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Uzungwa Scarp Nature Forest Reserve: a unique hotspot for reptiles in Tanzania

John Valentine Lyakurwa[1,2,*], Kim Monroe Howell[2], Linus Kasian Munishi[1], Anna Christina Treydte[1,3]

[1] *Department of Sustainable Agriculture and Biodiversity Ecosystem Management, The Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Arusha, Tanzania*
[2] *Department of Zoology and Wildlife Conservation, University of Dar es Salaam, P.O. Box 35064, Dar es Salaam, Tanzania*
[3] *Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany*
* *Corresponding author*. Email: johnlyakurwa@udsm.ac.tz

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Abstract. While knowledge of African vertebrate biodiversity has increased dramatically in recent years, the field of herpetology which encompasses many threatened and endemic species, has lagged behind, and many areas have not been adequately explored. Intensive field work was conducted during the rainy season from December 2017 to April 2018 to assess reptile occurrence mostly in previously unexplored areas of the Uzungwa Scarp Nature Forest Reserve (USNFR) which is part of the Udzungwa Mountain ranges in the Eastern Arc Mountains (EAM), and adjacent agricultural areas. Bucket pitfall traps, funnel traps, night transects and opportunistic search methods were used to sample reptiles across four zones: in lowland, submontane and montane forests of the USNFR, and in neighboring farmlands. Forty-five reptile species across 14 families were recorded, mostly concentrated on the lowland and submontane forests. The number of endemic and threatened species in the USNFR reaches 20 and 14 respectively, and most are found in the submontane forest. Nineteen species were new records for the USNFR, five of them representing range extensions. Reptile species richness, abundance and diversity differed significantly across the four zones, except between montane and farmland zones and between lowland and submontane. However, farmland zone was discordant from other zones in terms of species composition. This study adds to the importance of the EAM not only in harbouring large numbers of species but also as an important hotspot for endemic and threatened reptiles. It also calls for proper land-use practices in farms adjacent to protected areas for sustainable conservation of biodiversity.

Keywords. Eastern Arc Mountains, Farmland, Elevation, IUCN threatened species.

INTRODUCTION

In spite of the alarming trends on the loss of species (Lawton and May, 1995; Baillie et al., 2004; Pimm et al., 2014), little has been done to assess patterns of biodiversity and threats facing reptiles in Africa (Meng et al., 2016). A recent assessment by Meng et al. (2016) shows that 321 reptile species occur in Tanzania, of which about 13% and 28% are threatened with extinction and are endemic, respectively. Most of these highly fragile reptile populations are found in the Eastern Arc Mountains (EAM) (Meng et al., 2016; Spawls et al., 2018).

The Eastern Arc Mountains have faced a number of threats, with forest fires, agricultural encroachment, firewood collection, logging and climate change being the most important ones (Burgess et al., 2002; Newmark, 2002; Ehardt et al., 2005; Menegon and Salvidio, 2005; Meng et al., 2016). The mountains have lost over 70% of forest cover to agriculture within the last six decades (Newmark, 1998; Newmark, 2002; Hall et al., 2009) and

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currently support a large number of people (Ndangalasi et al., 2007; Platts et al., 2011). The same impacts have been reported from the Uzungwa Scarp Nature Forest Reserve (USNFR) (Zilihona et al., 1998; Menegon and Salvidio, 2005; Rovero et al., 2012), which encompasses the southern portion of the EAM. This reserve was recently upgraded from "forest reserve" to "nature reserve" category (URT, 2017), calling out for a higher protection status due to its unique biodiversity. Despite this upgrade, information regarding USNFR's biodiversity is extremely scant.

Since some reptile species possess very narrow distributional ranges and depend on highly-specific habitat requirements (Spawls et al., 2004; Meng et al., 2016), they become more vulnerable to the ongoing anthropogenic activities than wide-ranging species. Menegon and Salvidio (2005) showed that elevation determined distribution patterns of reptiles in the USNFR and reported most endemic species to be restricted to higher elevations. The same high elevation areas have faced severe agricultural expansion in the USNFR (Zilihona et al., 1998; Ehardt et al., 2005) and little is known on how reptiles utilize the transformed areas. As some of the farms are found on high elevations, the latter generally hosting more endemic species compared to lower elevations, these reptile species might extend to the farms close to the forest edge on the plateau side.

While there are several reports on reptiles of the USNFR (e.g., Menegon and Salvidio, 2005; Lyakurwa, 2017), most of these surveys were limited to the southern part of the reserve and to our knowledge, no study has ever investigated how reptiles utilize the agricultural areas bordering the USNFR. Since the previous reports examined both amphibians and reptiles simultaneously (except Lyakurwa, 2017), the surveys were limited to methods which could capture both species groups. A project on Uzungwa Scarp hyper-endemic amphibians has revealed a number of new records in the same area, especially with respect to the distribution extension of the hyper-endemic species and of new species of *Nectophryoides* (Tonelli et al., 2017), that were mostly found in the previously unexplored areas, and has emphasized the need for detailed surveys for reptiles. This study focused on assessing reptile occurrence in the least explored areas of the USNFR and adjacent agricultural lands. Our results on how endemic and threatened reptiles utilize the USNFR and the nearby areas dominated by human activities can be used for local and long-term conservation planning in this and other protected areas of Tanzania.

MATERIALS AND METHODS

Study site

This study was carried out in the Uzungwa Scarp Nature Forest Reserve (USNFR) and adjacent areas. The USNFR covers the southeastern part of the Udzungwa mountains and lies between 7°39'-7°51'S, and 35°51'-36°02' E (Ndangalasi, 2005). With an altitudinal range of 300 m.a.s.l to 2,068 m a.s.l it covers a total area of 207 km^2 (Shangali et al., 1998; Ndangalasi et al., 2007; URT, 2017). It borders the Chita River to the south, the Kidete River to the north and the Ruaha, Iwolo and Lukosi rivers to the west (Ndangalasi, 2005). Average rainfall in the USNFR is unimodal (from November to May) and ranges from 1,800 mm to 3,000 mm per year (Shangali et al., 1998; Ndangalasi, 2005). The average temperature varies seasonally and is estimated to range from 15 to 20 °C on the highlands and 19 to 27 °C in the lowlands (Ndangalasi, 2005). The nature reserve is comprised of lowland (< 800 m a.s.l), submontane (700-1,400 m a.s.l) and montane forests (> 1,400 m a.s.l), with areas of seasonally inundated grasslands and grassland with bushes (Shangali et al., 1998; Zilihona et al., 1988).

Data collection

Data were collected during day and night for five consecutive months in the wet season, from mid-December 2017 to the end of April 2018. Selection of sampling sites was primarily based on elevation, vegetation types (Shangali et al., 1998; Zilihona et al., 1998) and land use type. Other factors known to influence reptile abundance and distribution were also considered at each site. These factors included the amount of leaf litter, availability of rotten logs, distance from water bodies and from rock crevices, following Howell (2002) and McDiarmid et al. (2012). The study area was divided into four zones; three inside the USNFR, i.e., lowland forest, submontane forest and montane forest following Shangali et al. (1998) and Zilihona et al. (1998) with some slight modifications. The fourth zone was set in farmlands bordering the USNFR. These farms were located on the plateau side of the reserve (with elevation range similar to that of a montane zone) and were of interest to this study to verify if the observed pattern of endemism in the reserve would extend beyond the protected area. Each zone consisted of three sites (12 sites in total), each with a radius of 1 km, and placed at least 2 km apart. Data collection took place for ten days at each site (alternated between zones to reflect the timing and commonality of the season between sites throughout the data collection period), making a total of 120 days (90 and 30 days in and outside the USNFR, respectively). Several methods (bucket pitfall traps with drift fences, funnel traps, night transects and opportunistic searches) were used following Howell (2002) and McDiarmid et al. (2012) in order to maximize captures. One bucket pitfall trap line (Howell, 2002) consisted of a 55 m long drift fence, eleven 20-L buckets, set at an interval of 5 m and 10 double-ended funnel traps placed alternately between each bucket. Two bucket pitfall trap lines were established at each site, summing up to a total of six trap lines (66 buckets, and 60 funnels traps) per zone. Trapping was done for eight consecutive nights, in which trap monitoring was done immediately following sunrise and late afternoon, following Stanley et al. (1998) and Howell et al. (2012). A total of 176 bucket pitfall trap nights and 160 funnel trap nights were carried at each site leading to 2112 and 1920 bucket pitfall trap nights and funnel trap nights, respectively, for the entire study.

In addition, a total of four 50 m night transects were set at each site (total of 48 transects for the entire study), encompassing a range of micro-habitats (*sensu* Menegon et al., 2008). Each transect was located, marked in advance and searched thoroughly following Lyakurwa (2017). Since pitfall traps and night transects alone cannot adequately sample all species of reptiles, these methods were supplemented by opportunistic searching, during which reptiles were searched for in their possible hiding/ basking places. All reptiles encountered casually or in locations apart from the 12 sampling sites but within the study area were also recorded as opportunistic encounters. Species identification followed Spawls et al. (2018) while threat status followed Meng et al. (2016). Grouping of endemic/near endemic species based on their dependency to the forest followed Burgess et al. (2007). Kruskal Wallis test (Kruskal and Wallis, 1952) with Dunn's multiple comparisons was used to compare the overall reptile species abundance in the four zones while diversity was compared using Hutcheson's t-test (Hutcheson, 1970). Shannon Wiener index was used for species diversity. Species composition among the four zones and between the surveyed sites was compared using the Bray-Curtis similarity index (Legendre, 1998; Greenacre and Primicerio, 2013). Data were analyzed using R software version 3.5.0 and Paleontological Statistics software (PAST) version 2.17 (Hammer et al., 2001). Statistical significance was considered when P was less than 0.05. Voucher materials were deposited at the Department of Zoology and Wildlife Conservation of the University of Dar es Salaam (Appendix 1).

RESULTS

A total of 358 individual reptiles were recorded, representing 45 species in 14 families (Appendix 1). Thirty-three species were found in the USNFR alone, two in farmland alone, and 10 in both (Appendix 1). Seven species (*Kinyongia sp*, *Trioceros deremensis*, *Broadleysaurus major*, *Crotaphopeltis tornieri*, *Dendroaspis angusticeps*, *Gonionotophis nyassae* and *Lycophidion uzungwense*) were single observations while three were double observations (*Urocotyledon wolterstorffi*, *Trioceros tempeli* and *Afrotyphlops nigrocandidus*). Most individuals were found on trees (40.3%), understorey (25.5%), underground (9.1%), dead logs (6.6%), rocks (3.3%) and in farmlands, some individuals were found on house walls (0.8%). Among reptiles which were found above the ground (n = 177), 52.0% were found at 50-100 cm height, 32.2% between 100-300 cm height, and 15.8 % above 300 cm from the ground.

Nineteen species were new records for the USNFR, five of them representing range extensions (Appendix 1) from previously known distributional ranges. This raised the number of species in the USNFR and surrounding areas to 60 species across 16 families (Table 1 and Appendix 1). We documented that the USNFR harbours about 21% (20 species) of reptiles that are endemic/near endemic to Tanzania (Appendix 1). About 69% of reptiles endemic/near endemic to EAM are now confirmed to occur in the USNFR (Appendix 1). A large number of these endemics were chameleons (7 species), a number which is equivalent to 29% of all Tanzanian endemic chameleons.

Table 1. Total number of reptiles species, number of IUCN threatened species, per families found in and around USNFR (Sources: Menegon and Salvidio 2005; Lyakurwa 2017, this study). NT = Near threatened, VU = Vulnerable, EN = Endangered

Family	Total	Endemic	NT	VU	EN
Agamidae	1	0	0	0	0
Atractaspidae	2	0	0	0	0
Chamaeleonidae	9	7	2	0	1
Colubridae	12	2	1	0	1
Elapidae	2	0	0	0	0
Gekkonidae	8	2	0	2	0
Gerrhosauridae	1	0	0	0	0
Lamprophiidae	3	1	0	0	0
Natricidae	1	0	0	0	0
Psammophiidae	2	0	0	0	0
Pseudoxyrhophiidae	2	0	0	1	0
Pythonidae	1	0	0	0	0
Scincidae	9	2	0	1	1
Typhlopidae	1	1	0	1	0
Varanidae	1	0	0	0	0
Viperidae	5	1	0	3	0

The number of species considered as globally threatened/ near threatened with extinction (Near Threatened, Vulnerable, Endangered or Critically Endangered) reached 14 in the USNFR (Appendix 1), equivalent to 33% of all reported threatened/ near threatened reptile species in Tanzania. Most of these species were found in the submontane forest (Appendix 1; Fig. 1 and 2).

