-metric multidimensional scaling figure depicting the bacterial community composition of salamanders associated with naturalistic (black) and clinical (grey) environments: A. At month 1, B. At month six (B), stress values = 0.06 and 0.10, respectively



**Figure 5.** Non-metric multidimensional scaling figure depicting the bacterial community composition of salamanders and their environments (data represents samples collected on 'day 7' of sampling at 6 months after the start of the study). Black circles = salamanders in naturalistic environment; grey circles = salamanders in clinical environment; black diamonds = naturalistic substrate; grey diamonds = clinical substrate, stress value = 0.07

Our data show that naturalistic and clinical substrates in salamander enclosures are very different in terms of the culturable bacterial communities developing within them. Naturalistic substrates hold more diverse and stable bacterial communities, while the clinical substrate is much less stable and rapidly becomes dominated by a single morphotype. These findings reflect both the nature of the substrate and the maintenance regimes that they necessitate. The chemically and structurally complex nature of the naturalistic substrate likely allows for the regulation of a temporally stable microbiota, as well as providing a greater variety of environmental niches through variation in particle size and composition. Movement of bacteria through soil is often poor and this, along with highly localised co-evolution with bacteriophages, can lead to the development of a highly heterogeneous distribution of bacterial genotypes over a matter of centimeters (Vos et al., 2009). In addition, there is evidence that bacterial communities with a greater diversity exhibit higher temporal stability (Flores et al., 2014), and therefore such communities are, in effect, self-regulating.

Paper towels rapidly degrade and must be replaced frequently - in this case, weekly. This process resets the environment and therefore the associated bacterial community. This may have prevented the development of more complex microbiotas by interrupting succession and the development of complexity through competition; instead the faster reproducing morphotypes may have been favoured continuously. Moreover, the paper towel offered a more homogenous environment with a smaller range of niches for bacteria to grow, as well as probably also facilitating the movement of bacteria throughout the substrate - especially as when dampened there would be a continuous aqueous environment throughout the towel. These characteristics likely created an environment favouring lower diversity and reduced stability of microbiota.

As well as differing in stability and diversity of bacterial communities, the clinical substrate generated much higher peak abundances of bacteria during its weekly replacement cycle. The dominant bacterial morphotype became several orders of magnitude more abundant than total abundance of bacteria in the naturalistic substrate over the same timescale. Such blooms of bacteria may represent a health hazard for captive salamanders, as they may overwhelm immune responses (Seidel & Gerhardt, 2017). The combination of low diversity and high abundance of dominant bacteria may also allow invasion of pathogens more easily than a complex community. This result indicates the importance of frequent replacement of paper towel substrates.

We also demonstrated that the different substrates used for salamanders influenced the microbiotas of the animals themselves. Loudon et al. (2014) showed that organic matter was important for maintaining a "core" microbial community after moving adult salamanders (Plethodon cinereus) from the wild into captivity. Our data suggest that it is also important for maintaining the microbial community of captive bred animals that have not been exposed to microbiota in their natural habitat. Salamanders maintained on the naturalistic and clinical substrates also hosted significantly differentiated microbiotas. Four bacterial morphotypes occurred at much greater relative abundances on salamanders maintained on the clinical substrate compared to those maintained on the naturalistic substrate, for which only one bacterial morphotype predominated (Figures 3A and 3B). This pattern is the reverse of that found in the actual substrates, and may indicate that the fluctuating environment provided by the clinical substrate promoted diversity by creating temporal niches suited to different species. This situation differs from the substrate itself, as the salamander microbiome was not reset at the time of paper changes. However, despite these environmental fluctuations, salamanders reared on a clinical substrate maintained a broadly similar bacterial community in terms of the morphotypes present, if not the relative abundances of each bacterial type, compared to those reared on a naturalistic substrate (Figure 4). This supports the notion that amphibians regulate their skin microbiota, potentially through the production of anti-microbial peptides (Küng et al., 2014).

Species composition and community dynamics of microbiotas associated with *Salamandra* spp. are of particular interest given the recent emergence of a second lethal *Batrachochytrium* fungus (*B. salamandivorans*) in northern Europe, as a result of which massive population declines in this host species have been observed (Martel et al., 2013; 2014; Spitzen et al., 2013). However, the propensity for symbiotic bacteria of amphibians to mediate this pathogen is currently poorly understood.

In some other amphibian taxa, changes in microbiota caused by different husbandry approaches have correlated with differences in growth, such that conditions that promoted ostensibly more advantageous microbiotas also promoted growth (Michaels, et al., 2014). This is possibly mediated by effects of husbandry on behaviour and environmental stressors, which may have implications for the health and fitness of amphibians. In the present study, however, we detected no effect of substrate on growth rates in salamanders, which suggests that while the substrate may be important for determining patterns in microbiota, it does not translate to other measures of health and fitness in this case. All salamanders grew at rapid rates, on average increasing mass 12-fold in 6 months, suggesting that all the animals were in good general health rather than poor health which in amphibians is often associated with limited growth.

The results presented here are based on morphotypic identification and culturing techniques, which do not allow inclusion of portions of the microbiome that are not culturable under the conditions used, and do not allow specific/strain identification of bacteria. Although molecular techniques are required to more fully characterise microbial communities, the data presented here offer convincing evidence that a given subset of the environmental and host-associated bacterial communities are susceptible to differences in substrate, and it is likely these differences are also seen in the non-culturable portion of the microbiota. Moreover, these data represent only a 6-month window into the effects of substrate on a species that can live for more than 30 years and it is as yet unclear if the differences detected here in skin microbiota might have longer-term implications for hosts not detected in this short period, or if additional effects of substrate on skin microbiota may emerge over a longer period.

We did not measure the accumulation of waste products in substrates. Salamanders produce nitrogenous waste, partially in the form of solid faeces, but also liquid waste containing ammonia. Despite the manual removal of faeces, waste products accumulate in substrates and can eventually become toxic (Seidel & Gerhardt, 2017). While frequent changing of clinical substrates as they degrade may influence environmental and skin microbiome microbial communities, it also avoids accumulation of toxic waste products. Given the relative benefits of both types of enclosure, it is possible that a hybrid approach could be used whereby a small amount of naturalistic substrate is provided in a container within an otherwise clinical enclosure, to act as a bacterial reservoir.

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## Ecology and colour variation of *Oreophryne monticola* (Anura: Microhylidae) with reference to vocalisation and predicted habitat suitability

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**ABSTRACT** - The Lombok cross frog (*Oreophryne monticola*) is endemic to the islands of Bali and Lombok. We describe the colour variation and associated microhabitats of this endangered species, and provide some notes on its call characteristics and predictions of its distribution in Bali and Lombok. We surveyed sites on both islands with similar altitude but slightly different microhabitats. The frogs were more likely to be found in forests dominated by dipterocarp trees, many epiphytic ferns, and ground covered with leaf litter. Possible differences in colour variation and call suggest that there may be cryptic species within *O. monticola* that could be resolved by morphological and molecular study. The predicted distribution suggests nine isolated areas at around 1100 m a.s.l. on both islands that may be home to this frog.

#### INTRODUCTION

The islands of Bali and Lombok are part of the Lesser Sunda region known to be home to a rich biodiversity with several endemic species that are threatened with extinction (Reilly, 2016; Reilly et al., 2019a; Tänzler et al., 2016). A major threat to the fauna is habitat loss and disturbance from infrastructure and tourism since both islands are popular tourist destinations (Masseti, 2009; Simay et al., 2009).

One threatened amphibian species, the Lombok cross frog *Oreophryne monticola* (Boulenger, 1897), is endemic to both islands even though Bali lies to the west of Wallace's line and Lombok to the east. All other species of the genus *Oreophryne* are to the east of the line, suggesting that they are of Australasian rather than Asian origin (Reilly et al., 2019b). The islands are separated from each other by very strong ocean currents so that the two populations of *O. monticola* are isolated (Evans et al., 2003; Reilly et al., 2019b). Such isolation, may give rise to specific variations of perhaps colour, vocalisation and other characters (Kurniati, 2013; Amezquita et al., 2009).

The Lombok cross frogs is categorised as an endangered species (IUCN SSC Amphibian Specialist Group, 2018; Iskandar & Mumpuni, 2004), occupying a land area of less than 5,000 km<sup>2</sup> (Iskandar & Mumpuni, 2004), and restricted to elevations from 1000 to 1400 m a.s.l. (Iskandar, 1998). This small frog, never usually more than 25 mm long, has unusual reproductive habits as like others of the same genus its tadpoles are not free living but remain within the egg from which the froglet emerges.

In this study, we have investigated the frog on both Bali and Lombok with the objective of adding to the knowledge of its ecology, colour variation, and call characteristics. In addition, as a contribution to conservation planning, we have estimated its likely distribution on the two islands.

#### MATERIALS AND METHODS

#### Study sites and surveys

In 2017, single night time surveys were undertaken on Bali (Batu Karu, 1000-1450 m a.s.l.) on 28th January and Lombok (Sembalun, 1000-1480 m a.s.l.) on 3rd February; each study site was approximately 0.5 km<sup>2</sup> (Fig. 1). From 18.00h to midnight at each site, four surveyors used the visual encounter survey (VES) methods (Campbell & Christman, 1982; Pradhan et al., 2014) to search for frogs in forest litter, leaves, bushes, twigs, debris, ferns, and decayed tree trunks. To increase the chances of finding O. monticola, any sound of the frog's call was pursued. The location of each frog was recorded using a GPS Garmin 64s and descriptions made of the habitats and microhabitats occupied. The snout-ventlength (SVL) of each frog was measured using a caliper with 0.01 mm accuracy and frogs' body colour was recorded by photographing the dorsal and ventral surfaces using a DSLR Canon 70D camera. These photographs and descriptions were compared with the existing literature (Iskandar 1998; Iskandar & Mumpuni, 2004; McKay, 2006). All the captured individuals were then released back into the habitat where they had been found.



Figure 1. Map showing *O. monticola* sampling sites on the two islands (above), and the center point of sampled sites with the surrounding land use (below): A) Batu Karu (Bali), B) Sembalun (Lombok)

#### **Call characteristics**

Attempts were made to record frog calls at both sites but only on Lombok were calls successfully recorded. The surveyors got as close as possible to the source of frog calls and then made a recording with a SONY ICD-PX240 using WAV format, with a note of the time of day. Calls were analyzed using Adobe Audition version 3.0 software. The call characteristics are based on variation in several parameters including pulse duration, pulse period, dominant frequency, and bandwidth. For each of these parameters, the coefficient of variation (CV) was determined in order to categorize the calls. To make comparisons between the frog calls between the two islands, we used calls from Bali analyzed during a previous study by Kurniati & Hamidy (2014).

#### Distribution probability analysis

To estimate the likely distribution of *O. monticola*, we used MaxEnt v.3.4.1 (Phillips et al., 2017) species distribution modeling system (Sarker et al., 2019), maximum entropy approach. Several studies have shown that this model can provide meaningful results with as few as three GPS records (Proosdij et al., 2016). We modeled the potential distribution based on the GPS records of four individuals, two individuals from each island. The analysis was supported with 19 environmental variables provided by WorldClim v2.0

(http://worldclim.org/version2) datasets containing average monthly climate data, temperature, and precipitation (Fick & Hijmans, 2017), and 27 arc-second digital elevation modeling data points (DEM) from Badan Informasi Geospasial (http:// tides.big.go.id/DEMNAS). The spatial data were masked to the shapefile from (http://tanahair.indonesia.go.id) using ArcGIS (ArcGIS v10.3; ESRI, Redlands, CA). The autoco-linearity was not analyzed, because we were limited to predicting the probability of current range distribution without a clear understanding of ecological factors and the natural history of this species (Braunisch et al., 2013). The Jackknife method was used to asses best predictor variables for the distribution. We used 25 % of data for test and the remainders for training following Hu et al. (2016). Default regularisation was performed following Phillips et al. (2006). Four replicates were generated by subsample replication with a maximum of 1000 iterations for each replication. We present the data under the receiver operating characteristic (ROC) curve (by the AUC value). The closer the AUC value is to 1.0 the higher is the probability of the model predicting the distribution (Phillips & Dudik, 2008).



Figure 2. The frequency of O. monticola encountered in a variety of terrestrial and arboreal microhabitats

#### RESULTS

#### Microhabitat characteristics of O. monticola

On Bali, we found six frogs (one female and five males) located close to each other at an elevation of 1400 m to 1410 m a.s.l.. The female was found in terrestrial habitat among leaf litter, which was 30-40 mm deep around the buttress roots of a dipterocarp tree (Fig. 2). In contrast, all the males were found 0.8 to 3 meters above the ground either among the rhizomes and fronds of nest ferns (*Asplenium* sp.), which grow as epiphytes on dipterocarp trees (Fig. 3A), or inside the holes in the midrib of decaying tree ferns (*Cyathea* sp.). Overall, the site sampled on Bali can be categorised as tropical dry forest with steeply sloping open areas ( $\pm 20^{\circ}$ ) with or without leaf litter but with ferns in several locations, with trees about 5-7 meters apart but no water sources nearby. We suspect that the breeding sites of *O. monticola* were on the rhizome of the epiphytic nest ferns (*Asplenium* sp.).

On Lombok, 15 individuals (nine males and six females) were found at elevations ranging from 1447 m to 1475 m a.s.l.. Individuals were gathered in groups of 2 to 6, each individual about 2-8 meters apart, and each group was more than 100 m from others. All individuals, whether male or female, were found in a range of microhabitats which were either terrestrial, hiding amidst leaf litter at the base of tree trunks, or arboreal on nest ferns (1.5 m above the ground), decayed nest fern, or above the leaves of Araceae plants (Fig. 3B). The site sampled in Lombok was a tropical dry forest, more gently sloping than in Bali. It was dominated by dipterocarp trees and several gymnosperms with a leaf litter thickness of 50-60 mm; a greater depth than Bali. The environment was more humid and dense than on Bali with diverse vegetation such as perennial plant, shrubs, nest fern, abundant wet mosses. There were masses of O. monticola eggs embedded in a longitudinal strand of mucous observed on three substrates (nest fern, tree trunk, leaf litter) where they were guarded by the parents. On the nest fern, the mucous resembled water droplets hanging 20-30 cm from the fronds.



**Figure 3.** Preferred microhabitats of *O. monticola*: **A.** Batu Karu (Bali), dipterocarp tree with attached nest fern (*Asplenium* sp.), **B.** Sembalun (Lombok) a more humid environment with more dense and diverse vegetation

#### Body colour variation of O. monticola

The species is highly variable in pattern and coloration (Fig. 4). Dorsally, individuals from Bali were dark or light brown or redbrown (Fig. 4 A to F); the light brown frog had a darker broad mid-dorsal marking extending to the vent (Fig. 4B). Likewise from Lombok, there was great colour variation (Fig. 4 G to U). Some striking coloration was observed including uniformly brick red with black-spots (Fig. 4H); light to dark brown with striking nodules dorsally (Fig. 4 I & N); dorsum predominant grey with thin longitudinal mid-dorsal line from snout tip extending to the back of the femur (Fig. 4J); dorsum uniformly brackish brown (Fig. 4K); and dominant yellow mottled with black spots dorsally with yellow longitudinal mid-dorsal line, a dense light yellow from snout tip to interorbital on the head dorsally with 2 bold transversal yellow bars across the tympanum (Fig. 4 L & R). The venter colour of the Bali and Lombok frogs are similarly highly diverse, varying from dark brown and orange with white spot on the throat and belly, predominantly brick orange with light yellow on part of the belly, to uniformly black.



**Figure 4.** Dorsal colour variation in *O. monticola* from Bali and Lombok. Frogs are shown slightly smaller than life size (x0.9)

#### Call characteristics of O. monticola

The only call type we recorded on Lombok was a simple pulse repetition which covered a narrow spectral band with a dominant frequency in the range of 2147 – 2744 Hz giving a 597 Hz bandwidth (Fig. 5) (Table 1). The characteristics of the calls are static indicated by dominant frequency (CV: 7 %), maximum dominant frequency (CV: 3 %), minimum dominant frequency (CV: 3 %), and bandwidth (CV: 11 %) (Table 1). The call of *O. monticola*, recorded previously on Bali by Kurniati & Hamidy (2014), also covered a narrow spectral band with a dominant frequency of approximately 3000 – 3937 Hz and 937 Hz bandwidth (Table 1). However, the calls are more dynamic, with quite variable pulse period (CV: 26 %) and duration (CV: 47 %) (Kurniati & Hamidy, 2014) (Table 1).



Figure 5. Spectrogram (above) and oscillogram (below) of a call of *O. monticola* from Sembalun (Lombok) based on our field recording

**Table 1.** Call parameters for *O. monticola* on Bali (from Kurniati &Hamidy, 2014) and on Lombok

Call parameters	Bali	Lombok
Pulse duration	1.91 ms (CV*: 47 %)	-
Pulse period	7.49 ms (CV: 26 %)	-
Dominant frequency	-	2476 Hz (CV: 7 %)
Maximum dominant frequency	3937 Hz (-)	2744 Hz (CV: 3 %)
Minimum dominant frequency	3000 Hz (-)	2147 Hz (CV: 3 %)
Bandwidth	937 Hz (-)	597 Hz (CV: 11 %)

\*CV = coefficient of variation

## Distribution probability and elevational distribution of *O. monticola*

The average test AUC score for four replicate runs was 0.965, with a standard deviation of 0.0001. Our jackknife



Figure 6. A) Map showing the probability of the presence of *O. monticola* on Bali (left) and Lombok (right) by maximum entropy approach (purple dots = study sites), B) elevational distribution of *O. monticola* encountered in Bali (left) and Lombok (right) (white stars = study locations)

variable contribution test shows that among all the variables used for the modeling, the maximum temperature of the warmest month (28.9 %) contributed most significantly, followed by isothermality (mean diurnal range/temperature annual range) (25.7 %), and elevation (22.7 %). Our model predicted many isolated and fragmented environmental niche envelopes especially on Bali (Fig. 6A), at somewhat lower elevations than we encountered the species (Fig. 6B).

#### DISCUSSION

On both Bali and Lombok, O. monticola was found at a similar altitude but in slightly different microhabitats, more likely to be found in a forest dominated with dipterocarp trees, many epiphytic ferns, and ground covered with leaf litter. Compared to our findings on Bali, the frogs on Lombok were more evenly distributed between terrestrial and arboreal habitats. Ferns have previously been shown to create small-scale temperature and moisture refuges that buffer sensitive ectotherms, protecting not only the frogs but also many of their prey species from excessive heat and desiccation (Scheffers, 2014; Ellwood & Foster, 2004; Beaulieu et al., 2010). Ferns also offer suitable breeding habitat. We also found the eggs of O. monticola placed on nest ferns (Asplenium sp.) suggesting that they may present a more suitable microhabitat for arboreal frog breeding than other epiphytic plants (Inger, 1954). Other congeneric species such as O. furu (Gunther et al., 2009), O. sibilans, O.

unicolor, and O. clamata (Gunther, 2003), also use ferns as breeding sites. Leaf litter is also one of the most important microhabitats for terrestrial frogs by providing refuge against predators, great densities of arthropod prey, as well as adequate conditions for egg-laying (Stoler & Rick, 2011; Sluys et al., 2007). Oreophryne monticola has been categorised as a terrestrial breeder (IUCN SSC Amphibian Specialist Group, 2018), however, we doubt that leaf litter is the main microhabitat for O. monticola. Although leaf litter can be sufficiently humid, O. monticola still uses nest ferns as breeding sites, indicating arboreal adaptations. In other amphibians, placing the eggs in between the canopy and ground vegetation is an attempt to avoid both arboreal and ground-dwelling predators (Stewart, 1985). These findings suggest O. monticola is a semi-arboreal frog.

In our survey, we found the species at about only 1400 m a.s.l. (Figure 6B). A previous rainy season survey in Lombok (Rinjani mountain) recorded seven *O. monticola* at an altitude of 1250 m a.s.l. (Septian, 2016). This species was previously found as low as 1000 m a.s.l. (Iskandar, 1998), and we heard the calls of this frog at 1000 m a.s.l. in sampled sites in Lombok. This indicates that if suitable forest exists at lower elevations then the species can persist there.

The call recordings of *O. monticola* from the two islands showed differences in dominant frequency, although this may be significantly affected by variables such as altitude, humidity, and temperature (Goutte, 2013). There was also a difference in bandwidth which may result from differences in body size of the calling frogs (Kime, 2004). Call characteristics can be defined by the coefficient of variation (CV), where the static type has a CV of less than 12 % while dynamic type has a CV of greater than 12 % (Gerhardt, 1991). Based on the CV value, male individuals from Lombok may just be engaging with other males, while males in Bali were probably calling to attract females to make a mate choice (advertisement calls). These differences add urgency for further call recordings to compare these populations to ensure the vocalisations are comparable given the same behavioral context.