Except for *Afrotyphlops nigrocandidus*, all strictly forest dependent endemic/ near endemic species were found only in the protected areas of the USNFR (Appendix 1). Similarly, other endemic reptiles were found in areas inside the USNFR with the exception of *Trioceros tempeli* and *T.werneri* which were found both inside and outside the reserve and *Lycophidion uzungwense* which was only found outside the reserve (Appendix 1). Outside the USNFR, *A. nigrocandidus* was found in a farm plot while *T. tempeli*, *T.werneri* and *L. uzungwense* were found in natural forest fragments, in fruit trees (the former two)

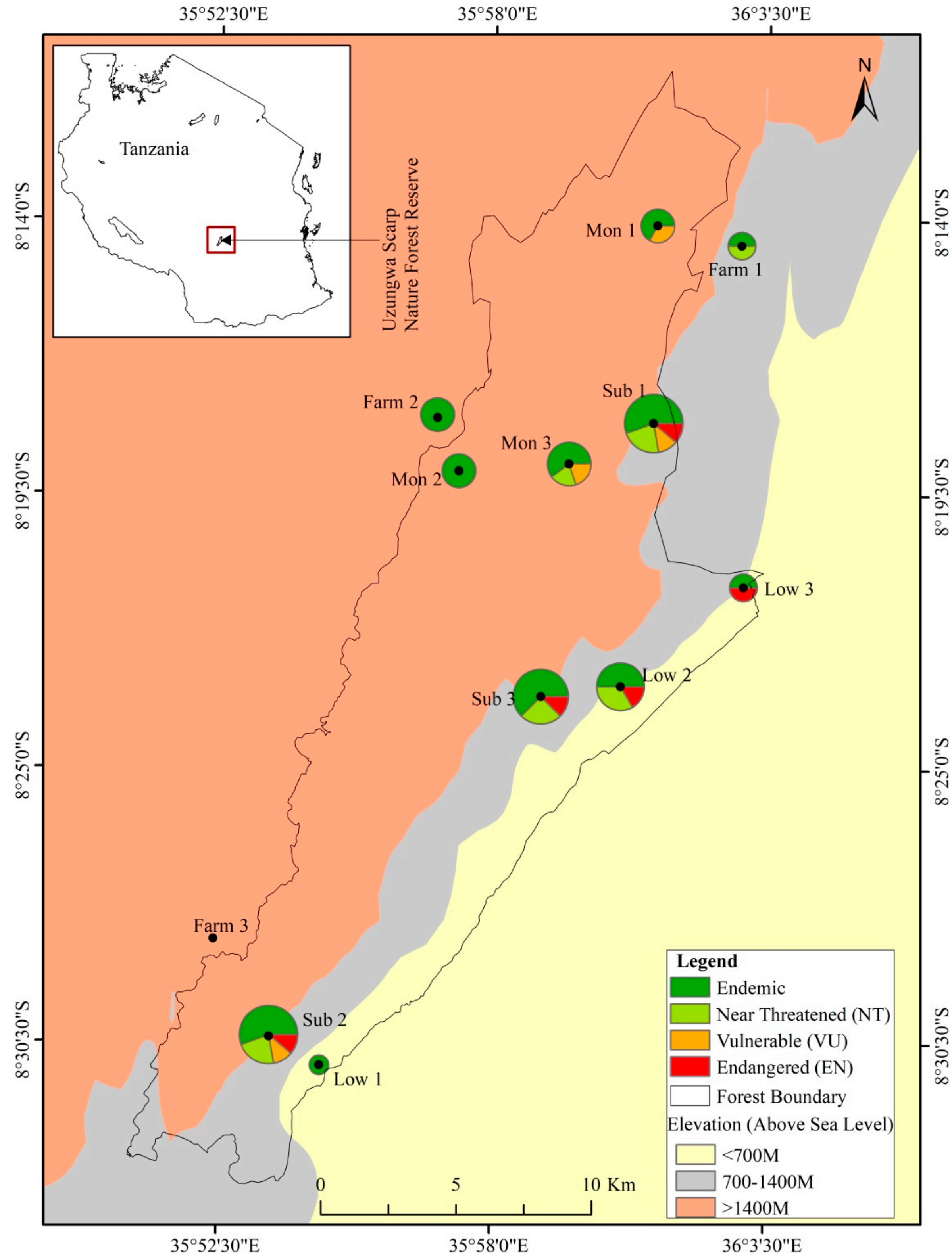

Fig. 1. Distribution of endemic, Vulnerable, Near Threatened and Endangered reptile species in the Uzungwa Scarp Nature Forest Reserve and adjacent areas as assessed from December 2017 to April 2018. Low 1,2,3 = Lowland sites, Farm1,2,3 = Farmland sites, Sub1,2,3 = Submontane sites, Mon 1,2,3= Montane sites.

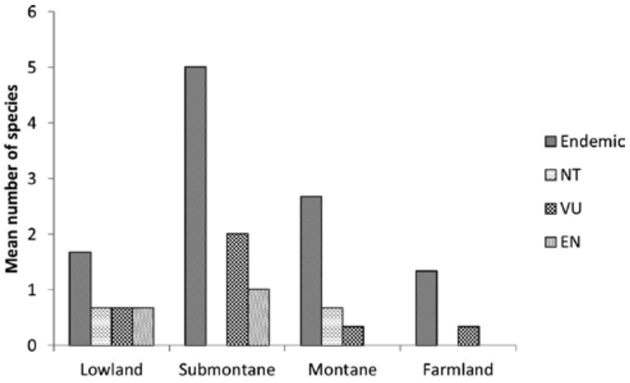

Fig. 2. Mean number of endemic, Near Threatened (NT), Vulnerable (VU) and Endangered (EN) reptile species in the four surveyed zones of the Uzungwa Scarp Forest Nature Reserve and adjacent agricultural areas.

and commercial forests dominated by *Cupressus sp.* and *Pinus sp*. Maize and bean fields were poor in reptile species, with only *Philothamnus hoplogaster*, *Lygodactylus grotei* and *Trachylepis varia* being the common residents.

All reptiles observed by Lyakurwa (2017) were also recorded during this study, except for *Buhoma procterae*, *Natriciteres variegata*, *Python natalensis* and *Xyelodontophis uluguruensis* (Appendix 1). Nine species recorded by Menegon and Salvidio (2005) in the same area were not found during this study (Appendix 1). *Lycophidion uzungwense*, previously found inside the USNFR by Menegon and Salvido (2005), was only found in a natural forest patch outside the USNFR. These patches, together with commercial forests and fruit trees in agricultural lands, also proved to be important for chameleons (especially *Trioceros tempeli* and *T. werneri*) (Fig. 3A). *Kinyon-*

Fig. 3. Some of the reptile species recorded in the USNFR from December 2017 to April 2018. A male *Trioceros werneri* on a commercial plant outside the USFNR (A), *Kinyongia sp* (B), *Aparallactus sp* (C), *Cnemaspis sp* (D), Male (A) and Female (B) *Urocotyledon wolterstorffi*. The above *Cnemaspis, Aparallactus and Kinyongia* could not be identified with certain to species level using Spawls et al. (2018).

gia sp (Fig. 3B) is believed to be a new species similar to *Kinyongia fischeri* based on morphological grounds. Similarly, *Aparallactus sp* (Fig. 3C) needs further studies as the currently available identification key by Spawls et al. (2018) was not sufficient to identify it to species level. The genera *Lygodactylus*, *Cnemaspis* (Fig. 3D) and *Urocotyledon* (Fig. 3E and 3F) encompass individuals with highly varying morphology and our findings likely represent more than one cryptic species in these genera.

Lowland and submontane forests contained a similar number of species, which decreased more than half towards montane forest and farmlands (Appendix 1). Overall reptile abundance differed significantly across the zones (H = 18.187, P = 0.0004). Further analysis using Dunn's multiple comparison showed no significant difference between farmland and montane forest and between lowland and submontane forests (Table 2). All other pairs were significantly different in overall reptile abundance (Table 2). However, Bray-Curtis similarity index showed farmland to be the most discordant zone (Fig. 4), with lowland and submontane zones being more similar in species composition. Lowland, submontane and montane zones contained more forest dependent species than farmland zone (Appendix 1). Sites in the farmland zone were very similar in species composition than when compared with sites in the protected area (Appendix 2). Also, sites close to each other were more similar in species composition than distant sites, whereby some distant sites showed complete dissimilarity (Appendix 2). Species

Table 2. Dunn's multiple comparison of overall reptile abundance in the four sampled zones of the USNFR and surrounding areas

Comparison	P value
Farmland vs Lowland	0.001
Farmland vs Montane	0.467
Farmland vs Submontane	0.004
Lowland vs Montane	0.001
Lowland vs Submontane	0.281
Montane vs Submontane	0.003

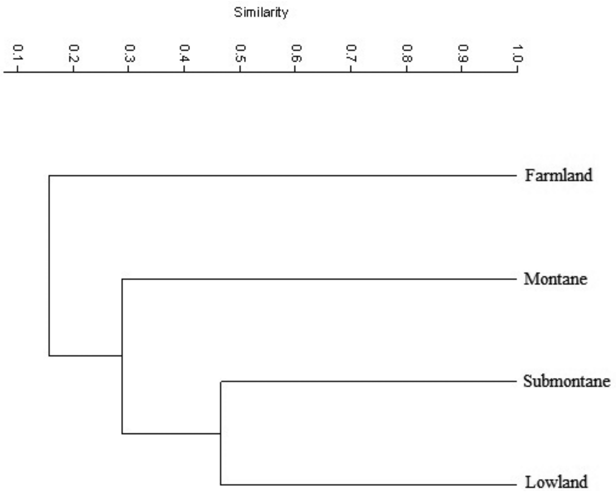

Fig. 4. Similarity cluster among the four zones of the Uzungwa Scarp Nature Forest Reserve and adjacent areas based on Bray-Curtis similarity index (Single Average Link) as per the current study.

Table 3. Hutchesons' t test summary of species diversity for the four surveyed zones of the USNFR and the surrounding areas.

Comparison	t value	DF	P value
Farmland vs Lowland	8.854	181	<0.001
Farmland vs Montane	1.678	124	0.096
Farmland vs Submontane	8.148	179	<0.001
Lowland vs Montane	5.971	98	<0.001
Lowland vs Submontane	0.912	196	0.363
Montane vs Submontane	5.324	97	<0.001

Table 4. Species richness, diversity and Chao richness estimator (±SE) for the four sampled zones

	Lowland	Submontane	Montane	Farmland
Species observed	26	24	9	11
Chao Estimator	32.17±5.13	43.97±17.26	9.98±2.22	17.19±7.48
Shannon diversity	2.23	2.16	1.17	0.79

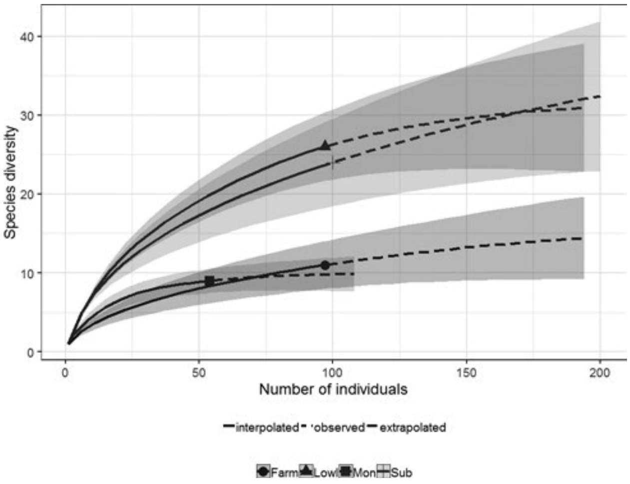

Fig. 5. Rarefaction curves for species recorded in the four sampled zones of the USNFR and surrounding areas from December 2017 to April 2018. Farm= Farmland (circle), Low=Lowland (triangle), Mon=Montane (square), Sub=Submontane (plus sign). Shaded region surrounding each line represent 95 % confidence levels

diversity in lowland and submontane was significantly higher than farmland and montane (Table 3). However, species rarefaction curves for the zones did not reach an asymptote (Fig. 5). The mean (±SE) number of species for Chao estimator was higher than the observed species in all zones (Table 4).

DISCUSSION

With our study, we were able to almost double the number of reptile species for the USNFR, from 33 species (Menegon and Salvidio, 2005) and 38 species (Lyakurwa, 2017) to 60 species. Such a result pinpoints the Udzungwa mountains as biologically the richest mountain block in the EAM in terms of herpetofauna, harboring the highest number of endemic and near endemic reptile species (34), followed by East Usambara (32), Uluguru (29) and Nguru (19) (Burgess et al., 2007). Previously, in terms of herpetofauna, this mountain range was ranked after Usambara and Uluguru mountains (Howell, 1993; Burgess et al., 2007). Three species out of the nine classified as globally threatened, endemic to Tanzania and climate change-vulnerable by Meng et al. (2016), are now confirmed to occur in the USNFR. This result highlights the importance of protecting these mountains and calls out for more long-term surveys in other parts of the Udzungwa and the EAM.

Although faunal surveys are recognized as one of the most critical steps in assessing forest biodiversity (Stanley et al., 1998), little attention has been given to African herpetology (Spawls et al., 2004; Largen and Spawls, 2010; Meng et al., 2016; Tolley et al., 2016). There are many areas in East Africa which are yet to be explored in a herpetological context (Spawls et al., 2004) and this study shows the need for detailed surveys even in previously visited areas, supporting Howell (1993) and Spawls et al. (2004), who showed the possibility of getting new records in most areas of East Africa, due to lack of intensive surveys in most parts of the region. The overall shortage of information adds more risk to the conservation of African biodiversity (Tolley et al., 2016), particularly herpetofauna and may lead to misallocated conservation priorities (Pimm et al., 2014), especially in a biodiversity hotspot country like Tanzania.

Contrary to previous studies, we found that most endemic, near endemic and IUCN threatened species were concentrated in the submontane forest of the USNFR. Menegon and Salvidio (2005) and Menegon et al. (2008) reported that the number of endemic and near endemic reptile species increases with altitude. Similarly, Burgess et al. (2002) reported more endemic vertebrates in montane forests of the EAM and fewer in lowland, submontane and upper montane forests. A large number of endemic and threatened species in the submontane forest areas might be due to the intermediate environmental conditions in the mid-elevation zones, which accommodate both high and low elevation specialists (McCain, 2010). However, the same zone has suffered from severe forest loss in recent years (Burgess et al., 2002) and it is not clear how this has been affecting reptiles. We hope that the recent upgrading of the protection status of the reserve will reduce the destruction activities that have been going on in submontane forests.

Farmland zone had fewer forest dependent species compared to other zones which agree with Burgess et al. (2007) who reported most EAM endemic species as specialists of dense forests. Our findings also highlight how the type of farming (e.g commercial tree plantation and some natural vegetation around/in the farm plots) might influence reptile assemblage and shows the potential of the farms surrounding the USNFR in buffering the montane forests. Some strictly forest-dependent species were found at the forest edge and can act as important indicators of ecosystem health following more studies. Therefore, land-use planning is highly important, particularly in the farmlands as the endemic species were found mainly in natural forest patches, fruit trees and commercial tree plantations near the USNFR, of which the species might decline in the future without proper land management.

While we have gathered data on reptiles from many more sites and over a prolonged period in the wet season compared to any other study in the Udzungwa mountains, there is still a need for subsequent surveys in the area, both in the dry and wet seasons. This is especially important, as the current species accumulation curves have not yet reached an asymptote showing the possibilities of getting new records. Some reptile species are highly secretive, have low population densities and/or are locally distributed (Spawls et al., 2004; Meng et al., 2016), making it possible to miss them when sampling only in one season. Since we found only a few reptiles (especially chameleons in the genus *Trioceros*) more than 10 m high, we recommend more efforts on sampling canopy dwellers (e.g., use of arboreal traps in future studies). Similarly, sampling all zones simultaneously might provide more meaningful data, which, was not possible in our study due to logistical constraints. Three to five years of consecutive trapping (McDiarmid et al., 2012) across various seasons (Stanley, 1998; Howell, 2002) has been recommended in order to increase the probability of recording rare species. Subsequent surveys will also enable documenting species that this study failed, adding to the conservation value of not only the USNFR and Udzungwa mountains but the entire EAM region. This article has shown the importance of re-assessing the herpetofauna of EAM, and adds to the importance of conserving these mountains.

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APPENDICES

Appendix 1. Reptile species, threat category, and endemism per families recorded in Uzungwa Scarp Nature Forest Reserve and surrounding areas. Note; * = species that were recorded by Menegon and Salvidio 2005, φ = recorded by Menegon and Salvidio but not surely in USFNR (either from general bibliography or from surrounding villages); ‡ = collected by Lyakurwa 2017 and not found by this study, Y = endemic, f = forest visitor, F= mainly forest, FF= strictly confined to forest.

Species	Voucher	Lowland	Submontane	Montane	Farmland	Threat category	Forest dependency	Endemic/ Near endemic
Agamidae								
Agama mossambica Peters, 1854		X				LC		
Chamaeleonidae								
Kinyongia sp	JVL 1709		X			NT	FF	Y
Kinyongia oxyrhina (Klaver & Böhme, 1988)			X	X		NT	FF	Y
Rhampholeon moyeri Menegon, Salvidio & Tilbury, 2002			X	X		LC	FF	Y
Rieppeleon brevicaudatus (Matschie, 1892)		X	X			LC		
Trioceros deremensis (Matschie, 1892)	JVL 1718		X			LC	FF	Y
Trioceros laterispinis (Loveridge, 1932) φ						EN	F	Y
Trioceros tempeli (Tornier, 1899)				X	X	LC	F	Y
Trioceros werneri (Tornier, 1899)				X	X	LC	F	Y
GEKKONIDAE								
Cnemaspis cf dickersonae (Schmidt, 1919)	JVL 1735, JVL 1733, JVL 1733		X			LC		
Cnemaspis uzungwae Perret, 1986	JVL 1712	X	X			VU	FF	Y
Hemidactylus mabouia (Moreau de Jonnès, 1818)	JVL 1724	X				LC		
Hemidactylus platycephalus Peters, 1854	JVL 1725	X				LC		
Hemidactylus sp	JVL 1723	X						
Lygodactylus capensis (Smith, 1849)		X			X	LC		
Lygodactylus cf angularis Günther, 1893	JVL 1701,	X	X		X	LC		
Lygodactylus grotei Sternfeld, 1911		X			X	LC		
Urocotyledon wolterstorffi (Tornier, 1900)	JVL 1737, JVL 1722	X	X			VU	FF	Y
Gerrhosauridae								
Broadleysaurus major (Duméril, 1851)	JVL 1727				X	LC		
	Opportunistic in lowland farms							
Scincidae								
Leptosiaphos kilimensis (Stejneger, 1891)	JVL 1707, JVL 1706		X			LC		
Melanoseps loveridgei Brygoo & Roux-Estève, 1982 *						LC		
Melanoseps uzungwensis Loveridge, 1942	JVL 1710, JVL 1711, JVL 1731, JVL 1732, JVL 1731, JVL 1732	X	X			EN	FF	Y
Mochlus afer (Peters, 1854)	JVL 1715, JVL 1716		X			LC		
Mochlus sp	JVL 1719	X						
Scelotes uluguruensis Barbour & Loveridge, 1928 *						VU	FF	Y
Trachylepis maculilabris (Gray, 1845)	JVL 1719	X	X			LC		
Trachylepis striata (Peters, 1844)		X				LC		
Trachylepis varia (Peters, 1867)			X	X	X	LC		
Varanidae								
Varanus niloticus (Linnaeus, 1766)		X				LC		
Atractaspidae								
Aparallactus sp	JVL 1729, JVL 1721	X	X					
Atractaspis aterrima Günther, 1863	JVL 1708, JVL 1720	X	X			LC		

Species	Voucher	Low-land	Sub-mon-tane	Mon-tane	Farm-land	Threat category	Forest depend-ency	Endemic/ Near endemic
Colubridae								
Boaedon fuliginosus (Boie, 1827)		X		X	X	LC		
Crotaphopeltis tornieri (Werner, 1908)			X			LC	FF	Y
Dasypeltis medici Bianconi, 1859 *						LC		
Dipsadoboa werneri (Boulenger, 1897) *						NT	FF	Y
Philothamnus hoplogaster (Günther, 1863)	JVL 1703	X	X	X	X	LC		
Philothamnus macrops (Boulenger, 1895)		X	X			LC	F	Y
Philothamnus punctatus Peters, 1867			X			LC		
Philothamnus semivariegatus (Smith, 1840) φ						LC		
Telescopus semiannulatus Smith, 1849		X				LC		
Thelotornis kirtlandii (Hallowell, 1844) *						LC		
Thelotornis mossambicanus (Bocage, 1895)		X	X			LC		
Xyelodontophis uluguruensis Broadley & Wallach, 2002 ‡						EN	FF	Y
Elapidae								
Dendroaspis angusticeps (Smith, 1849)		X				LC		
Naja cf melanoleuca Hallowell, 1857		X	X			LC		
Lamprophiidae								
Gonionotophis nyassae (Günther, 1888)	JVL 1724	X				LC		
Lycodonomorphus whytii (Boulenger, 1897)	JVL 1713			X	X	LC		
Lycophidion uzungwense Loveridge, 1932					X	LC	F	Y
Natricidae								
Natriciteres variegata (PETERS, 1861) Ϯ						LC		
Psammophiidae								
Psammophis tanganicus Loveridge, 1940		X				LC		
Psammophylax variabilis Günther, 1893	JVL 1704, JVL 1705		X	X	LC			
Pseudoxyrhophiidae								
Buhoma procterae (Loveridge, 1922) Ϯ						VU	FF	Y
Duberria lutrix (Linnaeus, 1758)				X		LC		
Pythonidae								
Python natalensis Smith, 1840 ‡						LC		
Typhlopidae								
Afrotyphlops nigrocandidus (Broadley & Wallach, 2000)	JVL 1702		X		X	VU	FF	Y
Viperidae								
Atheris barbouri Loveridge, 1930 φ						VU	F	Y
Atheris ceratophora Werner, 1896			X	X		VU	F	Y
Bitis arietans Merrem, 1820 φ						LC		
Bitis gabonica Duméril, Bibron & Duméril, 1854 φ						VU		
Causus defilippii (Jan, 1863) φ						LC		

Appendix 2. Bray-Curtis species similarity index summary for the 12 sites surveyed in the Uzungwa Scarp Nature Forest Reserve and adjacent areas from December 2017 to April 2018. Note; 0 represents no similarity (100% dissimilarity) while 1 represents 100% similarity. Low=Lowland, Sub = Submontane, Mon= Montane, Farm= Farmland. Numbers in bold indicate more strongly related sites (>50%) while those italicized indicate 100% dissimilarity.

	Farm 1	Farm 2	Farm 3	Mon 1	Mon 2	Mon 3	Sub 1	Sub 2	Sub 3	Low 1	Low 2
Farm 2	**0.5634**										
Farm 3	**0.8400**	**0.6575**									
Mon 1	0.0625	0.0364	0.0588								
Mon 2	0.1951	0.2813	0.1861	0.3200							
Mon 3	0.0377	0.0264	0.0364	0.2703	0.3044						
Sub 1	0.0364	0.0513	0.0351	0.2051	0.2083	0.4333					
Sub 2	0.0615	*0.0000*	*0.0000*	0.0408	*0.0000*	0.0286	0.2222				
Sub 3	0.1200	0.0882	0.0769	0.2353	0.2791	0.2182	**0.5263**	0.2887			
Low 1	0.0377	0.1316	0.0364	0.0541	0.0435	0.0345	0.0333	0.1714	0.1091		
Low 2	0.0526	*0.0000*	*0.0000*	*0.0000*	*0.0000*	*0.0000*	0.2169	**0.5591**	0.2308	0.2716	
Low 3	0.1000	0.0317	0.0952	0.0833	0.0606	0.0444	0.1277	0.1053	0.0952	0.2667	0.1765

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Experience of predacious cues and accessibility to refuge minimize mortality of *Hylarana temporalis* tadpoles

Santosh Mogali*, Bhagyashri Shanbhag, Srinivas Saidapur

*Department of Zoology, Karnatak University, Dharwad-580 003, Karnataka State, India. *Corresponding author*. E-mail: santoshmogali@rediffmail.com

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Abstract. We explored the effect of a prior experience of predation threat and access to the refuge sites on larval mortality in *Hylarana temporalis* with a 2 × 2 factorial design. The design included predator-naïve or predator experienced prey subjects, and presence or absence of a natural shelter. Water scorpion adult individuals (*Laccotrephes* species) provided predation threat and also an opportunity to the prey to experience a direct danger of predation. Both previous experience with predators and availability of shelters affected larval survival independently and not conjointly. A prior encounter with predators enabled prey tadpoles to escape predation more effectively with a significant increase in their survival in comparison to the predator-naïve subjects. Similarly, access to refuge sites significantly increased survival of predator-naïve as well as predator experienced tadpoles compared to that in the absence of shelters. Clearly, ability to sense water borne predacious cues in the vicinity and use refuge sites plays a key role in escaping from predation in the bronze frog tadpoles.

Keywords. *Hylarana temporalis*, *Laccotrephes* sp., predator, refuge availability, tadpole mortality.

Predator-prey interactions invariably lead to an evolutionary arms race in which early detection of either party is often the key for success. Progressive elimination of prey individuals from the ecosystem by the coexisting predators can have far reaching impact on their population dynamics. Most anuran amphibians show a biphasic mode of life cycle involving aquatic larval phase. In the aquatic phase of life the larval mortality is often due to desiccation of the ephemeral ponds before completion of the metamorphosis, infection, and to predation by the coexisting aquatic invertebrate and vertebrate predators. Therefore, adoption of phenotypic plasticity and diverse strategies become essential for their survival. Many anuran larvae including *Hylarana temporalis* are known to alter their behavior and the life-history traits under predation risk (Relyea, 2007; Sharma et al., 2008; Saidapur et al., 2009; Mogali et al., 2012, 2015, 2016; Mogali, 2018). For example, *H. temporalis* tadpoles metamorphosed early and at a larger body size under predation threat, when provided by a caged dragonfly larva (*Pantala flavescens*), compared to those reared under predation threat-free environment (Mogali et al., 2016). They also decreased swimming activity, by remaining still for longer times, to avoid being detected and increased burst speed on encounter with the predacious cues in the vicinity (Mogali et al., 2012). Taking shelter under the leaf litter, aquatic vegetation and other objects that provide refuge and utilization of benthic habitat can help tadpoles by reducing the risk of being detected by predators (Lima and Dill, 1990; Eklov and Persson, 1996; Hossie and Murray, 2010). Besides, predators may find difficult to capture a prey residing inside a refuge; at the same time, prey may have a better opportunity to assess the actual risk of predation by the predators residing in close vicinity (Hemmi and Zeil, 2005) and, consequently, evoke defensive behaviors. Nevertheless, hiding and stay-

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ing still by prey involve trade-offs. These strategies may reduce encounter rates with predators but also tend to reduce time dedicated to foraging activity. Indeed, often the habitats that are energetically very profitable are also the most dangerous, since the distribution of predators tends to match their prey's resource distribution (Lima, 1998). Besides, the refuge may not always be the best place for feeding. For instance, inadequate time spent in feeding leads to alteration in the metamorphic traits in *Bufo melanostictus* (Mogali et al., 2011).

The bronze frog (*Hylarana temporalis*) tadpoles are found along the gently flowing streams and isolated pockets of water along sides of the streams during the post-monsoon season (October-January) in South-Western Ghats of India (Mogali et al., 2012). They are bottom dwellers and thrive on detritus and algal matter (Hiragond and Saidapur, 2001). In these water bodies visibility is low due to shadows of the dense vegetation, brownish-dark color of the benthic area that is typically covered by the leaf litter and detritus matter (Veeranagoudar et al., 2004). The water bodies are also inhabited by several types of invertebrate predators that include aquatic beetles, dragonfly larvae, crabs and water scorpions that prey upon anuran tadpoles (our personal observations). During field visits we have observed water scorpions (*Laccotrephes* sp., Nepidae) actively feeding on bronze frog tadpoles. They detect their prey visually and by mechanical cues. The *H. temporalis* tadpoles have poor vision (Veeranagoudar et al., 2004) as in most anuran tadpoles (Hoff et al., 1999). However, they possess an innate ability to detect predators by chemoreception (Mogali et al., 2012) and evoke antipredator behavior. Further, predator-experienced *H. temporalis* tadpoles show enhanced defensive behaviors on subsequent encounter with predators (Mogali et al., 2012).