The body colour variations and vocalisations of *O.* monticola suggest the possible presence of cryptic species. The populations of the two islands have most likely be isolated for a long time since the two islands have been separated for ~12.7-24.4 million years, according to the biogeographical history of Limnonectes in Lesser Sunda isles (Reilly et al., 2019b). Further comprehensive assessment of morphological and molecular characteristics may help to resolve this issue.

According to our spatial analysis prediction, there are 9 isolated areas at around 1100 m a.s.l. on both islands that may be home to *O. monticola* provided that they have not already been extirpated since fragmented and isolated populations are prone to terminal decline. Further studies are required to confirm the predicted distribution and the results of such study will improve the understanding of this species' conservation status and what steps should be taken to secure its future.

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## First report of reproduction in captivity of the Central American bushmaster (*Lachesis stenophrys*) in a European zoo

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The Central American bushmaster (*Lachesis stenophrys*) (Fig.1) is a large species of pit viper. The males are a little longer than the females, averaging of 2-2.10 m and 1.9-2.05 m respectively (Solórzano, 2004). The species lives along the Caribbean versant of Nicaragua to western and central Panama (Campbell & Lamar, 2004), and in Costa Rica is found in tropical wet and subtropical rainforest on the Caribbean versant (Corrales et al., 2014) where the rainfall is very high (3500-6000 mm annual range). Although it can be found at altitudes of 100-700 m its preferences are thought to be 200-400 m (Ripa, 2002).

Lachesis spp. are the only pit vipers on the American continent that lay eggs instead of giving birth to live young (Campbell & Lamar, 2004; Solórzano, 2004). The eggs are deposited in subterranean cavities and the shelters of other animals, with clutches of up to 18 eggs (typically 9 - 13), hatching in 75 – 90 days (Solórzano, 2004). In captivity, females reach sexual maturity at approximately 1.6 m in total length and at about 3.5-5 years of age (Ripa, 2002). There have been several publications describing the husbandry and breeding of *L. stenophrys* in captivity (Ripa, 1994; Ripa 2002; Hohmeister, 2004; Chacón & Valverde, 2004; Corrales, et al., 2014) but we give here the first description of captive breeding in a European zoo.

#### MATERIALS AND METHODS

#### **Breeding Pair**

The breeding pair in this study arrived at Faunia zoo, from Costa Rica, in 2012, as members of a future 1:2 (males/ females) breeding project. Their addition to the collection was a great opportunity for us to have a new blood line. The individuals were healthy and growing slowly, the male reached a weight of 4.5 kg the male and the female 3.7 kg immediately before the breeding started in 2019.

#### Husbandry

The breeding pair were kept separately from each other in two identical glass terraria measuring  $200 \times 65 \times 85$  cm (L x W x H). Ventilation was provided by a longitudinal metallic



Figure 1. L. stenophrys, Parque Nacional Braulio Carrillo, Costa Rica

mesh on the top and two circular meshes either side of the terraria. The female was kept behind the scenes, while the male was exhibited to the public. The terraria were equipped mainly with big tree roots, and live plants (*Epipremnum* sp. and *Monstera deliciosa*) offering hiding places. The female's terrarium also had a restraint area (45 cm x 65 cm x 85 cm) which provides a dark and dry place to rest, destined to become the future nesting site. The substrate consisted of coconut fiber, mulch and a layer of 1-2 cm of dry leaves.

Ambient lighting was provided by two fluorescent tubes (58 W) and for basking one halogen lamp (50 W) during the spring-summer and a GU10 LED 6500 k during the mid-autumn and winter. The basking lights were on an approximately 12:12 h on/off cycle, however the ambient lights (fluorescent tubes) were timed independently, turning on and off simulating natural cloud cover and sunset. Ambient temperatures fluctuated between 21-24 °C during the autumn/winter and 27- 29 °C with a maximum temperature up to 30 °C directly beneath the basking lamp in spring/summer. The temperature and humidity were measured by a TFA 30.5013 digital thermo-hygrometer.

In order to trigger sexual behaviour, we simulated the fluctuation in the environmental conditions described by Corrales et al. (2014). The ambient humidity was held at

around 50-60 % during the coldest months but in spring a fogger was installed that increased relative humidity to 90-95 % and, at the same time, the temperature was increased by 5-6 °C. The fogger used was an Orieme Airsano 3L, located above the terrarium, modified with two large plastic tubes which conducted the moist air inside. In addition, a daily misting was made with a 5 L hand sprayer for the care of the plants but the animals themselves were only sprayed when shedding. An IR nocturnal camera (WiFi Cube Tenda C5S HD 30fps 120 degrees) was installed to record any mating behavior during the night.

#### Diet

At a frequency of about every 20 days (Ripa, 1994; 2002), the snakes were fed with 1 or 2 small rats (120-200 g, to avoid regurgitation) that were either defrosted or had been freshly killed. However, when the female was younger (about 1 m length) it refused anything other than chicks.

#### Veterinary monitoring during the gestation period

During gestation the embryos were monitored by both X-ray and ultrasound. The X-ray equipment used was a portable Uni -Travel 70/100, power rating 70 mA-99KV. The snake was held 40 cm from the machine giving 50 mA / 70 KV y 8 mA/s. The ultrasound machine was an Edge II Sonosite system, with a linear transducer that works from 6 to 13 MHz.

#### Incubation of eggs

The eggs were placed in an incubator ( $35 \times 45 \times 60$  cm) adapted from one that had been in a wine bar. It had heating as well as an effective cooling system, which is essential during the hot conditions of the Madrid summer time. Within the incubator three eggs were held in a plastic box ( $28 \times 17 \times 8$  cm) half-filled with vermiculite that had been moistened with water in a 1:0.6 (volume/weight ratio). The eggs were maintained at 26-26.5 °C and the humidity ranged between 75-85 %. Eggs were not turned and were partially embedded in the vermiculite (Fig. 2). In addition, as the eggs shells were somewhat decalcified, to help reduce dehydration we dusted the whole eggs with calcium carbonate powder, a procedure we use frequently when incubating the decalcified eggs of other species.



**Figure 2.** Eggs of *L. stenophrys* being incubated in vermiculite and dusted with a calcium carbonate to reduce dehydration

#### OBSERVATIONS

#### Mating behaviour and copulation

Mating started when the ambient humidity and temperature were increased in mid-February. A change in behaviour was noticed in the male, who was abnormally nervous, moving around the terrarium and flicking his tongue morning and evening. Consequently, on 20 February we decided to put him together with the female. The male was observed approaching the female following her for some hours, rubbing his ventral scales over her body in a fierce way, as previously described Corrales et al. (2014) and Ripa (1994). The first copulation was recorded on the same day inside the restraint area of the female's terrarium. Early in the morning and for the next three days the pair was seen resting close to each other. The male and the female were placed together twice more (13 March and 3 April) to ensure successful mating. On 13 March we observed the same behavioural pattern seen before, but no copulation was recorded and on the third occasion neither snake appeared interested in the other.

#### **Gestation period**

During gestation, the female was as usual resting and only moved when needing to drink, feed, defecate or shed. Four years previously, a breeding female had died due to egg retention (dystocia), so the gestation period this time was highly controlled with reference to previous published studies (Ripa, 2002; Hohmeister, 2004; Corrales et al., 2014) and the opinions of other specialists. The female was fed every 20-25 days during pregnancy and only 5-6 days before oviposition did she refuse food. Clues indicating gestation, or that forthcoming oviposition was imminent, included the female looking for the best place to make a nest and gathering leaves.

#### Veterinary monitoring

After 100-115 days of gestation, X-rays and ultrasound scans were used to check for signs of dystocia. X-rays were taken of the distal third of the animal, which showed the presence of four masses, presumably eggs that were 4.2-4.8 cm by 3.1-3.5 cm oval shapes with perfectly defined profiles (Fig. 3). Although they had relatively high radio-opacity they are overshadowed by the more radio-opaque intestine. The presence of granular radio-dense material compatible with food in transit was seen in the intestine. The egg masses alternated with each other in order and had a smooth surface but not always perfectly regular. Ultrasound scanning of the distal third of the animal, as expected, showed the presence of several eggs.

#### Egg laying and incubation

On 26 June (126 days since 1st copulation) a necklace-like group of five tiny follicles (Fig. 4) were seen on the substrate suggesting pre-ovulatory follicular stasis in one of the ovaries. On both the 3th and 4th of July, normal sized but infertile egg was found. Then on 11th July (141 days since 1st copulation) the female was found to be guarding a clutch of five eggs (Fig. 5). A trans-illumination procedure with a



**Figure 3.** X-ray examination of the distal third of the female *L. stenophrys,* with elipses drawn to show the position of the eggs against the relatively radio-opaque intestine

mobile flashlight revealed a small group of capillaries in two eggs suggesting they were fertile, the other three of poor appearance. All eggs were white to yellowish in colour, and had a soft, but irregular and decalcified shell. For incubation, three of the eggs were removed, coated in calcium carbonate powder, and then carefully placed in the incubator in the plastic box with vermiculite. One week later one egg began to rot (148 days since 1st copulation) and then after 184 days (23rd August) one of the two eggs identified as fertile was found to have become rotten, leaving only a single egg to be incubated. Unexpectedly, the female apparently laid two more eggs on 9th September (201 days after 1st copulation) but these were discarded because they were dehydrated, probably due to long retention (Fig. 6).



Figure 4. A necklace-like group of very small follicles ejected from the female *L. stenophrys*, 126 days after first recorded copulation



Figure 5. The female *L. stenophrys* protecting a newly laid clutch of five eggs



**Figure 6.** Dead embryo of *L. stenophrys*, note the dehydration of the vitellus, in an eggs that was retained for 60 days beyond the time that other successfully fertilised eggs were laid

After 78 days of incubation, on 26th September (218 days after 1st copulation), hatching from the single remaining egg began. Longish slits were made at the head end of the egg (Fig. 7), the snout of the hatching neonate protruded for some hours, and hatching was completed during the night. The neonate weighted 45g and was 41 cm long.



**Figure 7.** At early hatching stage, only the snout of the juvenile *L*. *stenophrys* is protruding

#### CONCLUSIONS

Ex situ breeding of *L. stenophrys* proved to be a difficult task as evidenced by the low fertilisation rate, long retention of some eggs, and apparently some pre-ovulatory follicular stasis associated with one ovary. At least in the case of pre-ovulatory follicular stasis the cause often relates to problems with husbandry (DeNardo, 2006). For future breeding attempts, veterinary monitoring of the process, especially between copulation and egg laying, is recommended as it enabled us to confirm that the female was pregnant. Consequently, we were able to decide when to take husbandry decisions such as to stop feeding, provide a bigger nest, and leave her undisturbed. We could also 'estimate' the egg laying date and if there been signs of distocia then surgery could have been undertaken while the female was still healthy. The high proportion of infertile eggs observed on this occasion could be related to this being the first breeding attempt of this female or low fertility of the male. The final number of eggs (9) laid is well within normal limits for this species (Ripa, 2002; Corrales et al., 2014) but the single neonate obtained had a weight and length at the lower end of the normal range (Hohmeister, 2004; Corrales et al., 2014).

The timing of mating may fluctuate between February to April (Corrales et al., 2014) and the snakes are best paired at some time in this period. Mating can be stimulated by increasing ambient humidity and temperature and the timing of pairing made more precise by watching out for changes in male behaviour. Normally, eggs are deposited from June to August and birthing takes place from August to October. Consequently, the typical period from copulation to hatching takes about 180 days. In our case the single neonate that emerged did so 218 days after first copulation, somewhat later than might be expected.

The humidity and temperature of the incubation substrate used in this study would seem to be the best recommendation for this species (Ripa, 2002; Corrales et al., 2014). Covering decalcified eggs with a thin calcium layer could help to protect them against natural dehydration and opportunistic pathogens, but further tests with a larger sample size are required before any definite recommendations can be made.

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## Mass displacement of Korean clawed salamanders (Onychodactylus koreanus) and the threat of road-kill

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**ABSTRACT** - Little is known about the ecology of the Korean clawed salamander (*Onychodactylus koreanus*) but populations are declining due to human activities. We surveyed a mountainside road in the Republic of Korea and recorded the sex and life history stage of the salamanders crossing the road. Our results present the first evidence of mass movement in this species. We also show that road-kill is greater than estimated in previous studies. The males observed had fleshy hind-limb extensions and all females except one were gravid, indicating they were in reproductive condition and that mass movement of this species is probably linked to seasonal conditions and reproduction. Consequently, we argue that road-kill may have a large impact on this species by eliminating reproductive adults. We call for road-kill mitigation measures on mountainside roads and further research into the migration patterns of this species.

#### INTRODUCTION

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Amphibians are particularly prone to being killed on roads because they are slow-moving and many species migrate long distances seasonally to breed (Mazerolle, 2004; Brzeziński et al., 2012). Consequently, roads cutting through habitats can become significant threats to migrating populations (Jackson, 1996; Marsh et al., 2005).

In several amphibian species, mass movements associated with reproduction have been documented (Bovbjerg & Bovbjerg, 1964; Holland et al., 1990; Woolbright & Martin, 2014). Mass movements are associated with annual migration of adults to and from breeding sites and the dispersal of juveniles (Bovbjerg & Bovbjerg, 1964; Woolbright & Martin, 2014). When amphibians cross roads en masse, road traffic may kill a large number of individuals which may impact populations through genetic isolation, unequal sex-ratio, and result in extirpation (Puky, 2005). However, despite such negative consequences, the effects of roads have not been evaluated in detail for many amphibian species (van der Ree et al., 2015). This is partly due to the lack of background ecological information to make assessments as is the case with the Korean clawed salamander (Onychodactylus koreanus). This species is endemic to the Korean Peninsula and has a patchy distribution along the main mountain ranges (Poyarkov et al., 2012; Lee & Park, 2016). This pattern of patchy distribution is due to the species being lungless, requiring cool and humid forests and unpolluted, low-temperature mountain creeks to survive and breed (Lee et al., 2012; Lee & Park, 2016).

Due to anthropogenic causes, populations of *O. koreanus* are already shrinking (Suk et al., 2017; Maslova et al., 2018) and the risk of population decline is increased as subpopulations are already likely to be isolated. However, the effects of known threats on populations have not been properly evaluated

and other potential threats are yet to be investigated (Hong, 2017). Herein, we report the first evidence of mass movement and associated high road-kill of *O. koreanus*.

#### MATERIALS AND METHODS

#### **Field observations**

Observations were made by driving a car along a 2.6 km stretch of a two-lane mountainside road in Wangsan-ri, Gangneung, Republic of Korea (37.6357°N, 128.7782°E). The altitude of the roads ranged between 532 m a.s.l. at the first point of observation to 418 m a.s.l. northward to the last point of observation (Fig. 1). Alongside this road, and separated from it by a wall of granite boulders approximately two meters high, was a closed maintenance road. To the east, the roads were flanked by deciduous and conifer forests and to the west by a creek. The distance from the forest edge to the creek was approximately 150 m (Fig. 2).

We collected data during the nights of 11 and 12 October 2019. The air temperature was 12 °C on both nights and the weather was windy with light drizzle. We drove the car slowly (< 12 km/h) on the two-lane road and searched visually for salamanders by their movement and shape against the headlights. We recorded the sex and life history stage of all the identifiable road-kills and any live individuals, and photographed each of them for future reference. Detailed ecological information such as snout-to-vent length, accurate ground temperature, and humidity were not recorded. For the live individuals, we moved them across the two-lane road in the direction of the creek where they were heading. For all but seven individuals, we uploaded pictures to a citizen science network iNaturalist (GBIF Occurrence Download https://doi.org/10.15468/dl.kavt2n). Statistical analysis



**Figure 1.** The stretch of two-lane road in Wangsan-ri, Gangneung, Republic of Korea, where mass movement of *O. koreanus* was observed on 11 and 12 October 2019. Map was generated using Google Earth Pro (Google Inc., California, USA), with data credits to SIO, NOAA, U.S. Navy, NGA, GEBCC. Image Landsat/Copernicus.



**Figure 2.** Structure of the roads where the observations of *O. koreanus* were made, seen from above **A.** and in cross section **B.**, the yellow arrows denote the general movement direction of observed salamanders

We analyzed a total of 43 observations of adult salamanders to test for sex ratio imbalance and for difference in road-kill between sexes in moving salamanders. As the data were not normally distributed our analysis was confined to a chi-square test (RStudio, RStudio Inc.; Boston, USA). We excluded five juveniles and individuals of unknown sex from the analysis.

#### RESULTS

Over two nights, we observed a total of 48 individual *O. koreanus* on the road (25 males, 18 females, two juveniles, and three damaged specimens of indeterminate sex; Fig. 3). On 11 October 2019 between 00:13 h and 00:46 h, we observed 15 males, 12 females, two juveniles and two individuals of indeterminate sex on the two-lane road. On 12 October between 20:20 h and 21:10 h, we observed 10 males, 6 females and one specimen of indeterminate sex on the same stretch of two-lane road. The total survey time over the two nights was about 85 minutes. Sex ratio was not significantly different within the migrating *O. koreanus* ( $\chi^2$  = 1.13, df = 1, p = 0.285). All females except one were gravid (n = 17) and contained well developed ova in their bodies (Fig. 3).

Among the 48 individuals observed, 18 were road-kills (three females, 12 males, three indeterminate) and 30 individuals were found alive (15 females, 13 males, two juveniles). Thus, the total road mortality rate over two nights was 37.5 %. Road-kill was significantly higher in males (25 %) than in females (6.25 %;  $\chi^2$  = 5.40, df = 1, p = 0.020). **DISCUSSION** 



**Figure 3.** Some of the *O. koreanus* individuals observed on 11 and 12 October 2019 in Wangsan-ri, Gangneung, Republic of Korea-**A.** Two *O. koreanus* found sitting on the middle of the two-lane road (white arrows), **B.** Gravid female killed by a car, with developing eggs visible (yellow arrows), **C.** Gravid female found alive on the road with eggs visible through the transparent skin

Our observations are the first evidence of mass movement in *O. koreanus*, a salamander species endemic to the Korean peninsula. We also highlight the risk of road-kill associated with this event. We found that *O. koreanus* were coming down the mountain slope and crossing the road westward, towards the creek. In this process, the salamanders were forced to cross a two-lane road. As a result, many individuals were victims of road-kill. For safety reasons, the traffic on the road during our surveys prevented us from a thorough examination of the opposite lane (uphill / southward direction). It is likely that the salamanders we observed were not the only ones moving during those two nights and thus our figures for salamanders on the road and mortality are probably underestimated.

During a three year study of ten Korean national parks (Song et al., 2009), a total of four *O. koreanus* road-kills were recorded among 24 stretches of roads each 188.9 km long. The authors considered that road-kill risk of *O. koreanus* was low. In another study of vertebrate road-kill, 14 *O. koreanus* were recorded among 368 amphibian road-kills over five years on a 18 km stretch of road in Odaesan National Park, Gangwon province (Min et al., 2012). This area is in the same general region as our study site where we recorded a significantly higher number of *O. koreanus* road-kill on a significantly shorter distance and time span. Consequently, our results show that the road mortality of the species can be greater than previously estimated during mass movement.

We could not identify the causes of this mass movement. However, the area was under the indirect effects of typhoon Hagibis during the time of observation (SBS News, 2019). Rainfall brought on by the typhoon could be one of the causes of this event (Gleeson et al., 2018). Another possible reason for this mass movement is reproduction, which is known as a major factor of amphibian migration (Semlitsch, 2008). Although the known breeding season of *O. koreanus* is between April and July, the species may breed once or twice a year depending on the region and populations (Lee et al., 2008; Lee & Park, 2016). Therefore, the individuals observed may have been migrating to the creek to breed since most females were gravid and the males still retained fleshy hind leg extensions (Park, 2005; Lee & Park, 2016).

Amphibians are usually active after sunset and night time when traffic volumes on rural mountainside roads are unlikely to be high (Gibbs & Shriver, 2005). However, the particular road in this study is the only one leading to a highland plateau, a popular area where many people visit to photograph stars and night sky, giving higher than normal traffic volumes. In such situations where roads cutting through habitats have high traffic volumes, road-kill can have a significant impact on migrating populations by eliminating large numbers of individuals in a short time. Considering the patchy distribution and occurrence of mass movements in the species, we argue that the effects of road mortality present an additional potential threat to the species.

Habitat modifications in general and edge effects associated with road constructions have clear detrimental effects on amphibian populations (Jochimsen et al., 2004; Marsh & Beck, 2004; Rytwinski & Fahrig, 2015; Bae et al., 2019). In our observations, the roads themselves, the wall of granite boulders and the maintenance road all present obstacles for migrating salamanders and expose them to greater risk of mortality due to desiccation and predation. Despite this, we could not find any devices or installations on this road to mitigate road-kill.

In conclusion, we suggest that road mortality is an additional threat to *O. koreanus*. However, detailed information on movement patterns is needed to properly evaluate the extent of this threat. Also, further investigations on the frequency and duration of mass movements are needed to better understand the associated threats. Meanwhile, we call for mitigation measures to decrease the risk of road mortality.