The natural habitats in which *H. temporalis* tadpoles live is full of detritus material and leaf litter. Therefore, we hypothesized that tadpoles of the bronze frog profitably use these shelters (leaf litter) to reduce predation risk of the coexisting predators. An additional assumption was that tadpoles having prior experience of predacious cues will be more successful in escaping predation by using shelters compared to the those facing the predators for the first time (predator-naive). To test our hypotheses, tadpoles of *H. temporalis* were exposed to free hunting water scorpion (*Laccotrephes* sp). The prey subjects were either predator-naïve or predator experienced. They were exposed to predator in laboratory set ups with or without the refuge sites (leaf litter) for a predetermined period to record survival/mortality of prey.

Eight clutches of *H. temporalis* were collected in November, 2013 from a stream in the Western Ghats near Anmod village (15.430888°N, 74.373601°E), Karnataka State, India. Each clutch was individually reared in plastic tub (32 cm diameter and 14 cm deep) containing 5 L of aged tap (dechlorinated) water. Hatching time occurred six days later, at Gosner stage 19 (Gosner, 1960). The tadpoles of different clutches were mixed to normalize genetic differences among the groups. After mixing, tadpoles were reared in two separate glass aquaria (90 cm L × 30 cm W × 15 cm H) each containing 25 L of aged tap water. Upon reaching stage 25, tadpoles were supplied with boiled spinach *ad libitum*. The water scorpions (*Laccotrephes* sp.) were collected from the same location from where the eggs of *H. temporalis* were obtained and were reared individually, to avoid cannibalism, in small plastic bowls (14 cm diameter and 7 cm deep) with 500 mL of aged tap water. Prey tadpoles served as food for water scorpions.

The tadpoles born in laboratory, and never exposed to predator, served as the predator-naïve subjects. To obtain predator-experienced tadpoles we exposed groups of 30 tadpoles to a 48 h starved predator for 8 h (09:00-17:00 h). After the 8 h trial period the predator and injured tadpoles were removed from the test bowl. On an average, predator ate 3 ± 0.3 tadpoles and injured 4 ± 0.5 (x ± SE) during the trial period. Predator-threat experienced and uninjured tadpoles obtained from 80 trials were used in the experiments the following days, in order to assess their performance against predators on the subsequent encounter with them.

The leaves of *Aporosa lindleyana* were collected from the same sites from which *H. temporalis* eggs were collected. Structural refuge was made using water soaked (2 days) leaves (dry mass 15 g ± 1.8; x ± SE) chopped to have ~ 1 cm^2 pieces. These were spread at the bottom of the testing bowls to serve as shelters/ refuge site).

In all trials, prey tadpoles were of comparable sizes (length 20.48 ± 0.09 mm, width 5.31 ± 0.07 mm, and weight 62.0 ± 0.64 mg; x ± SE; n = 30) and developmental stages (stages 28-30). The water scorpions used in various trials were comparable in size (length 31.0 ± 0.08 mm, width 10.11 ± 0.05 mm, and weight 622.0 ± 5.2 mg; x ± SE; n = 30). The size of prey and predators was measured using a digital caliper (accuracy 0.01 mm) and weight was recorded using an electronic balance (accuracy 0.001 g).

The experiment involved using a 2 × 2 factorial design with the following groups.

1. Predator-naïve tadpoles were exposed to predator in the absence of refuge
2. Predator-naïve tadpoles were exposed to predator in the presence of refuge
3. Predator-experienced tadpoles were exposed to predator in the absence of refuge

4. Predator-experienced tadpoles were exposed to predator in the presence of refuge

Each treatment consisted of 30 trials (overall 120 trials).

For each trial the test tadpoles were released (n = 30) into the plastic tub (32 cm diameter and 14 cm deep) containing 3 L of aged tap water and with or without the structural refuge and allowed to familiarize themselves to the tub for 15 min. Then one water scorpion starved for 48 h was introduced gently into the tub and left there. The trials were terminated after 24 h. The number of surviving tadpoles in various trials was recorded to compute the number of tadpoles lost due to predation. Data were analyzed using two-way analysis of variance for assessing the overall consequence of a prior exposure to predaceous cues and the access to the refuge and their interaction on prey survival. Data on number of tadpoles consumed between the two treatment groups were analyzed by independent-samples t test.

Two-way ANOVA showed significant main effect of predator exposure (P < 0.001, Table 1) and refuge availability (P < 0.001, Table 1) but not of their interaction (P = 0.253, Table 1).

Accessibility to the refuge significantly reduced the larval mortality in predator-naïve tadpoles when compared to that without refuge (t_{58} = 4.402, P < 0.001, Fig. 1A). A similar trend was also evident in the case of predator-threat experienced tadpoles (t_{58} = 5.648, P < 0.001, Fig. 1B). The larval mortality was significantly lower in predator-experienced tadpoles compared to predator-naïve tadpoles regardless of the availability (t_{58} = 4.144, P < 0.001) or unavailability (t_{58} = 4.353, P < 0.001) of refuge sites (Fig. 1).

The present study shows the importance of available shelters and of a prior experience with predator in evoking defensive behaviors and enhances escape predation in *H. temporalis* tadpoles. Both these factors independently and not conjointly affect the larval survival following their encounter with water scorpion. The results reveal that accessibility to refuge sites and prior experience of predation threat are key determinants of survival from the aquatic insect predators in *H. temporalis* tadpoles. Previous studies have shown that bronze frog tadpoles exhibit antipredator defense behaviors in response to water borne chemical cues of predator by staying away from the predator and reducing their swimming activity (Mogali et al., 2012). Our study revealed a heightened defense response in predator-experienced tadpoles in the subsequent encounters with the predator (Mogali et al., 2012) in conformity with the observations reported on other anuran species (Semlitsch and Reyer, 1992; Hettyey et al., 2011). The results of the present study show that predator-experienced tadpoles learn to escape predation and become less vulnerable to predator compared to the predator-naive individuals regardless of the accessibility to structural refuge. This clearly demonstrates that prior experience with predator plays a key

Table 1. Results of two-way ANOVA for exposure and refuge availability and their interactions. The response variable is mean number of *Hylarana temporalis* tadpoles lost due to predator. Significant values are bolded.

Source	df	MS	F	P
Exposure	1	108.300	35.382	**<0.001**
Refuge availability	1	132.300	43.222	**<0.001**
Exposure × refuge availability	1	4.033	1.318	0.253

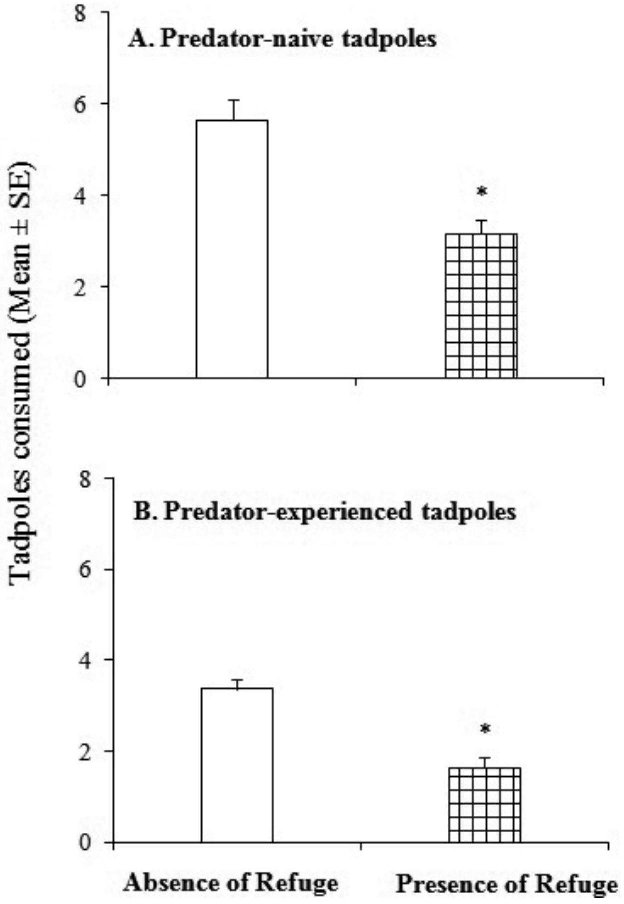

Fig. 1. Number of predator-naïve and predator-experienced *Hylarana temporalis* tadpoles consumed by the predator, *Laccotrephes* sp., in relation to accessibility to refuges in trials of 24 h. An asterisk over the bar indicates a significant difference between two treatments. Data represents x ± SE. Each trail consists of 30 tadpoles of either predator-naïve or predator-experienced. 30 trials were carried out for each treatment and total all together 120 trials.

role in enhancing survival chances of tadpoles by escaping predation. Healey and Reinhardt (1995) made similar observation on Coho salmon against the predacious Rainbow trout. Álvarez and Nicieza (2006), who studied *Rana temporaria* tadpoles, showed that 48 h association with the predator was a sufficient period of time to enhance their ability to escape predation. The present study on *H. temporalis* shows that even mere 8 h exposure to predatory cues is sufficient to evoke a stronger effective defense behavior on subsequent encounters with the predator. Possibly, even a shorter time exposure might be sufficient for prey tadpoles to evoke their defensive behavior on following encounters with predators. However, additional studies are needed to establish the minimum period of exposure to predators that is required to condition the prey and modify their future performance of defensive behaviour. Further, releasing predacious cues that remain effective for longer periods may not be in the interest of the predator. Indeed, several studies showed that predaceous cues are labile in nature (Peacor, 2006; Sharma et al., 2008) and their half life ranges from 0.2 h to a few days (Van Buskirk et al., 2014). Thus, it appears that persistence of predatory cues and their ability to evoke defense behaviors in prey may vary among anuran tadpoles.

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Tonal calls as a bioacoustic novelty in two Atlantic Forest species of *Physalaemus* (Anura: Leptodactylidae)

Thiago R. de Carvalho[1,2,*], Célio F.B. Haddad[1], Marcos Gridi-Papp[2]

[1] *Laboratório de Herpetologia, Departamento de Zoologia e Centro de Aquicultura (CAUNESP). Universidade Estadual Paulista, Rio Claro, SP, Brazil. *Corresponding author*. Email: thiago_decarvalho@yahoo.com.br
[2] *Department of Biological Sciences, University of the Pacific, Stockton, CA 95211, USA*

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Abstract. The frog genus *Physalaemus* has almost 50 species with vocalizations that are mostly composed of a single note. This note tends to have a broad harmonic structure or a pulsed structure. The sister species *P. lateristriga* and *P. olfersii* have pulsed advertisement calls that have been described as a noisy and long-lasting warbling sound. We provide the first account of inclusion of tonal sounds as part of the vocal repertoire of these species. Pure tones can (1) be long and form the entire call; (2) form prefixes of variable length separated by silence from the advertisement call; (3) be brief and form the onset or the offset of the regular advertisement call. Tonal calls may be an evolutionary novelty and they are not known from other populations of *P. olfersii* and *P. lateristriga*. Identification of the mechanism of sound production and of the behavioural roles of these unique calls may help elucidate the evolution of call complexity in frogs.

Keywords. Animal communication, bioacoustics, Leiuperinae, vocal repertoire.

INTRODUCTION

Most anurans communicate using stereotyped signals with relatively simple acoustic structure (Gerhardt and Huber, 2002; Köhler et al., 2017). Various call traits can be shared among the species of a clade and yet enough variation is present, in most cases, to make the advertisement call of each species unique. Advertisement call differences can form major prezygotic isolation barriers and can be used to separate morphologically cryptic species (Blair, 1958; Johnson, 1966).

The vocal repertoires of frogs in the Neotropical genus *Physalaemus* are almost always simple with calls composed of a single note (Tárano, 2001; Giaretta et al., 2009). The calls of this group are of particular interest because its sister genus *Engystomops,* which used to be included in *Physalaemus*, contains the túngara frog (*E. pustulosus*; Lynch, 1970; Nascimento et al., 2005; Lourenço et al., 2015), a model organism in behavioural and acoustic communication studies. A variety of studies has explored the evolution of an optional secondary pulsed sound (chuck) that the male túngara frog can add to the end of his regular advertisement call (whine; Ryan, 1985). The laryngeal mechanism underlying the addition of the chuck has not been fully described, however, and further insight may be gained from comparisons with other species in the group.

In both *Physalaemus* and *Engystomops*, advertisement calls tend to be long and have a pronounced harmonic structure with a smooth amplitude envelope (Cannatella et al., 1998; Wilczynski et al., 2001; Provete et al., 2012). Descending frequency modulation throughout the call is very common with most of the change concentrated at the beginning. Some species, however, have pulsed calls

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Table 1. Acoustic terminology adopted in this study.

Traits	Description
Temporal traits	
Call length (sec)	Time from initial 10% to final 10% of amplitude of one call
Rise time (%)	Point of maximum amplitude relative to call length
Call rate per minute	(Total number of calls - 1) / time from beginning of first call to beginning of last call
Spectral traits	
Dominant frequency (Hz)	Frequency containing the greatest energy in one call. It matches the fundamental frequency in tonal calls.
Frequency Modulation (Hz)	Dominant frequency at 10% final minus that at initial 10% of one call

and lack the descending frequency modulation (Bokermann, 1966; Padial and Köhler, 2001; Weber et al., 2005b). *Physalaemus olfersii* and *P. lateristriga* are examples of this latter type of advertisement call (Drewery et al., 1982; Giaretta et al., 2009; Cassini et al., 2010). Based on acoustic structure and laryngeal morphology, their call was speculated to be possibly produced in the same manner as the chuck encountered in the call of the túngara frog (Drewery et al., 1982).

Here, we report on previously unknown call complexity in the genus *Physalaemus* generated through the incorporation of tonal sounds. We document its occurrence in *P. olfersii* and *P. lateristriga*. We also discuss the potential role and the mechanism underlying this striking acoustic novelty based on the vocal biomechanics of the group.