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## Sea turtles in Lake Bardawil, Egypt - size distribution and population structure

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**ABSTRACT** - We investigated the size distribution, sex ratio, and proportion of sexually mature green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) turtles in Lake Bardawil, a large coastal lagoon. During the study 30 green turtles (8 males, 4 females, and 18 juveniles / sub-adults) and 14 loggerheads (1 male, 8 females, and 5 sub-adults) were captured. Forty percent of the green and 64 % of loggerhead turtles were believed to be sexually mature. The green turtles had a mean curved carapace length of 65.23 cm (15 – 100 cm range) and the loggerhead turtles 68.79 cm but with a much narrow range (60- 80 cm) reflecting the absence of juveniles. This study provides evidence that Lake Bardawil is an important feeding and development area for green turtles and feeding area for loggerhead turtles and expands our knowledge of such important sites in the Mediterranean basin.

#### INTRODUCTION

ake Bardawil of North Sinai, Egypt has recently been Lrecognised as being a major feeding ground for sea turtles in the Mediterranean Sea (Nada et al., 2013; Rabia & Attum, 2015; Bradshaw et al., 2017). It is known that the majority of post-nesting green turtles (Chelonia mydas) from Cyprus originate from the North Sinai feeding grounds and that these females have high fidelity to their feeding site (Bradshaw et al., 2017). Two man-made channels (Boghaz 1 and 2) in Lake Bardawil (Fig. 1) have lessened the Lake's environmental severity by reducing the salinity. This has probably increased the food supply and may have created a new foraging ground for green (Bradshaw et al., 2017) and, presumably also, loggerhead turtles (Caretta caretta). However, there is concern regarding the conservation of sea turtles in North Sinai, given the high rates of sea turtle mortality (Nada et al., 2013; Rabia & Attum, 2015, 2018).

Practically nothing is known regarding the population or size structure of sea turtles in the Lake Bardawil. The sand bar that separates it from the Mediterranean Sea is a nationally important nesting site for both green and loggerhead turtles (Rabia & Attum, 2015) and in the Lake itself, green and loggerhead turtles are the largest herbivore and consumer respectively. They may thus have an essential ecological and trophic role as do turtles in other coastal feeding grounds (Moran & Bjorndal, 2005, 2007). Knowledge of population structure is vital to determine the status of sea turtle populations (Heppell et al., 2003), hence the objective of this study was to determine the size distribution, sex ratio, and proportion of sexually mature green and loggerhead turtles in Lake Bardawil.



**Figure 1.** Map of Lake Bardawil, North Sinai, Egypt. The black circles represent locations of sea turtle capture. Boghaz 1 and 2 are manmade channels and Boghaz Zaranik is the natural seasonally open inlet.

#### **METHODS**

#### Study site

Lake Bardawil, a coastal lagoon, is located about 35 km to the west of El Arish city and covers an area of 600 km<sup>2</sup> (length 90 km, average width of 10 km, Anufriieva et al., 2018). There are extensive sea grass beds in the Lake that act as a nursery and spawning ground for several commercially important fish species (Mehanna & Hegazi, 2013). The Lake is substantially separated from the Mediterranean Sea by a

long, narrow sand bar (300 m – 2000 m wide) but connects with it by a small, natural and seasonally open inlet (Boghaz Zaranik) and two man-made channels (Boghaz 1 and 2) that allow the exchange of water and organism (Fig. 1). The minimum widths of Boghaz Zaranik, Boghaz 1, and Boghaz 2 are roughly 80 m, 215 m, and 345 m respectively. The water in the Lake differs from the coastal Mediterranean Sea in being shallower (mean depth 1.2 m, maximum depth 6.5 m), warmer and with much higher salinity (Krumgalz et al., 1981; Khalil et al., 2013; 2016; Anufriieva et al., 2018). Vegetated coastal dunes and salt plains are the dominant mainland habitat surrounding the Lake and the eastern corner is within the Zaranik Protected Area (ZPA).

#### Survey method

In 2019, from 08.30h to 16.00h for 68 days (29th April to 23rd December), we surveyed for turtles in an area of the Lake where previously turtle observations had been most numerous (Rabia, unpublished data). We travelled by boat to our general study area and then moved in a 0.5 km radius around the different survey points. If a turtle was observed, it was encircled with small trammel nets and then placed in the boat for examination. We identified the species, noted its gender, measured the curved carapace length (CCL), and recorded water depth in 0.5 m increments (Bolten, 1999). The turtles were sexed by reference to the longer tails of males (Casale et al., 2018).

We also estimated whether individuals were sexually mature although the growth rate and age of sexual maturity of sea turtles is variable and thus there is some ambiguity. When a medium sized turtle is assessed it could be a large sub-adult or a small sexually mature adult (Casale et al., 2018). We used 78 cm CCL as the threshold for sexual maturity of green turtles (Broderick & Godley, 1996), and 65 cm for female loggerheads based on data from a previous study that reported the minimum size of nesting females (Broderick & Godley, 1996). We categorised the frequency of CCL length of each species in 10 cm increments to allow for comparison with other data sets.

Turtles were tagged on their left front flipper with uniquely coded metal tags (National Band and Tag Company, Newport, KY, USA). Tagging was undertaken as part of the routine work of the Protected Area, of which the first author is manager, and according to current best practice (Balazs, 1999).

#### RESULTS

Turtles were captured on 19 of the 68 surveys days and all were captured in depths ranging from 0.5 – 3 m. There were some days when turtles were almost captured but then escaped, especially at the beginning of the study, and we found it more difficult to detect turtles on cloudy days because of the lower contrast between the turtles and lake bottom. We captured 30 green turtles (8 adult males, 4 adult females, and 18 juveniles and sub-adults) giving an adult sex ratio of 2:1 M:F and 14 loggerhead turtles (1 adult male, 8 adult females, and 5 sub-adult turtles) giving an adult sex ratio of 1:8 M:F. The distribution of curved carapace length (CCL) between the green and loggerhead turtles differed somewhat (Fig. 2). The mean ( $\pm$  SD) minimum length for all green turtles was 65.23  $\pm$  22.85 cm with a range of 15 – 100 cm; for adult females 88.00  $\pm$  9.09 cm, 80-100 cm; for adult males 84.38  $\pm$ 4.17 cm, 80 – 90 cm; and, for juveniles and sub-adults 51.67  $\pm$  19.49 cm with a range of 15 – 77 cm. The mean minimum length for all loggerhead turtles was 68.79  $\pm$  7.00 cm, 60-80 cm); for the for sub-adults 61.09  $\pm$  2.24 cm, 60 – 65 cm; for adult females 72.88  $\pm$  4.52 cm, 70 - 80 cm; and for the single male 75 cm.

#### **Green turtles**



Figure 2. Curved carapace length distribution among adult male, adult female, and unsexed juvenile and sub-adult green and loggerhead turtles

We did not have any turtle recaptures during our study. We captured a female loggerhead turtle that had a flipper tag from Dalyan beach, Turkey and observed on 25 May, 10 June, and 26 June, 2018 at the same site (Yakup Kaska, pers. comm.). A fisherman also reported seeing a green turtle with a satellite transmitter on the carapace December 22, 2019. In addition, a pair of green turtles were observed mating in the Lake on April 30, 2019. We observed evidence of injury or predation, with one green turtle missing a front flipper, one green turtle had evidence of impact trauma to the carapace that had healed, one loggerhead turtle had a large semicircle part of its shell missing, comprising about 25 % of its body length that had healed and resembled an animal bite, and

one loggerhead turtle was captured with blunt trauma on the top of the head that had healed as a notable depression. We also observed one fresh, dead adult male green turtle during the study. No obvious cause of death was discernable.

#### DISCUSSION

Our study is the first to describe the population structure of green and loggerhead turtles in Lake Bardawil. Despite our relatively small sample sizes, we believe we were still able to infer useful information regarding the populations of both species. Sex ratio can be useful to understanding the status of a population (Bender, 2006). Unequal sex ratio is typically interpreted to reflect sexual segregation in differential habitat use between males and females or different survival rates between the sexes, with usually lower survival among males (Bender, 2006). We believe the unequal sex ratios favoring female loggerhead and male green turtles in our study is not the result of sexual segregation in habitat use as this is apparently rare or not well documented in sea turtles (Hamman et al., 2006).

Sea turtles often have female biased sex ratios at birth (Heppell et al., 2003), which could be used as a baseline to compare adult sex ratio and any difference could be interpreted as different survival according to sex (Bender, 2006). The male biased sex ratio of green turtles could suggest that there is lower survival of females. Fresh water turtles often have lower female survival due to increased mortality from travelling to nesting sites or predation during nesting (Steen et al., 2006). However, increased mortality of female sea turtles as result of travelling to or nesting in Mediterranean is not documented.

Male loggerhead sea turtles could have lower survival than females as a result of males being smaller than females and more vulnerable to predation, males spending more energy and time travelling in search of females and competing with other males for access to females, higher vulnerability to predation because of lower male vigilance, and the increased search effort by males for females during the breeding season could make males more vulnerable to being captured in nets. However, a few studies from other populations have suggested that male and female green and loggerhead sea turtles have similar survival rates (Chaloupka & Limpus, 2002 & 2005). It is also difficult to interpret the unequal sex ratios as a result as different mortality rates of male and female sea turtles, because studies usually report survival of nesting females and combine the sexes together to estimate adult survival (Heppell et al., 2003; Campbell & Lagueux, 2005; Troeng & Chaloupka, 2007; Casale et al., 2018).

The size distribution and proportion or number of animals at sexually maturity can be used to infer size segregation of habitat use, potential reproductive growth, and viability of sea turtle populations (Heppell et al., 2003; Mazaris et al., 2005; Rees et al., 2013). There was a wide range in the distribution of size classes of green turtles, from 15 cm to 100 cm, suggesting that Lake Bardawil is a feeding ground for all size classes and both sexes, unlike other feeding grounds in which there is size segregation according to depth and habitat (Koch et al., 2007; Bresette et al., 2010). In addition, the size distribution of green turtles in our study was also wider than reported previously in Lake Bardawil and in other feeding sites in the Mediterranean (Nada et al., 2013, Casale et al., 2018). We were unable to determine if there was fine scale separation of depth utilised according to size class due to our small sample size and lack of more detailed sampling at depth. The inclusion of all size classes in a sea turtle population may indicate a healthy population (Bjorndal, 1985, Bjorndal et al., 1999). In contrast, the size distribution of loggerhead turtles consisted of mostly sexually mature and sub-adult turtles. This suggests that juvenile loggerhead turtles may use other areas to feed as size based segregation of sea turtles occurs according to ontogenetic shifts in food and/or habitat requirements and predation risk in feeding habitats (Limpus et al., 1994; Kock et al., 2007; Bresette et al., 2010; Rees et al., 2013).