MATERIAL AND METHODS

Vocalizations were recorded in the field using analogue recorders (Marantz PMD 420 with Sony Metal-SR 100 Audio Cassette) and supercardioid microphones (Sennheiser ME-80) positioned at an approximate distance of 2 m from the calling males. The recordings were digitized at a 44.1-kHz sampling rate and 16-bit sample size. Calls were recorded from two localities in the Brazilian state of São Paulo (south-eastern Brazil): *Physalaemus olfersii*—Parque Estadual da Serra do Mar, Núcleo Santa Virgínia, São Luiz do Paraitinga (-23.332200°, -45.096828°; 979 m a.s.l.); *Physalaemus lateristriga*—Ribeirão Branco (-24.358600°, -48.743000°; 840 m a.s.l.). Voucher specimens and recordings are housed at the Célio F. B. Haddad collection (CFBH). Information on sound recordings is as follows: *Physalaemus lateristriga*—(1) CFBH 63, recorded on 4 February 1995, at 22:20 h, air temperature 19°C; (2) MGP 5011401, recorded on 14 January 2005, at 19:30 h, air temperature 19°C, water temperature 22°C; call voucher is one male of the series CFBH 16564-16567, 16569-16575, 16577-16582, 16584-16585. *Physalaemus olfersii*—(1) MGP 4111701-4111705, recorded on 17 November 2004.

The acoustic analysis was conducted in Soundruler (Gridi-Papp, 2007), a package of MATLAB scripts (Matlab, 2004) that allows for unbiased quantification of acoustic traits using automated procedures. Call rate, however, was measured manually in Audacity (Audacity Team, 2017). The settings used in the frequency analysis included FFT size = 1024 samples, FFT overlap = 90%, window type = Hanning, contrast = 70%. Settings for automated recognition of pulses were (in samples): pulse detection (smoothing = 250, resolution = 25); pulse delineation (smooth factor = 1, smoothing = 25, resolution = 1). Settings for frequency tracking range: 1000 Hz each step. Critical amplitude ratio: CFBH 63 (-1; disabled); MGP 5011401 (5.0). A 500-Hz high-pass filter at 48 dB was applied to the sound file CFBH 63 in Audacity to reduce background noise. Temporal traits were measured from oscillograms and spectral traits from spectrograms. The acoustic terminology is summarized in Table 1. A call-centred approach (*sensu* Köhler et al., 2017) was used for the acoustic characterization of acoustic signals described in the present study.

RESULTS

Tonal calls were recorded from two males of *P. lateristriga* that only produced tonal calls during the recording. Three males of *P. olfersii* were also recorded making tonal calls but these were shorter and positioned as prefixes or suffixes of the regular (pulsed) advertisement calls.

The two males of *P. lateristriga* that only produced tonal calls were recorded at the same locality and temperature but with a separation of 10 years. Both exhibited an interruption within the call but it corresponded to a frequency shift in one individual and to a silent gap in the other. The tonal calls (n = 8; Fig. 1A) of the first male *P. lateristriga* (CFBH 63) lasted 0.83–1.40 sec (1.18 ± 0.09; min–max, x ± SD) and were emitted at a rate of 17.8 calls/min. Rise time was 8.3–89.3% (62.4 ± 27.1) of the call's length. The dominant frequency of the call was 1572–1701 Hz (1641.9 ± 51.2). The call started at about 1500 Hz and gradually modulated up to approximately 1750 Hz. An abrupt shift in frequency separated the initial part of the call from the last 10–20% (see Fig. 1A). The final section of the call had a downward frequency modulation approximately from 1300 Hz to 1100 Hz.

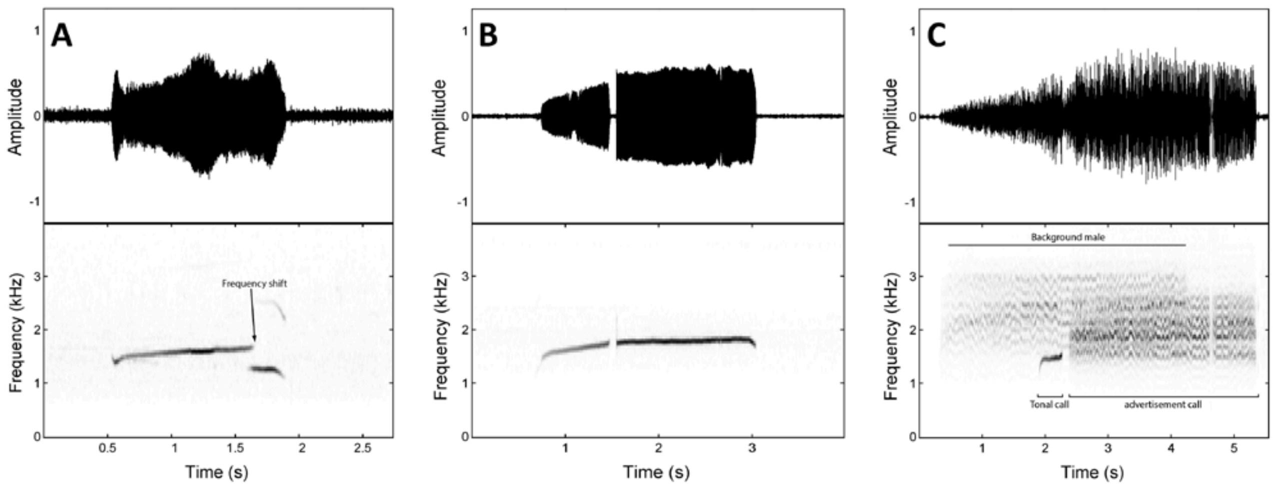

Fig. 1. (A-B) Two males of *Physalaemeus lateristriga* recorded from Ribeirão Branco (São Paulo, Brazil): (A) Tonal call from the recording CFBH 63; (B) Tonal call from the recording MGP 5011401. (C) Male *Physalaemus olfersii* recorded from Santa Virgínia (São Paulo, Brazil). A vocal bout from the recording MGP 4111701 depicting a short tonal call followed by a typical advertisement call (overlapped by another male calling in the background).

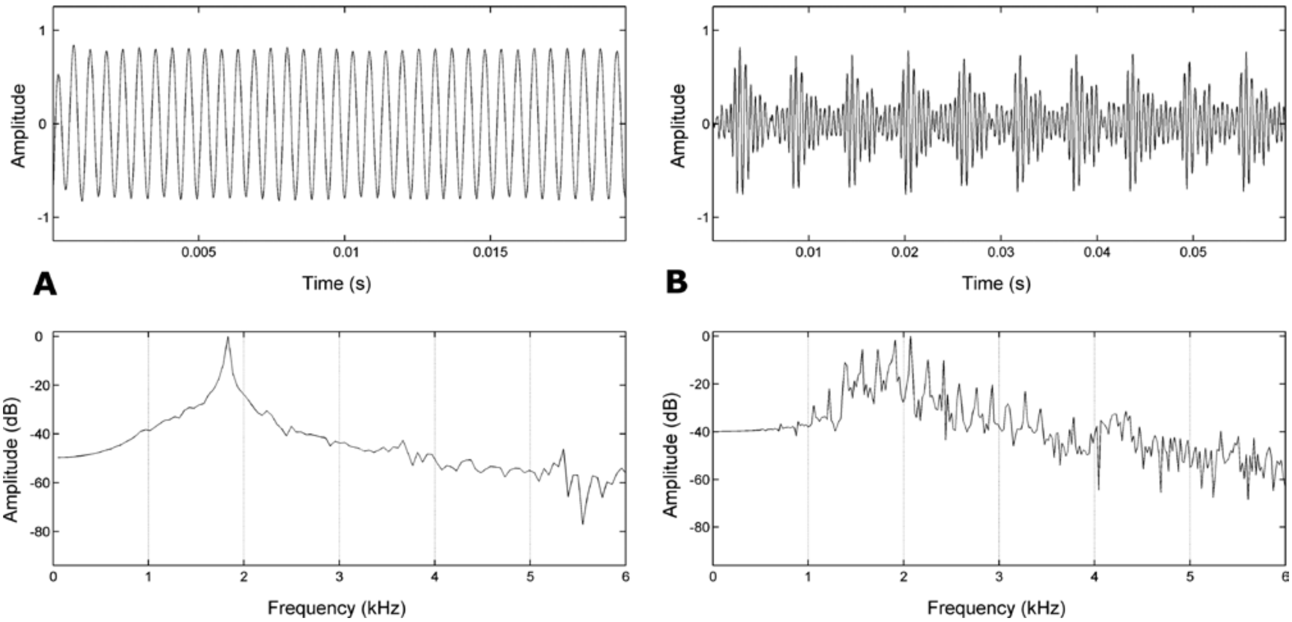

Fig. 2. Waveform and 4096-FFT amplitude spectrum of (A) a 20-msec section from a tonal call (recording MGP 5011401; *P. lateristriga*), and a 60-msec section from (B) a typical advertisement call (MGP 4111704; *P. olfersii*), respectively.

The tonal calls (n = 14; Fig. 1B) of the second male *P. lateristriga* (MGP 5011401) lasted 0.90–2.57 sec (2.12 ± 0.39) and were emitted at a rate of 8.84 calls/min. Rise time was 33.5–95.2% (66.1 ± 20.4) of the call's length. The calls had a silent gap of 0.0689-0.1076 sec (0.0817 ± 0.0105) near the first third or half of the call's length (see Fig. 1B). The duration of the first part of the call varied from 0.33 to 1.05 sec (0.78 ± 0.19), while that of the second part varied from 0.22 to 1.63 sec (1.09 ± 0.46). The dominant frequency was 1787–1830 Hz (1811.9 ± 22.1). Ascending frequency modulation occurred throughout the call, stabilizing in the last 25%, with a drop in frequency of only 50-100 Hz in the last 10%. The dominant frequency started at 1450–1600 Hz (1470.4 ± 55.0) and ended at 1700–1750 Hz (1728.8 ± 21.4).

Unlike *P. lateristriga*, males of *P. olfersii* did not produce call bouts containing only tonal calls in our recordings. Three male *P. olfersii* (MGP 4111701-3) emitted (Fig. 1C) a tonal call shortly followed by the typical pulsed advertisement call. However, the tonal portion was consistently shorter than those described earlier for *P. lateristriga*. All three males of *P. olfersii* were recorded while calling in antiphony with neighbouring conspecific males (Fig. 1C). Two other recorded males of *P. olfersii* (MGP 4111704-5) did not emit tonal calls, but changed to a tonal-like structure at the very final portion of a few calls: 50-70 msec in duration and dominant frequency of 1250-1750 Hz.

In both species, the tonal calls were very narrowly tuned whereas the pulsed calls had energy distributed through a wide frequency range (Fig. 2). The dominant frequency was always centred near 1700 Hz but in the tonal calls the amplitude of the fundamental frequency was 40 dB higher than that of any other harmonic, whereas in the pulsed calls the amplitude of the dominant frequency was less than 5 dB higher than that of the closest side-band.

DISCUSSION

The acoustic structure of the tonal calls diverged sharply from that of the pulsed advertisement calls in *Physalaemus lateristriga* and *P. olfersii*. The striking contrast between these structures made *P. olfersii* males stand out acoustically from the chorus when they added tonal sounds to their calls. Tonal calls also made *P. lateristriga* stand out in their diverse acoustic assemblage of tropical anurans and insects due to the rarity of tonal sounds in nature (Rossing, 2007).

A single other species of *Physalaemus* (*P. fernandezae*) has been reported to produce tonal sounds (Barrio, 1965). This species, different from *P. lateristriga* and *P. olfersii*, appears to always produce tonal-only advertisement calls and no other call types have been described. The brief acoustic description provided by Barrio (1965) indicates other similarities with the calls recorded in our study. In addition to being tonal, the calls of *P. fernandezae* have high fundamental frequency and ascending frequency modulation. These shared characteristics are unlikely to reflect common descent because *P. fernandezae* is not closely related to the *P. olfersii* group, within which *P. olfersii* and *P. lateristriga* are sister taxa (Lourenço et al., 2015). Tonal calls are therefore likely to have evolved independently in these two groups and the accompanying acoustic similarities may reflect a common laryngeal mechanism for the production of tones rather than homology.

The addition of a tonal prefix or suffix adds complexity to the advertisement call of *P. olfersii*. A complex vocal repertoire has been described for *P. spiniger* but it did not include tonal sounds (Haddad and Pombal, 1998; Costa and Toledo, 2013). In *Engystomops*, some species (*E. pustulosus, E. petersi*) are known to optionally add sounds with a distinct acoustic structure to their advertisement call as ornaments that make the call more attractive to females (Ryan and Rand, 1993).

The behavioural significance of the tonal calls in the *P. olfersii* group is unclear. All recorded individuals of *P. olfersii* and *P. lateristriga* vocalized from the ground within a few meters from the water. Neither females nor other males were observed to interact with the callers during shifts between calls including or not tonal sounds. The callers themselves did not change position, orientation, or indicate any change of behavioural context when switching between emitting or not tonal sounds. In other anurans, the second most commonly reported call type is emitted during aggressive interactions (Wells, 2007), but in *Engystomops pustulosus* the second most common sound (the chuck) increases the attractiveness of the call to females (Ryan, 1985). The tonal call could also be an accidental acoustic consequence of variation in the structure or mechanics of the larynx and may lack an exclusive role.

The mechanistic origin of such distinctive acoustic signals is intriguing. The dominant frequency in both the pulsed advertisement calls and the tonal calls is about 1700 Hz and the main difference between them is the presence or absence of pulsing. This aspect is also present in the advertisement call of *E. pustulosus* in which the chuck is pulsed while the whine is not. Large laryngeal fibrous masses have been described in some species of leiuperine frogs including *E. pustulosus* and *P. olfersii* (Ryan and Drewes, 1990). Such structures attached to vocal cords have proven to be necessary for the production of the chuck but not of the whine (Gridi-Papp et al., 2006). The onset of the chuck in *E. pustulosus* has been suggested to be controlled by muscles that deform the larynx (Drewery et al., 1982; Ryan and Drewes, 1990). Alternatively, a mathematical model indicates that the mechanical interactions between the fibrous mass and the vocal cords may determine the onset of the chuck with base on laryngeal airflow (Kime et al., 2018). One way or the other, the lack of pulsing in the tonal calls of *P. lateristriga* and *P. olfersii* could result from halting the vibration of the laryngeal fibrous masses.