The mean CCL of green turtles in our study was smaller than that of dead green turtles washed ashore while that of the loggerhead turtle CCL was similar to the dead loggerhead turtles but smaller than the stranded, dead green turtles along the Mediterranean coast of North Sinai and other sites of the Mediterranean basin (Rabia & Attum, 2015; Casale et al., 2018). We believe that our data may under represent sexually mature individuals and potential bias sex ratio as a result of capture bias as there were about 20 instances in which really large male and female green and loggerhead turtles were captured but escaped during the struggle of lifting the heavy individuals to the boat, especially at the beginning of the study when capture technique was still novel. We know that the largest individuals were not recaptured, but do not know if the other large individuals were recaptured later in the study once implementation of capture technique was more successful.

The high proportion of juvenile green turtles, the smallest recorded CCL being 15 cm, suggest that Lake Bardawil is a benthic feeding area for all size classes of green turtles, while the Mediterranean coast of North Sinai is used as a nesting site (Rabia & Attum, 2015, Bradshaw et al., 2017). The lack of small juvenile loggerhead turtles captured and the adult female captured from a nesting site in Turkey suggests that Lake Bardawil is used as a feeding site by sub-adult and adult loggerhead turtles from other parts of the Mediterranean basin but could also include females that nest along the Mediterranean coast of North Sinai (Rabia & Attum, 2015). Although small juvenile loggerhead turtles were not observed in Lake Bardawil, they have been observed in the Mediterranean coasts of North Sinai prior to this study (Rabia, unpublished data), suggesting that the Mediterranean coast of North Sinai could possibly be used as neritic foraging habitat for juvenile loggerhead turtles. The long-term population viability of sea turtles in part depends upon high survival of sub-adult and adult individuals (Heppell et al., 2003) and thus the protection of green and loggerhead sea turtles in Lake Bardawil is vital to the conservation of sea turtles in Egypt and the greater Mediterranean Sea. In conclusion, this study provides evidence supporting the classification of Lake Bardawil as an important feeding and development area for green turtles and feeding area for

loggerhead turtles in Egypt and expands our knowledge of known sea turtle feeding and development sites in the Mediterranean basin.

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## Records of limb abnormalities in three anurans from eastern Amazon - Atelopus hoogmoedi, Allobates femoralis and Dendropsophys leucophyllatus

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solated reports of amphibians with malformed, extra, missing, or abnormal limbs in natural populations have been in the scientific literature for centuries (Ouellet, 2000). However, the increasing, sporadic appearance of such abnormalities over the last few decades has demonstrated a high susceptibility of this group to this type of abnormality (Ouellet, 2000; Lannoo, 2009; Henle & Dubois, 2017). The causes of deformities and abnormalities in amphibians are believed to be related to several factors, such as ultraviolet radiation, chemical pollution (Blaustein et al., 2003), climate change (Pounds, 2001), and epidemic disease such as chytrid fungus (Pounds et al., 2006). Herein we describe instances of limb abnormalities in three anuran species of the eastern Amazon.

In 2017, during monitoring studies of the herpetofauna of Parque Natural Municipal do Cancão (PNMC) (0.90275°N, 52.00497°W; Datum WGS84/SAD 69), municipality of Serra do Navio, Amapá state, Brazil, we observed three cases of limb abnormalities. These were in Atelopus hoogmoedi Lescure, 1974 (Anura, Bufonidae), Allobates femoralis (Boulenger, 1884) (Anura, Aromobatidae) and Dendropsophus leucophyllatus (Beireis, 1783) (Anura, Hylidae). Single specimens with morphological abnormalities were collected at the margin of a stream near the Amapari River, in the western portion of PNMC. We captured the live anurans under collection permits from ICMBio (48102), using a combination of the visual encounter surveys and audio strip transects. We fixed the voucher specimens in 10 % formalin and preserved them in 70 % ethanol (Heyer et al., 1997) and subsequently identified and classified the abnormalities following the nomenclature proposed by Henle & Dubois (2017). The specimens are now housed in the Herpetological Collection of Federal University of Amapá – CECCAMPOS 2901 - Atelopus hoogmoedi; CECCAMPOS 2177 - Dendropsophus leucophyllatus; CECCAMPOS 3457 – Allobates femoralis).

Two of the anurans, A. hoogmoedi and D. leucophyllatus, showed ectromely which is characterised by the absence, partial or complete, of a limb (Henle & Dubois, 2017). The abnormality in A. hoogmoedi consisted of a partially missing radio-ulna in the right forelimb, where the hand was



Figure 1. Ectromelia of the radio-ulna in the left limb in a male of A. hoogmoedi from the municipality of Pedra Branca do Amapari, Amapá state



Figure 2. Ectromelia of the radio-ulna in the right limb in a male of D. leucophyllatus from the municipality of Serra do Navio, Amapá state

absent (Fig. 1). Dendropsophus leucophyllatus presented ectromely of the radio-ulna in the left forelimb (Fig. 2), with a somewhat shorter radio-ulna than in A. hoogmoedi. While, in A. femoralis there were three types of malformations in the fingers: brachydactyly, polydactyly and syndactyly (Fig. 3). In the right hand of A. femoralis, we observed a fusion in the tissue between the two medial phalanges (syndactyly)



**Figure 3.** Brachydactyly, polydactyly and syndactyly in the right limb in a male of *A. femoralis* from the municipality of Serra do Navio, Amapá state

and in the distal finger two abnormalities a proximal fission duplicating the finger (polydactyly) but with the bones of the fingers reduced in length (brachydactyly) (Lannoo, 2009; Henle & Dubois, 2017).

Concerning the prevalence of these abnormalities, only a single specimen of each of the three species was detected with an abnormality although this accounts for a high proportion of the total number of specimens observed - *A. hoogmoedi* (1 of 22 or 4.5 %), *D. leucophyllatus* (1 of 13 individuals or 7.6 %) and *A. femoralis* (1 of 55 or 1.8 %). The area of the survey is a fully protected conservation unit but is within the Serra Navio municipality where for generations there has been manganese extraction resulting in contamination with chemical residues including arsenic (Queiroz, 2008) which might be an exogenous factor affecting anuran populations.

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## Occurrence, conservation status and threats to the Indian eyed turtle (*Morenia petersi*), in Purba Medinipur, West Bengal, India

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The Indian eyed turtle *Morenia petersi* (Anderson, 1879) (Chelonia: Geoemydidae) is a fresh water hard-shelled species rated as Vulnerable on the IUCN red list. However, very little is known about this species. It is believed to have a discontinuous distribution across Bangladesh, India and Nepal (Das & Sengupta, 2010). Historically, in India it was found in the tributaries of the river Ganges and along the western bank of the river Brahmaputra. It has also been reported from Assam (Baruah & Sharma, 2010; Ahmed & Das, 2009), Bihar (Das, 1995), Uttar Pradesh (Javed & Hanfee, 1995), and Uttarakhand (Bahuguna, 2010). Here we present the first report of *M. petersi* in West Bengal.

During a visual encounter survey of the local herpetofauna in Purba Medinipur District (West Bengal), we found the first specimen of *M. petersi*. This prompted us to intensify our search by random sampling around all closely located water bodies. The species was identified following Das and Sengupta (2010). Meristic and morphometric measurements were taken according to (Terentev & Chernov, 1949) using a digital calliper accurate to the nearest 0.1mm. The captured turtles were measured, weighed on an electronic scale, photographed, and then released at the site of capture. To find out more about the species we undertook an informal survey in seven villages, using photographs to aid recognition of *M. petersi*. We interviewed around 250 people about their understanding of the turtle and its socio-economic importance.

On 25 September 2018, we found a small population of M. petersi in a small pond (Fig. 1) in the middle of Panchrol village (21°49'57.7"N 87°27'20.0"E, 19 m a.s.l.). We were able to capture two of the turtles which were found out of the water. All the M. petersi observed were around the pond which was originally part of an irrigation canal (Figs. 2 to 7). Five years ago, it was used for agricultural purposes and is now filling rapidly with non-biodegradable domestic and commercial waste. Due to anthropogenic pressure and illegal construction most of the stretch of the canal is fragmented. With the help of the community survey we found that this species has been observed in fields and other local ponds, but sightings seemed rare. All the ponds were privately owned and used for fish farming and domestic purposes. It was suggested that M. petersi prefers vegetation-rich shallow water bodies. Some of the vegetation we documented and identified at the pond was Centella asiatica, Cynodon dactylon, Hydilla sp., Eichhornia crassipes, Pistia Sp., Ipomoea aquatica.



Figure 1. Habitat of *M. petersi* at Panchrol village of Purba Medinipur District



Figure 2. Morphology of the *M. petersi* observed in this study- A. Sub-adult male, B. Adult female, C. Sub-adult male carapace, D. Adult female carapace, E. Sub-adult male plastron, F. Adult female plastron

Mital et al. (2019) considers that habitat loss, pollution, and hunting are the three main threats to the survival of *M. petersi* across its range. We were informed that there are poachers and illegal traders who collect and smuggle *M.* 

petersi to other parts of country for meat and the pet trade.

Until now, a detail locality record of *M. petersi* in West Bengal has been lacking. We believe that this vulnerable species is being threatened with local extinction due to habitat loss and exploitation for meat and the pet trade. Lack of information on current status of *M. petersi* is likely the main reason for it not being protected under Indian Wildlife Protection Act of 1972 nor included under the CITES list (Turtle trade in India, 1994). The future of this little-known species will be strengthened by further scientific study, habitat restoration and conservation, and stronger legal protection.