Without pulses, the calls of *P. olfersii* and *P. lateristriga* could be expected to exhibit a pronounced harmonic structure like those of most leiuperine frogs, but instead, they are tonal. This raises the question of whether the tonal calls are being produced by vocal cord vibration or as whistles, by oscillations of airflow as it passes between the arytenoid cartilages. Some evidence in favour of a reduced role of vocal cords can be observed in the frequency modulation of the call. The *P. olfersii* group can present subtle ascending frequency modulation at the attack phase and descending frequency modulation during the decay phase of the call as best seen in *P. feioi*, *P. orophilus*, and *P. soaresi* (Weber et al., 2005a; Cassini et al., 2010). This matched modulation of amplitude and frequency is expected whenever the vocal cords do not block the airflow immediately before the onset and after the offset of the call (Gridi-Papp, 2014). The tonal calls could be produced by the vocal cords, however. While not the highest among leiuperines (see comparison in Provete et al., 2012: Table 5), the advertisement calls of most species in the *P. olfersii* species group have a high dominant frequency (Weber et al., 2005a; Cassini et al., 2010). Extensive filtering of the harmonics could concentrate all the energy in the fundamental frequency and result in tonal calls.

This study shows that males of *P. lateristriga* produce pulsed or tonal calls and males of *P. olfersii* can switch between these two structures within a call. These species offer a unique opportunity for study of the acoustic mechanisms that underlie two call modes that are fixed in other species of *Physalaemus*. Furthermore, tonal calls are not known from other populations of either species, indicating that this might be an opportunity to document the evolution of a novel acoustic signal. Finally, this case seems analogous to that of *E. pustulosus*, in which males optionally incorporate a categorically different signal into their calls. The study of this system may provide new insight into the evolution of diversity in acoustic communication.

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Scientific publication of georeferenced molecular data as an adequate guide to delimit the range of Korean *Hynobius* salamanders through citizen science

Amaël Borzée[1,*], Hae Jun Baek[2,3], Chang Hoon Lee[2,3], Dong Yoon Kim[2], Jae-Young Song[4], Jae-Hwa Suh[5], Yikweon Jang[1], Mi-Sook Min[2,*]

[1] *Division of EcoScience, Ewha Womans University, 03760, Seoul, Republic of Korea. *Corresponding authors.* E-mail: amaelborzee@gmail.com; minbio@yahoo.co.kr
[2] *Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, 08826, Seoul, Republic of Korea*
[3] *National Institute of Ecology, Seocheon, 33657, South Chungcheong Province, Republic of Korea*
[4] *National Park Research Institute, Korea National Park Service, Wonju, 26441, Gangwon Province, Republic of Korea*
[5] *National Institute of Biological Resources, 22689, Incheon, Republic of Korea*

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Abstract. Despite the importance of clearly assessing the distribution boundaries of species, it is not possible for scientists to acquire genetic information and conduct molecular analysis for all populations. Consequently, citizen science is of increasing importance for large scale data collection. In this study, we described the range boundaries of the four *Hynobius* species occurring in Korea based on genetic identification and refined their distribution through citizen science data. The genetic identification of individuals was extracted from the literature, while the citizen science data were extracted from iNaturalist through GBIF. Distribution boundary lines were drawn from the genetic data and consistency with citizen science datapoints was assessed through a comparative analysis with the points found beyond the established boundary lines. Depending on the species, 1.43 to 25.00% of the observations extracted from the citizen science data were located beyond the boundaries suggested by the molecular analyses, with average distances ranging from 3.51 ± 2.97 to 51.47 ± 30.87 km (mean ± SD). We considered these variations negligible in the view of the whole distribution of these species. In general, the distributions extracted from iNaturalist were accurate and adequately representative of the distribution of the species, with the exception of the recently split *H. quelpaertensis*. Additionally, citizen science data highlighted the absence of gaps in the distribution of these species. In conclusion, given the good accuracy of citizen science data, we recommend the publication of molecular based data so that citizen science platforms could help define accurately the range of species for which data is missing or outdated.

Keywords. *Hynobius*, range description, population presence, public engagement, iNaturalist, Republic of Korea, North East Asia, citizen science.

INTRODUCTION

Despite a newly renewed interest in citizen science over the last decades (Reed, 2008; Crain et al., 2014; Jordan et al., 2015), citizen science has been playing a major role in the advancement of natural and ecological sciences over the last centuries (Gray et al., 2017; McKinley et al., 2017), with demonstrated benefit to biodiversity conservation (Newman et al., 2012, 2017; McKinley et al., 2017). Because of the ubiquitous impact of human activities on biodiversity (Pimm and Raven, 2000; Steffen et al., 2011) and the species extinction rate, which could be a thousand

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times higher with respect to pre-human levels (Pimm et al., 1995; Scheffers et al., 2012), large-scale data collection is becoming more and more urgent. Since one of the strengths of citizen science is the quantity of datapoints collectable over a short period of time (Lintott et al., 2010), relying on this discipline to create conservation policies for the remaining species, through ever more convenient and accurate technologies (Sullivan et al., 2009; Joppa et al., 2012; Bowser et al., 2014) is therefore one of the potential ways to breach the wave of destruction.

Citizen science involvement takes several forms and it is assessed to be extremely efficient when conducted with clear protocols and objectives (Shirk et al., 2012; Gray et al., 2017), or combined with molecular analysis (Silvertown et al., 2011). Conversely, not everyone is satisfied by the quality of data collected through citizen science, as discussed by Cohn (2008), Conrad and Hilchey (2011) and Dickinson et al. (2010). It is however impossible for a single researcher to conduct large scale field surveys on the totality of a species' range. For instance, it took four years of field work to describe the comparatively small range of the Suweon Treefrog (*Dryophytes suweonensis*), a non-cryptic and highly detectable species, via a study conducted almost every day of the species' breeding season (Borzée et al., 2017). In comparison, as of 1 February 2018, the totality of the species' range, minus a single location, is available from the citizen science website iNaturalist (https://www.inaturalist.org). The species used as an example is comparatively well studied (Borzée, 2018) but there are other well-studied species which geographic ranges are poorly described (Jetz et al., 2012; Meyer et al., 2015). Additionally, ranges are dynamic and need regular updates, as they can show geographic shifts in response to climate change (Chen et al., 2011).

Given that technologies become increasingly user-friendly and convenient for citizen science, an increase in the quality and resolution of the data uploaded is expected. For instance, it is common to upload datapoints directly from the observation site, including GPS coordinates at the cm resolution, pictures and other metadata. A rising platform for the upload of observations is iNaturalist (www.inaturalist.org), and the platform success in recording species is demonstrated by the presence of data for about 75% of bird species and 35% of amphibian species (as of 1 February 2018). The particularity of iNaturalist is that, despite anyone being able to upload any observation, classified as "Need ID" if fulfilling minimum requirements, the observations are then cross-validated to obtain a "research grade". This requires the ID to be confirmed by at least two-thirds of the identifiers (i.e. anyone interested in confirming or reassigning the species/genera/clade ID of the observation). Therefore, the ID provided does not reflect the knowledge of a single person but that of the community, and therefore of a meta-brain, including scientist expert in their field (Joppa et al., 2012; He and Wiggins, 2015).

Here, we first defined the range of the four *Hynobius* salamander species occurring in Korea through traditional molecular tools, and then refined range and presence within ranges through the platform iNaturalist. The four species are *Hynobius leechii*, *H. quelpaertensis*, *H. yangi* and *H. unisacculus*. The secondary purpose of this work was to highlight the accuracy of citizen science in a region where it is still comparatively under-used (Roh et al., 2014).

MATERIALS AND METHODS

Species

Four of the described *Hynobius* species are present in the Republic of Korea, and three of these species are endemic (Min et al., 2016). *Hynobius leechii* is widespread on the Korean peninsula and North-East China, while *H. quelpaertensis*, *H. yangi* and *H. unisacculus* are restricted to the southern coastal area of the peninsula (Yang et al., 1997, 2001, 2005; Kim et al., 2003; Min et al., 2016). This coastal area is also populated by three candidate species (Baek et al., 2011a, 2011b), although these were not included in our analyses as the clades have not yet been given the species status. The species breed between February and May, both in natural streams and modified landscapes in the form of rice paddies. They are locally abundant species present under vegetation and litter of forested hills outside of the breeding season.

Molecular assessment

The *Hynobius* sequences used here were extracted from the literature (Kim et al., 2003; Yang et al., 2005, 2007; Baek et al., 2011a, 2011b; Min et al., 2016). Each data point for which molecular identification was available, based on any gene sequence, was incorporated in the dataset, resulting on N = 270 for molecular-based species assignment.

Citizen science data

Prior to data download, the citizen science data on iNaturalist (https://www.inaturalist.org) were curated on 15 October 2017 for obvious errors. A query for observations was created with the filters "*Hynobius*" and "South Korea", and a few observations were flagged as "captive" when coming from zoos or private collections, based on GPS coordinates. The citizen science datapoints were then downloaded through GBIF.org (https://doi.org/10.15468/dl.tb0v6j; accessed 5 February 2018), filtered for *Hynobius* observations in the Republic of

Korea and from iNaturalist only, dated up to 15 October 2017. Only the observations reaching "Research Grade" on iNaturalist are transferred to GBIF, and the research grade can only be reached when more than two-thirds of the identifiers agree on a taxon. The original download included 852 datapoints, but all the observations with known issues flagged by GBIF were removed, and duplicated records were deleted. Additionally, only datapoints geolocated with an accuracy of at least three decimal places (100 m resolution) were maintained. Finally, to avoid spatial autocorrelation, any point within 200 m of another point from the same dataset was deleted, in correspondence to the core range of several salamander species (Semlitsch, 1998; Semlitsch and Bodie, 2003). This selection resulted in 468 datapoints, collected between 18 April 2005 and 26 August 2017.

Spatial and statistical analysis

The two datasets where then uploaded on ArcMap 10.5 (Environmental Systems Resource Institute, Redlands, California, USA) and each species was colour coded (Fig. 1). Based on the genetic analyses data, we drew lines joining the border localities of each species. For localities close to the seashore, border lines which guaranteed the smallest distance between the locality and the coast were drawn. Given the geographically representative sampling, in relation to the low vagility of the species and of salamanders in general (Semlitsch, 1998; Semlitsch and Bodie, 2003), we can consider the drawn lines as adequate estimates of each species' range. In addition, since no large-scale hybrid zones are expected between the species in this study (Baek et al., 2011a; Min et al., 2016), we excluded the possibility of a significant misidentifications because of cytonuclear disequilibrium.

Once the boundary of the four species were established, here referred to as "distribution boundary lines", we counted for each species the number of localities identified by citizen science which were external to the distribution boundary lines and measured the distance between the focal locality and the closest distribution boundary line. We did not include datapoints located between DNA identified localities and the sea shore, as not all islands were genetically tested.

The presence points within the species boundary lines were then investigated in GIS through the distance tool for clear gaps in distribution. For this purpose, a gap was defined as one tenth of the longest diagonal crossing the range of the species, here limited to the Republic of Korea.

The distance between datapoints and distribution boundary lines was then statistically tested for differences between species. As the data was not normally distributed for each cell of the design (observation of Q-Q plots), and there were no significant correlations between the four species and distance to the boundary lines (Pearson Correlation; r = 0.09, n = 27, P = 0.663), we used an independent-samples Kruskal-Wallis test to assess the relationship among the distances between datapoints and distribution boundary lines. The statistical analyses were performed with SPSS v21.0 (SPSS, Inc., Chicago, USA).

RESULTS

The DNA based location map used to draw the distribution boundary lines included 270 samples (Fig. 1), divided into 164 datapoints for *H. leechii* (Fig. 2), 45 for *H. quelpaertensis* (Fig. 3), 19 for *H. unisacculus* (Fig. 4) and 43 for *H. yangi* (Fig. 5). The citizen science data included 468 samples, distributed into 350 datapoints for *H. leechii*, 92 for *H. quelpaertensis*, 12 for *H. unisacculus* and 14 for *H. yangi*. There were 27 (13.28%) citizen science datapoints that were external to the distribution boundary lines: 5 for *H. leechii* (1.43% of datapoints), 18 for *H. quelpaertensis* (19.57%), 3 for *H. unisacculus* (25.00%) and 1 for *H. yangi* (7.14%).