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## Defensive behaviour exhibited by the yellow-striped poison frog (*Dendrobates truncatus*) in response to simulated predation

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nurans use a diversity of behavioural strategies to avoid Abeing preyed upon. By adopting sudden postures or displays (deimatic behaviours) potential predators may be distracted or startled, and among aposematic anurans this may act to increase conspicuousness (Skelhorn et al., 2015; Umbers et al., 2017; Toledo et al., 2011). Body-raising is one type of deimatic behaviour in which anurans stretch out their appendages and raise their bodies off the ground (Toledo et al., 2011). This has been described in certain members of the Hylidae, Leptodactylidae, Leiuperidae, Bufonidae, and Dendrobatidae (Toledo et al., 2004 & 2011; Blanchette & Saporito, 2016 & 2017). Among dendrobatids, body-raising has been reported in the aposematic Ameerega flavopicta in the field (Toledo et al., 2004), and most recently in Dendrobates auratus in the laboratory and field (Blanchette & Saporito, 2016, 2017). Although present in two different genera of dendrobatids, body-raising behaviour among other species, and in particular other members of the genus Dendrobates, remains unknown. Therefore, to determine if other members of the genus Dendrobates (comprising five species, Frost, 2019) also exhibit body-raising, we performed simulated predation experiments identical to those of Blanchette and Saporito (2016) with two additional species: D. tinctorius and D. truncatus. These two species were chosen because of their phylogenetic relationship with D. auratus (a species known to exhibit body-raising), wherein D. auratus and D. truncatus are sister species and form a distinct clade within Dendrobates that is different from D. tinctorius (Grant et al., 2017).

We undertook two similar predation simulation experiments; one for each frog species. The frogs were held in four 38 L glass terraria, two for each species, with sphagnum moss bedding and two small plastic cover objects (11 cm x 7 cm). The terraria were maintained at around 23 °C, a relative humidity of greater than 85 %, and on a 12hour light:dark cycle. The frogs were provided with fruit-flies (Drosophila melanogaster) daily. Predation was simulated in the terraria by gently picking up and releasing individual frogs (three times, in succession) with a pair of 7.5 cm pressure sensitive forceps (Williams et al., 2000; Blanchette & Saporito, 2016). Experiment 1 involved four adult captivebred D. tinctorius (average SVL = 24.9 mm); two frogs in one terrarium were 'preyed upon' every day for 94 days (05 February 2018 - 09 May 2018), whereas the two frogs in the other terrarium were 'preyed upon' every other day for 133 days (05 February -17 June 2018). Experiment 2 consisted

of five adult captive-bred *D. truncatus* (average SVL = 20.2 mm), two in one terrarium and three in the other. All these frogs were 'preyed upon' every other day for 143 days (01 June - 21 October 2019). The simulated predation was performed at random times during the photophase (06.00h to 18.00h). No frogs were harmed during the study, and the experimental procedure was approved by the John Carroll University, Institutional Animal Care and Use Committee, protocol #1700.

None of the *D. tinctorius* exhibited body-raising behaviour; however, after 101 days, *D. truncatus* began to exhibit bodyraising behaviour in response to simulated predation. Bodyraising consisted of *D. truncatus* slightly extending their front and rear legs, arching their dorsal surface, and pointing their snout towards the ground (Fig. 1). The behaviour was observed initially in one individual, but by the end of the experiment, three of the five individuals exhibited the same behaviour. Although the behaviour was originally elicited only following simulated predation, once individual *D. truncatus* began body-raising, they also immediately bodyraised when they were exposed from under a cover object (similar to Blanchette & Saporito, 2016, 2017).



**Figure 1.** Adult *D. truncatus*: **A.** In a non-defensive stance, **B.** Exhibiting body-raising behaviour following simulated predation

Body-raising due to simulated predation was previously reported in captive, laboratory-raised *D. auratus* (Blanchette & Saporito, 2016), and is identical to the present report of body raising in *D. truncatus*. Although originally described in captive *D. auratus*, body-raising was also described in a natural population of *D. auratus* in Costa Rica (Blanchette & Saporito, 2017), suggesting the same is likely true of *D. truncatus*; however, this remains to be examined. As a defensive behaviour, body-raising in dendrobatids may function in more than distracting or startling potential predators. Aposematic dendrobatids are conspicuously coloured/patterned and also possess alkaloid defences, features that work together to deter potential predators (Saporito et al., 2012). Therefore, body-raising (and in particular, dorsal arching) in dendrobatids would increase the exposure of a frog's dorsal region, a body region with greater concentrations of alkaloids (Saporito et al., 2010), to potential predators, while also increasing their dorsal conspicuousness (Toledo et al., 2004; Saporito et al., 2010; Blanchette & Saporito, 2017).

Our findings that only *D. truncatus* exhibits body-raising are consistent with the hypothesis that *D. truncatus* and *D. auratus* are sister species, and suggests that this behaviour may be restricted to this lineage. Conversely, the absence of body-raising in *D. tinctorius* suggests this behaviour may be absent in other *Dendrobates*; however, this will require further study, with particular attention on its presence in *D. leucomelas*, the sister species of *D. tinctorius* (Grant et al., 2017). It will also be of interest to examine the occurrence of this defensive behaviour in other dendrobatids, including members of the genus *Adelphobates* (which is the sister taxon of the genus *Dendrobates*; Grant et al., 2017), as well as other members of the genus *Ameerega*.

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## A case of cannibalism in the false smooth snake *Macroprotodon cucullatus* on the island of Lampedusa

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The genus *Macroprotodon* (Colubridae, Colubrinae) includes four snakes species with a Mediterranean (Maghreb & S. Mediterranean) chorotype (Sindaco et al., 2013).

The only population of false smooth snake in Italian territory is located on Lampedusa Island (Province of Agrigento, Sicily), a limestone 'horst' belonging to the North African continental shelf and located in the Sicilian Channel, 113 km east from Tunisia and 205 km south from Sicily. Based on morphological characters (Wade, 2001), in recent times most studies attribute the Lampedusan false smooth snake to *Macroprotodon cucullatus textilis* (Duméril & Bibron, 1854) (Kreiner, 2007; Capula et al., 2011; Sindaco et al., 2013; Cattaneo, 2015; Di Nicola et al., 2019; Di Nicola, 2019). The genetic identity of this population has not yet been investigated, consequently its taxonomy requires confirmation and so here we will refer only to *Macroprotodon cucullatus* (Geoffroy St-Hilaire, 1827).

The false smooth snake is a secretive species with nocturnal or crepuscular habits (Capula et al., 2011; Speybroeck et al., 2016). Macroprotodon spp. feed mainly on reptiles; in Iberia M. brevis (Günther, 1862) feed primarily on amphisbaenians (Blanus spp., 50%) and lacertids (mainly Psammodromus spp., 21 %); in Morocco they mainly eat skinks (Chalcides spp., 42 %), lacertids (Psammodromus spp., 21 %) and amphisbaenians (Blanus spp., 14 %) (Pleguezuelos et al., 1994); in Balearic Islands M. mauritanicus seem to predate mainly small rodents (Pleguezuelos et al., 1994). Ophiophagy and cannibalism seem to be guite rare behaviours and are currently known only in the Balearic populations of *M. mauritanicus*, where a young *Malpolon* monspessulanus was predated (Pleguezuelos et al., 1994). Two cases of cannibalism have been observed in captivity (Wade, 1988) and a case between two fighting adult males in the field, documented only in the early stages of head swallowing (Capellà et al., 2011).

Information on the feeding behaviour of the Lampedusan *M. cucullatus* is still based on the observation of a few cases, which would indicate a diet of mainly lizards and arthropods (Corti & Luiselli, 2000; Cattaneo, 2015). However, most of the remains of the arthropods may have actually been ingested by the predated lizards (Corti & Luiselli, 2000). The current study reports the first observation of cannibalism detected in Lampedusan *M. cucullatus* and moreover the first in this

species, if its identity is confirmed by biomolecular analysis.

On 18th April 2019, near Taccio Vecchio (Lampedusa), a false smooth snake was caught in order to record some morphological characters. The snake was an adult male (Fig. 1A) with a snout to vent length of 315 mm, a tail length of 50 mm (injured tip) and a body weight of 20 g. During the measurements the snake defecated and the faeces were collected and preserved in absolute alcohol. The faecal remains were subsequently hydrated and then examined by stereomicroscope. Six pieces of snake skin were found, including dorsal and ventral scales (Fig. 1B, C & D). The dorsal scales were smooth and shortened with a single apical pit, consistent with the skin being from M. cucullatus. On Lampedusa, the false smooth snake is sympatric with only the Eastern Montpellier snake Malpolon insignitus, but this species has elongated and grooved dorsal scales (Di Nicola, 2019) quite unlike those of *M. cucullatus*. The intact ventral scales were stretched and measured and a maximum width of 6.0 mm was detected. This value was compared with a specimen (R771) of the Zoology Museum Pietro Doderlein (University of Palermo) on which higher values at midbody (6.6 mm) and similar values near the cloaca (5.8 mm) were found. Taking into account that the museum specimen was a sub-adult (SVL: 224 mm) and the measured scales were not totally stretched, it can be assumed that the remains belong to a juvenile or sub-adult snake.



Figure 1. False smooth snake: A. Adult male with cannibalistic behaviour, B. Overview of all the faecal remains, C. Detail with dorsal scales, D. Detail with ventral scales

Mainland populations of Macroprotodon spp. consume mostly lacertids and amphisbaenians both in Europe and in Africa (Pleguezuelos et al., 1994); consequently cannibalism was unexpected. However, there are two potential explanations for cannibalism, which are not mutually exclusive. The first concerns possible scarcity of suitable prey. Amphisbaenians are not present in the whole Italian territory and lacertids are generally lacking on Lampedusa (Padoa-Schioppa & Massa, 2001), except for a small population of Italian wall lizard Podarcis siculus currently located in the only urbanised area of the island, where they were introduced (Lo Valvo & Nicolini, 2001). In the same area, the Maltese lizard Podarcis filfolensis has also been observed (Lo Cascio et al., 2005), but they are apparently now absent (authors' unpublished data). On some other Mediterranean islands, in response to the availability of different resources, some species are known to shift their feeding habits to other prey (Corti & Luiselli, 2000; Pleguezuelos et al., 1994). The second explanation concerns population density. On Lampedusa M. cucullatus is not usually considered abundant (Corti & Luiselli, 2000; Capula et al., 2010), but during the authors' surveys the species was observed throughout the island and in some locations the population density was apparently high (detection of about 4-5 snakes / hour), with cases of communal sheltering among individuals of similar age. It is possible that this high local density increases the likelihood of cannibalism, in a situation where other colubrid species with cannibalistic tendencies appear to have developed behaviours to minimise intraspecific interactions (Kolanek et al., 2019). Further investigation of the Lampedusa population of *M. cucullatus* may help establish the frequency of cannibalism and give greater insight into why this occurs.