We did not find any gap in population presence that was higher than one tenth of the longest diagonal crossing the range of the species within the Republic of Korea. The average distance for datapoints beyond the distribution boundary lines was 37.17 ± 32.54 km (mean ± SD). *H. unisacculus* displayed the shortest average distance (3.51 ± 2.97; n = 3), followed by *H. leechii* (4.75 ± 2.99;

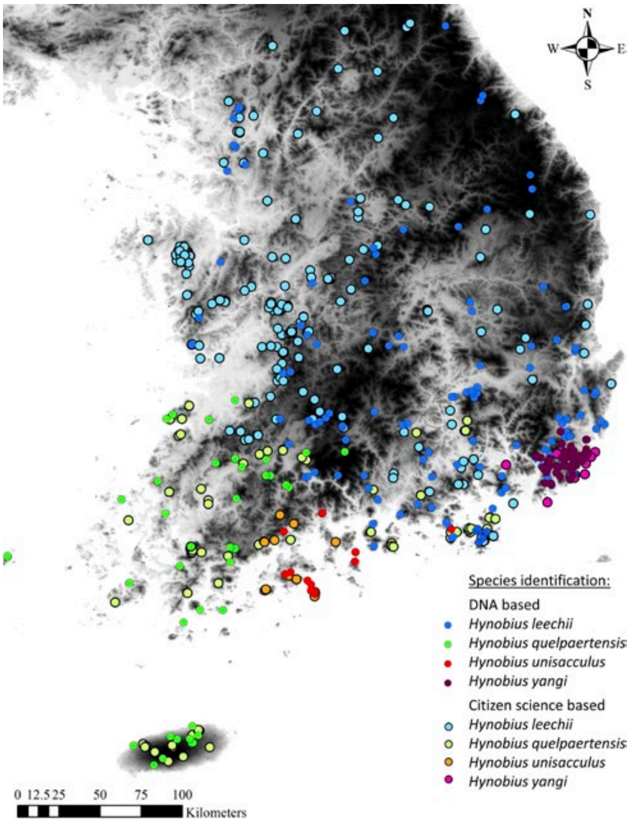

Fig. 1. Distribution of *Hynobius* spp. in the Republic of Korea. The map includes *H. leechii*, *H. quelpaertensis*, *H. unisacculus* and *H. yangi* data extracted from both mtDNA and citizen science (iNaturalist through GBIF; doi.org/10.15468/dl.tb0v6j).

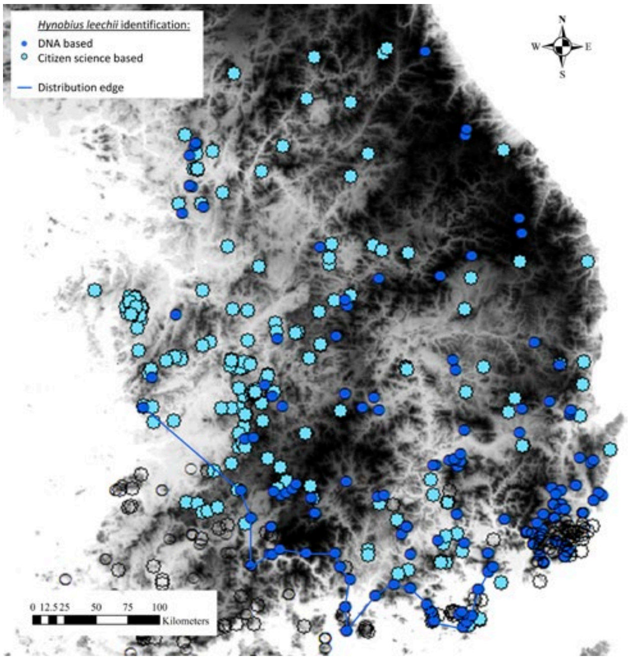

Fig. 2. Distribution of *Hynobius leechii* in the Republic of Korea. The map includes both mtDNA and citizen science datapoints (iNaturalist through GBIF; doi.org/10.15468/dl.tb0v6j), with the distribution boundary lines drawn from mtDNA data.

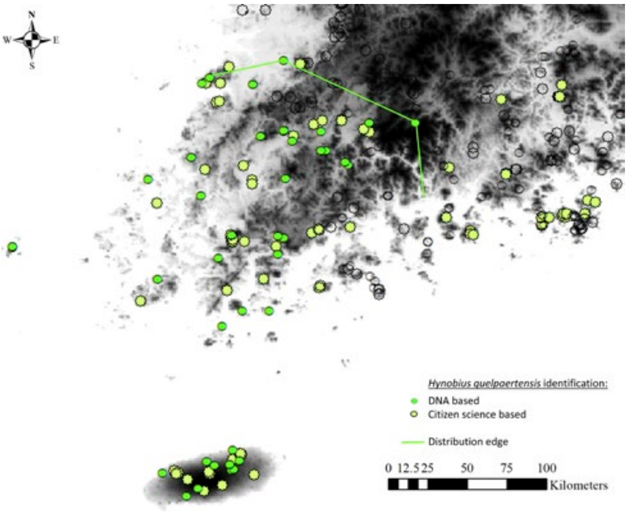

Fig. 3. Distribution of *Hynobius quelpaertensis* in the Republic of Korea. The map includes both mtDNA and citizen science datapoints (iNaturalist through GBIF; doi.org/10.15468/dl.tb0v6j), with the distribution boundary lines drawn from mtDNA data.

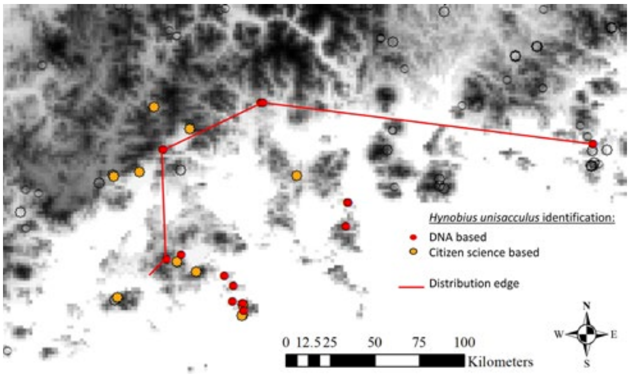

Fig. 4. Distribution of *Hynobius unisacculus* in the Republic of Korea. The map includes both mtDNA and citizen science datapoints (iNaturalist through GBIF; doi.org/10.15468/dl.tb0v6j), with the distribution boundary lines drawn from mtDNA data.

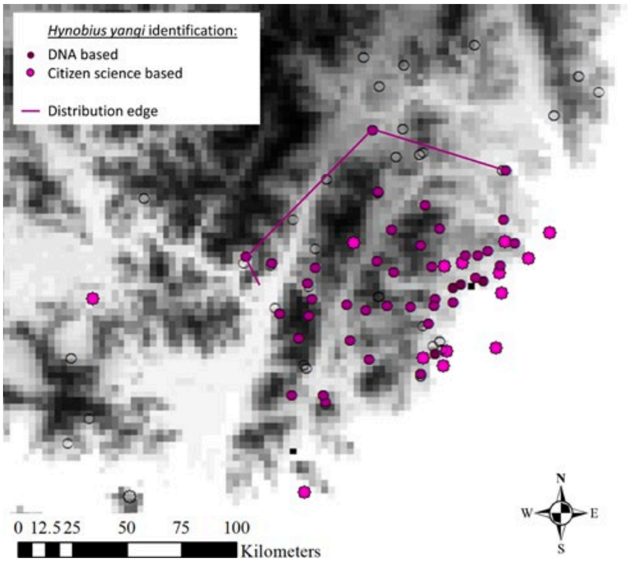

Fig. 5. Distribution of *Hynobius yangi* in the Republic of Korea. The map includes both mtDNA and citizen science datapoints (iNaturalist through GBIF; doi.org/10.15468/dl.tb0v6j), with the distribution boundary lines drawn from mtDNA data.

n = 5), by *H. yangi* (20.67 km; n = 1) and finally by *H. quelpaertensis* (51.47 ± 30.87; n = 18). The independent-samples Kruskal-Wallis test used to assess whether the distance between citizen science datapoints and the distribution boundary lines varied between species was significant (H = 1.49, df = 3, n = 27, P = 0.009). The largest divergence between distribution boundary lines and citizen science observation was observed for *H. quelpaertensis* (Fig. 6).

DISCUSSION

Through the integration of citizen science-based data collection following the guides provided by molecular tools, we refined the distribution of the four Korean

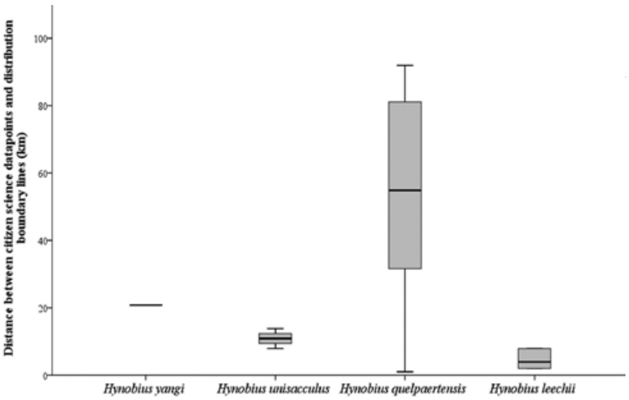

Fig. 6. Discrepancies between citizen science and mtDNA based range description for Korean *Hynobius*. The data presented here is the distance between the citizen science datapoints and the distribution boundary lines, for each Korean *Hynobius* species separately. The distance is significantly different between species.

as further genetic testing may narrow down the width of contact zones between species. Finally, we confirm that when citizen science data collection follows clear landmarks set by molecular analyses, the results provided are clear and accurate. However, we need to emphasize that citizen science is most efficient when conducted within a specified framework (Shirk et al., 2012; Gray et al., 2017). Our work follows the steps of other citizen science projects focused on amphibians (e.g., Mossman et al., 1998; Corn et al., 2000; Roh et al., 2014) and we recommend the broader development of this type of projects. If specific projects were set up for the distribution of *Hynobius*, instead of the opportunistic data collected here, even more precise results would be expected.

Regarding the discrepancy points between the two methods, the largest majority was located within the distribution range of *H. quelpaertensis* before the species was split into *H. quelpaertensis* and *H. unisacculus* (Min et al., 2016). Therefore, once these cases removed, only nine discrepancy points remained, highlighting the impact of recent taxonomic modifications onto natural science enthusiasts. Eventually, the combination of knowledgeable nature enthusiasts and the verification method for "Research Grade" on iNaturalist provides clear distribution patterns. Interestingly, the distribution patterns drawn here from citizen science are more accurate than the ones extracted from the red list of the International Union for Conservation of Nature (http://www.iucnredlist.org; as of February 2018). We therefore recommend the use of citizen science platforms, such as iNaturalist, to assess the distribution of species that have not been assessed yet, or those in need of an updates, such as the Korean *Hynobius* species.

Our results however call for a resolution of the taxonomic question regarding the overlapping ranges and potential hybridisation between the different Korean *Hynobius* clades. For instance, the known subclades within *H. leechii*, geographically located between the distribution of *H. unisacculus* and *H. yangi* (Baek et al., 2011a, 2011b; Min et al., 2016) could not be used in this study. However, the taxonomic resolutions are also expected to cause confusion in the identification of *Hynobius* individuals by nature enthusiast when and if new species are described. For this reason, the involvement of experts on citizen science platforms is essential and the resulting general education, together with the development of interests, is one of the best ways to reach conservation purposes on the long term (Cooper et al., 2007; Marshall et al., 2012).

An important point that also needs to be raised here is that citizen science accuracy is directly related to the taxon studied (Cohn, 2008; Crall et al., 2011; Gardiner et al., 2012). The identification of *Hynobius* sp. based on morphology is not considered easy (Kim et al., 2003), even for experienced researchers, and the molecular identification available was the most important contributing factor for the refinement of the ranges presented here. Additionally, a small group of dedicated users on the citizen science platform could make a significant difference. Most observations used for this study were confirmed by a group of dedicated users, allowing the observations to reach the "Research Grade", or oppositely, downgraded to the genus identification because of disagreements with the original observer. These people are therefore important in their own rights for the accumulation of knowledge on species, as already observed by Rotman et al. (2012) and Johnston et al. (2017).

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Mirrored images but not silicone models trigger aggressive responses in male Common wall lizards

Stefano Scali[1,*], Roberto Sacchi[2], Mattia Falaschi[1,3], Alan J. Coladonato[2], Sara Pozzi[2], Marco A.L. Zuffi[4], Marco Mangiacotti[1,2]

[1] *Museo di Storia Naturale, Corso Venezia 55, 20121 Milano, Italy. *Corresponding author.* Email: stefano.scali@comune.milano.it
[2] *Dipartimento di Scienze della Terra e dell'Ambiente, Università degli Studi di Pavia, Via Taramelli 24, 27100 Pavia, Italy*
[3] *Dipartimento di Scienze e Politiche Ambientali, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy*
[4] *Museo di Storia Naturale, Università degli Studi di Pisa, Via Roma 79, 56011 Calci, Italy*

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Abstract. Disentangling the effects of single releasers in animal communication is a demanding task because a releaser often consists of a combination of different key stimuli. Territorial communication in reptiles usually depends on visual, chemical, and acoustic stimuli, but the role of each of them depends on phylogeny. Lacertids are modern lizards that rely mainly on chemical cues for their communication, but they also use aggressive displays based on visual recognition. We experimentally tested the visual stimuli that release an aggressive response in the males of a typical lacertid, the common wall lizard (*Podarcis muralis*), testing the effects of silicone models and mirrored images in captivity. The response to models and control (a blank sheet) was not significantly different and these stimuli did not release any aggressive behaviour. On the contrary, the reflected image in a mirror caused overt aggression (i.e., bites against it) in 63% of tested individuals. The results clearly demonstrate the role of visual stimuli in territorial communication, but only as a combined effect of shape and motion, differently from other lizard families for which shape is enough to stimulate aggressive responses. Mirrors can be useful tools to investigate aggression related to physiological and morphological aspects in lacertid lizards.

Keywords. visual stimulus, aggression releaser, mirrored image, plaster model, Lacertidae.

INTRODUCTION

Animal communication systems have evolved so that individuals can make decisions based upon the behaviour, morphology, and physiology of other individuals (Endler, 1993). Communication depends on signal transmission between a signaller and a receiver and in order for the signal to be successful, it must be detected by the receiver against a background of other potential stimuli (Fuller and Endler, 2018). Signals act as releasing mechanisms, intended as the totality of all parts of the nervous system that are involved in the filtering of incoming stimuli and it ensures that only the "appropriate" stimuli release a specific behaviour pattern (Immelmann, 1983). The key stimulus or releaser may consist of single or complex cues and disentangling their effect to fully understand which of them releases certain behaviours could be challenging. The majority of releasers consists of a combination of motor patterns acting as a signal and a morphological structure enhancing the signal's effect (Lorenz, 1981), so a single portion of a key stimulus could be insufficient to release a particular behaviour. Notably, when the behaviour pattern is simple and the risk of error is low, releasers are extremely simplified, such as the pecking at the red spot on the beak triggering the regurgitation of food by adult herring gulls (Tinber-

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gen, 1951). By contrast, when costs associated with errors are high, the releasers are more complex to ensure that behavioural patterns start only when they are necessary. This especially applies to aggressive behaviours, whose primary function is releasing of aggression against fellow members of the species and to avoid unnecessary fighting against heterospecific opponents (Immelmann, 1983).