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## Why the long finger? Observation of male–male combat in African bush squeaker frogs, Arthroleptis stenodactylus (Anura: Arthroleptidae)

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Sexual dimorphism has been widely reported among Sanurans. In general, male frogs use advertisement calls to attract females and to defend territory (Duellman & Trueb, 1986). Male secondary sexual traits, such as spines, tusks and nuptial excrescences, are not uncommon in anurans and are associated with defensive behaviours and, in the case of nuptial excrescences, may increase grip during amplexus - they are best developed in stream breeders (Duellman & Trueb, 1986). Another form of secondary sexual trait is observed in males of the arthroleptid genera Arthroleptis and Cardioglossa. In most species of these genera, widely distributed across sub-Saharan Africa, sexually mature males present elongate third fingers and spines on the median surface of fingers (second and third) and groin (inguinal) region (Blackburn, 2009). In some cases the length of the male third finger can reach up to 30-40 % snout-vent length (SVL) (Blackburn, 2009), and in Arthroleptis stenodactylus Pfeffer, 1893 the third finger length varies with SVL (Schmidt & Inger, 1959). The function of this trait is unclear and the only account suggests that it can be related to combat behaviour. Amiet (1989) reported a malemale combat of A. poecilonotus Peters, 1863 from Cameroon: "...these frogs were standing on their hind limbs, with the fore limbs under the arms of the other, and with their elongate third finger vibrating against each others' backs....." (as translated by Blackburn 2009, p.555).

In northern Mozambique, combat between two male A. stenodactylus (Fig. 1; YouTube, 2020) was observed at night after rain in November 2018, at a campsite on the outskirts of Nampula town (15° 02'22" S, 39° 08'39" E; 390 m a.s.l.). Several males were heard calling at the same time as the two males were observed and filmed. Although three human observers were within 20-30 cm of the pair the entire time, they were not deterred by our presence, with their attention focussed solely on the fight. The behaviour reported here closely resembles the description of A. poecilonotus malemale combat (Amiet, 1989). From the time we first noticed the action the males engaged in combat twice - at first we thought it was a couple in amplexus. Only the second combat was filmed and lasted approximately 60 seconds. The two frogs were standing on their hind limbs, with fore limbs around each other, and their third finger can be observed vibrating against each other's back. The males seem to attempt to flip each other, disengaging and quickly reengaging, before the

defeated male left. The winner started calling again soon after the combat.



**Figure 1.** Two video screen shots of male-male combat in *A. stenodactylus* showing: **A.** The fight stance, and **B.** Grasping with the elongated finger

To our knowledge, this is the second report and first footage of this behaviour in *Arthroleptis*. It reinforces the idea that this unusual sexual trait has evolved as a form of weapon. Although *A. stenodactylus* is commonly found East Africa, including anthropogenic environments, the observation of this behaviour has gone unreported until now. Natural history observations are fascinating and may help us understand the evolution and diversity of male sexual characters. We thank the National Geographic Society-Committee for Research and Exploration (9934-16) and the Percy Sladen Memorial Fund for funding this expedition.

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### **Reptiles and amphibians of New Zealand: A Field Guide**

Dylan van Winkel, Marleen Baling & Rod Hitchmough Bloomsbury Wildlife, 2019, ISBN 9781472974990, pp 366 + x, flexiback



eaders who travelled to KAotearoa New Zealand (NZ) for the 9th World Congress of Herpetology in January 2020 may well have been accompanied by this UK republication from 2019. Other attendees may have purchased the original, NZ version (van Winkel et al., 2018) at a congress sales table. Either way, you are unlikely to have been disappointed. As stated on the back cover of the UK version, this book aims to be "the ultimate photographic field guide to New Zealand's

herpetofauna, and ... a gateway into the world of these fascinating tetrapods for amateur and expert herpetologists alike". Indeed, it is.

As the authors note, New Zealand's endemic herpetofauna is famous for its tuatara and leiopelmatid frogs (modern representatives of long-distinct lineages). However, it is also notable for its diversity of long-lived lizards, almost exclusively viviparous. Though only two families of lizards are present (diplodactylid geckos and skinks), over 100 species have been proposed. Much of this diversity is morphologically cryptic, recognised using molecular approaches and awaiting formal description; undescribed taxa are recognised here with 'tag names', following the approach taken by the NZ Department of Conservation's threatened species listings (Hitchmough et al., 2016). This field guide also includes the visiting marine snakes and turtles (migrants or vagrants), as well as several introduced frogs and a skink, all Australian in origin, that have naturalised in NZ (the skink is an 'Unwanted Organism' under biosecurity legislation).

Physically, this book is a suitable size for a backpack (about 22 x 14 cm, and just under 725 g). The cover of the UK version is attractive (though not in my view as fresh and compelling as that of the NZ version, with its greater number of endemic taxa on a white background). The inside covers provide a useful quick guide with page references to the different genera. The text begins with a generous and comprehensive introduction of 29 pages, covering the history of human attention from the arrival of Polynesian

settlers, diversity and origins, extinctions and introductions, conservation of endemic species, and NZ's ecosystems and geographic history. The legislation that makes it "... illegal to capture, collect, hold in possession or deliberately disturb..." native reptiles and amphibians without an authority under the Wildlife Act 1953 is explained, as is the process of application if one wishes to keep species in captivity. The introduction states the book's aim as to help "you, the reader, identify reptiles and amphibians ecountered in New Zealand, particularly in the field, where species identification can be challenging". Given the encouragement to take high-quality, close-up photographs for identification purposes (p. 26), it would have been useful to restate the legislative requirements at this point and resolve the tension in achieving a close-up examination without prior authority.

In the species accounts that follow, each major section (tuatara, geckos, skinks, frogs, marine turtles and marine snakes) is first introduced with a helpful biological overview. Each recognised taxon then receives an account of at least two pages, including a reasonably detailed physical description, a summary of known distribution, habitat, natural history and etymology. The text is crisp, clear and informative. Unsurprisingly for a field guide, no sources are given in the species accounts; thus, the expert will want to consult additional references, such as the more recent of those in the modest list of 'Further Reading' near the back of the book (for example, Chapple 2016 for NZ lizards). A distribution map that distinguishes predicted (based on database records plus a realistic buffer) from potential (crude estimates of adjacent areas where the taxon is considered also likely to be present) are helpful, without giving away sensitive information. I would have liked to see the release sites from recent conservation translocations distinguished from areas of natural occurrence. I'd also have liked an obvious rationale for the order of species within each section (it seems neither alphabetical nor geographical); this is particularly problematic for the >60 skinks in the genus Oligosoma where the index becomes crucial for finding a given species, and a page-holder ribbon would also have helped. Each section (other than for the monospecific tuatara) is followed by a key to species. Towards the end of the book there is a regional checklist of species (particularly helpful for NZ herpetologists) and a useful glossary.

A stand-out feature of this book is the superb photography, much of it by Dylan van Winkel. Many taxa are helpfully photographed against a uniform white background, often with additional supporting photographs illustrating colour variations, habitats or life-history stages other than adults. In the general accounts, there are also often excellent line diagrams of morphological features.

The authors are NZ wildlife ecologists and a conservationscience advisor, with expertise (collectively over 60 years) in herpetological restoration projects, biosecurity, taxonomy and conservation-status listings. They have produced a field guide that is both beautiful and useful - a major step up on earlier guides such as that of Jewell (2011). It comes as no surprise to learn that the original NZ version received a Certificate of Commendation for best field guide in the 2019 Whitley Awards of the Royal Zoological Society of New South Wales. Whilst I can't comment on the usefulness of the keys, or whether the taxonomy recognised here will be followed by formal descriptions that stand the test of time, I certainly recommend this book to all those who seek an introduction to the NZ herpetofauna. The retail price for the UK publication is 30 pounds; cheaper prices for this or other versions may be found on-line.

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## HERPETOLOGICAL BULLETIN REPORT

#### March 2020

All four issues of *The Herpetological Bulletin* were published on time during 2019 with 99 % of manuscripts appearing within three months of being accepted.

There were 105 manuscript submissions in 2019, twenty more than in 2018 (Table 1) but similar to the 108 received in 2017. Full paper submissions were only 17 % of the total but two manuscripts referred to *The Herpetological Journal* are not included in the statistics. The overall acceptance rate of 50 % was high compared with 2018 (Table 1 - 35 %) but closer to the 44 % accepted in 2017.

Table 1. Submission and acceptance rates for manuscripts received in 2019 (2018)

	No. submitted	No. accepted	% accepted
Full papers	18 (31)	13 (12)	72 (39)
Short Notes	12 (13)	5 (7)	42 (54)
Natural History Notes*	65 (41)	34 (11)	52 (27)
Totals	105 (85)	52 (30)	50 (35)

\*Also includes Short Communications

During 2019, *The Bulletin* has become more closely connected to social media. At the launch of each issue, a message is now posted on the BHS Facebook page that highlights the front cover illustration, comments on one or two items of interest, and links to the Issue's table of contents on the BHS website. Also for the first time in Issue 150, the PDFs of some articles have contained hyperlinks to illustrative video clips posted on the internet. The PDF version of *The Bulletin* is available to all BHS members and access to videos adds interest and understanding to their experience. Articles with hyperlinks are now made available to the general public from the time of upload to the BHS website rather than being embargoed for one year as 'subscription only'. The reason for this change is that the text accompanying a video clip, on the video hosting site, contains a link to the published article on the BHS website and this greatly increases the visibility (hit rate) of the BHS website.

Issue 150 celebrated the 40th year of *The Bulletin* and in that time 13 BHS members have held editorial responsibility for the final product. Distinguished among those is Roger Meek who has edited both *The Bulletin*, and for a time *The Journal*, since 2012. Roger will finally step down from his role with *The Bulletin* from the April 2020 AGM, with the Society's gratitude.

The outlook for *The Bulletin* is strong and currently the BHS Council supports the retention of both the electronic and printed versions. For the future there will likely be a move to full colour for the printed version. There is also a plan to upload all back issues of *The Bulletin* to the BHS website, currently they only go back to 2008.

The BHS is very grateful to the following people who gave their time and expertise reviewing manuscripts for The Bulletin in 2019:- Roger Avery, John Baker, Dirk Bauwens, Trevor Beebee, Xavier Bonnet, Andy Buxton, Nicolas Casewell, Tim Colston, John Cooper, Margaret Cooper, Andrew Cunningham, Roger Downie, Eleanor Drinkwater, Iri Gill, Chris Gleed Owen, Stuart Graham, Richard Griffiths, Jihene Ben Hassine, Rick Hodges, Robert Jehle, Jim Labesco, Miguel Landestoy, Steve Langham, Todd Lewis, Roger Meek, Robert Mendyk, Konrad Merbert, Anita Malhotra, Chris McInerny, Chris Michaels, Kanto Nishikawa, Eric Patterson, Alan Rees, Gordon Schuett, Christine Tilley, Romulus Whitacker, and John Wilkinson.

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