Aggressive behaviours are most common in territorial species, because individuals are forced to compete for limited resources, such as partners, food, shelters and reproductive sites, even if territoriality can be also assessed without overt aggression (Brown, 1964; Myrberg and Thresher, 1974; Van den Berghe, 1974; Stamps, 1977; Kaufmann, 1983). Among reptiles, many cases of territorialism are well documented for chelonians, crocodiles, tuataras, and, particularly, for lizards (Pough et al., 2004). Three main kinds of stimuli can be used for territorial communication by reptiles: visual, chemical, and acoustic. The latter is typical of species living in habitats where visual displays are ineffective and it is used only by nocturnal geckoes (Marcellini, 1977), terrestrial tortoises (Galeotti et al., 2005a; Galeotti et al., 2005b) and crocodilians (Vliet, 1989). Chemical cues are effective communication tools, used by most reptiles, but particularly developed in modern species, like snakes and most scleroglossan lizards, thanks to the evolution of a complex vomeronasal system that freed the tongue from its ancestral role and allowed the transformation to a chemosensory organ (Mason, 1992; Schwenk, 1993; Cooper, 1994).

Disentangling the role of intraspecific communication channels needs experiments analysing the single cues separately. For example, numerous studies have been done on iguanians (in particular on agamids and iguanids), a reptile clade that bases most of its territorial communication on visual stimuli, like posture, dewlap extension, colour patches and colour changes (Yang et al., 2001; Yang and Wilczynski, 2002; Van Dyk and Evans, 2008; Norfolk et al., 2010; Osborne et al., 2012; Dunham and Wilczynski, 2014; Yewers et al., 2016). On the contrary, information is still lacking on the role of visual cues in lacertids, a family of scleroglossan lizards that relies mostly on chemical communication by means of femoral pores to assess territoriality (Cooper, 1994; Martin and López, 2015; Mangiacotti et al., 2017; Baeckens et al., 2018).

Visual stimuli can be tested using four kinds of methods: silicone models that mimic shape and colours of the species, the reflected image in a mirror of the subjects involved in the experiments, video playbacks showed to lizards, and direct staged encounters with another male. The latter method has been often used, but many factors can affect results, such as opponent size, residence status, and individual motivation (Sacchi et al., 2009). Video playbacks have been successfully used for some species (Macedonia and Stamps, 1994; Yang et al., 2001; Ord et al., 2002; Van Dyk and Evans, 2008; Frohnwieser et al., 2017), but they require a long preparation time to acquire video sequences or to prepare animated images after 3D scanning. Hence, it requires a substantial *a priori* knowledge of the species' stereotyped behaviours to present a complete sample set to the subjects. Silicone models have been used, for example, with *Stellagama stellio* and *Pseudotrapelus sinaitus* (Norfolk et al., 2010), *Platysaurus minor* and *P. monotropis* (Korner et al., 2000) and they proved to be adequate cues for territorial behaviours both for agamids and cordylids.

Mirrors are the most used visual stimuli in experimental designs because they are easy, cheap, and typically stimulate aggressive behaviours against the reflected image (Balzarini et al., 2014). Furthermore, the signal is enhanced by positive feedback, because an aggressive posture or behaviour is immediately replicated by the mirrored lizard. Numerous species have been successfully tested using this methodology, particularly from families Agamidae, Phrynosomatidae, and Dactyloidae (Korzan et al., 2000; Brandt, 2003; Farrell and Wilczynski, 2006; Osborne et al., 2012; Dunham and Wilczynski, 2014).

Podarcis muralis, our model species, is a typical representative of this family. It is a small (snout to vent, SVL up to 7.5 cm) and sexually dimorphic lizard, with males stouter and with bigger heads than females; males show a marked territorial behaviour (Edsman, 1990; Sacchi et al., 2009), as supported also by data about testosterone levels and homing behaviour in previous works (Scali et al., 2013; Sacchi et al., 2017). As a consequence, intraspecific communication in this species has been intensively studied as far as chemical cues are concerned (Martin et al., 2008; Heathcote et al., 2014; Pellitteri-Rosa et al., 2014; Baeckens et al., 2017; Mangiacotti et al., 2017, 2019), but no information is available about visual stimuli and communication during intraspecific encounters (but see Zagar et al., 2015). Since aggressive displays and postures of *Podarcis muralis* have never been detailed before (but see Sacchi et al., 2009 and Abalos et al., 2016 for some information), we discarded playback videos and chose a dual experimental approach based on silicone models and mirrors as visual stimuli to boost and record aggressive behaviours. The specific aim of our work was to assess if visual stimuli can trigger an aggressive response in a typical lacertid lizard that bases most of its intraspecific communication on chemical cues. We did this by comparing the aggressive response to: i) a static and oversimplified visual cue (i.e., a silicone model); ii) a more complex and realistic visual stimulus combining movement, behaviour, and posture (i.e., a mirrored image).

MATERIALS AND METHODS

Ninety *P. muralis* adult males (SVL > 50 mm) were captured by noosing in various localities in Lombardy (northern Italy) between April and June 2016, to maximize territorial response in accordance with reproductive season (Corti and Lo Cascio, 2002; Sacchi et al., 2017). The capture sites were located within 50 km each other and had similar ecological conditions, being all peripheral urban habitats, with comparable habitats, presence of predators and densities. Lizards were carried to the Natural History Museum of Milan and housed in individual plexiglas boxes (40 × 40 × 30 cm) with a refuge positioned near one box's wall, water *ad libitum* and fed with three mealworms (*Tenebrio molitor*) per/day. A sheet of absorbent paper was used as substrate, to keep the resident odour in each terrarium and thus lizards could consider it as their own territory. The vertical sides of the boxes were also covered externally with white paper sheets, to avoid external stimuli and wall reflectance. The room was exposed to a natural day/night cycle.

After an acclimation period lasting between three and seven days, lizards were tested in the same terrarium where they were kept, after removing water and food. A heating lamp (ZooMed Repti Basking spot lamp, 150 W) was turned on for 15 minutes to achieve a plateau body temperature similar for all the individuals (Sannolo et al., 2014), then it was turned off and a cold led lamp (Greenergy, 8 W, 600 lm) was lighted to ensure uniform lighting in the terrarium. A surveillance camera (SONY Super Night Vision Camera, M020-s53-001, located near the heating lamp) was turned on and lizard behaviour was recorded for 15 minutes immediately after inserting a visual stimulus in the box. Individuals were randomly assigned to the following three different stimuli (n = 30 for each treatment without replicates): i) a white paper sheet covering one side of the box, and used as control to simulate the insertion of an object by researcher's hand; ii) a silicone model simulating a new lizard invading the resident's territory; iii) a mirror covering one side of the box, reflecting lizard image and movements. All the stimuli were positioned inside the terrarium near the wall of the box opposite to the refuge.

The lizard model was a silicon-rubber cast prepared by the museum taxidermist using a dead male *P. muralis* specimen that was painted brown on the back and white on the belly and throat using water-based tempera colours (Fig. A1 in Supplementary materials). The model was painted one month before starting the experiment to perfectly dry the paint. A push-up stance was obtained inserting an iron wire in the model to simulate a territorial posture with the anterior part of the body raised and showing throat colouration (Molina Borja, 1981).

Since difference in individual size has been proved to affect the outcome of male-male combats in *P. muralis* (Sacchi et al., 2009) and the lizards tested against the model did not always had the same size as the model (SVL = 67 mm, see Table A1 in Supplementary materials for lizard mean size), we performed a preliminary test to investigate the potential inhibitory effect of such a difference. Latency (i.e., the time between the insertion of the model in the terrarium and the first movement of the focal lizard) was used as a proxy for the potential inhibitory effect (the longer the latency, the larger the effect) and it was regressed against the signed difference between lizard and model size ($SVL_{lizard} - SVL_{model}$). The regression was not significant (one-way ANOVA: $F_{1,28} = 1.27$; P = 0.27), so we assumed that model size did not affect lizard aggressive response.

The videos were analysed in the platform BORIS (Behavioral Observation Research Interactive Software, Friard and Gamba, 2016, freely available at www.boris.unito.it). All the behaviours addressed to the stimulus were scored as follows: 1) no interest (i.e., walking across the terrarium without any interactions with the stimulus); 2) interest without aggression (i.e., observing or tongue-flicking towards the stimulus); 3) interest with overt aggression (i.e., biting against the stimulus).

Scores for the three treatments were compared using a Kruskal-Wallis test and Mann-Whitney tests were used as *post-hoc* tests. Analyses were performed under the R rel. 3.4.2 statistical environment (R Development Core Team, 2017) and, otherwise stated, reported values represent means and standard errors.

RESULTS

The higher aggression score (i.e., 3) was observed only for the mirror treatment, where 63.3% (19 out of 30) of males bit the stimulus. The highest score achieved by males in the other two treatments was 2, with 66.7% and 56.7% in control and silicone model treatments respectively. These differences were highly statistically significant (Kruskal-Wallis: $\chi^2 = 26.021$, d.f. = 2; P < 0.001). Mann-Whitney *post-hoc* tests showed that aggression scores did not differ between control and silicone models (P = 0.44); on the opposite, both comparisons involving

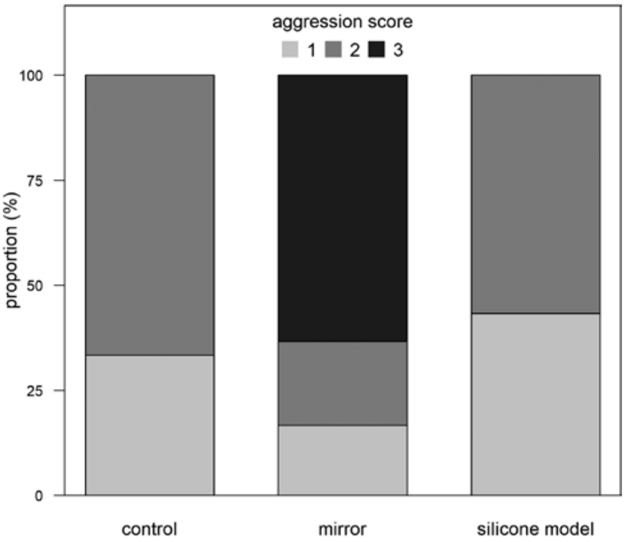

Fig. 1. Proportions of aggressive responses against the stimuli (control, plaster model, and mirror respectively) by common wall lizards (see methods for scoring details).

mirror were significant ($P < 0.001$ in both cases), being the score of the mirror treatment always higher than the other two (Fig. 1).

DISCUSSION

Our experiment demonstrated that visual stimuli are important releasers in triggering aggressive behaviours in the common wall lizard. It is often difficult to disentangle the effect of single key stimuli in a complex stimulus, but the approach used in this study allowed us to separate the effect of a simple cue, the shape of a lizard, by the composite effect of shape and motion. The result is not as trivial as it might seem, because some lizard species actively respond to simplified and motionless models by activating some territorial behaviours, demonstrating that they do not necessarily need more complex visual stimuli. This is true for some Agamidae, such as the tawny dragon lizard, *Ctenophorus decresii*, whose territorial behaviour has been intensively studied. Indeed, males of this polymorphic species engage in complex displays to defend territories and they use the same behavioural patterns also against models, responding differently even to throat colours (Yewers et al., 2016). Also cordylids, such as *Platysaurus minor* and *P. monotropis*, show overt aggression behaviours against models, demonstrating poor species recognition when models of different congenerics were proposed (Korner et al., 2000). All the above examples support the hypothesis that oversimplified visual stimuli can provoke an aggressive response in these taxa.

The common wall lizards were not interested in the presence of models within the enclosures and their only possible reaction was moving sometimes around and on the fake lizards and tongue-flicking at a certain distance. These behaviours were adopted even when the control stimulus (i.e., the white paper sheet) was inserted in the enclosure, so no conclusion can be inferred because they could be due to simple exploration activity. By contrast, the mirrored image always caused an alert posture, such as "freezing" in front of the mirror, repeated tongue-flicking or push-up displays, often culminating in overt aggression against the image with multiple bites or jumps. Numerous authors proved that mirrors are efficient stimuli able to release aggressive behaviours for many species belonging to different families (Agamidae, Phrynosomatidae, and Dactyloidae) (Korzan et al., 2000; Brandt, 2003; Farrell and Wilczynski, 2006; Norfolk et al., 2010; Osborne et al., 2012; Dunham and Wilczynski, 2014). Interestingly, in all these cases the species belong to visually-oriented lizard clades, making such a result expectable. On the opposite, to our knowledge this is the first time that the same kind of visual stimulus (i.e., a mirrored image) releases aggressive behaviour in a lacertid lizard, which is thought to be more chemical- than visual-oriented (but see Garcia-Roa et al., 2017; Baeckens et al., 2018). One main objection to the reliability of our results could be the different smell between the proposed stimuli and a real lizard, but we chose to exclude chemical stimuli in our experiment to disentangle the effect of shape, movement, and chemical hints. Our results confirm the observations by other authors that aggression releasers often are not single visual stimuli, such as a still image, but the combination of different stimuli, such as shape and motion (Macedonia and Stamps, 1994; Yang et al., 2001; Ord et al., 2002; Yang and Wilczynski, 2002; Van Dyk and Evans, 2008; Frohnwieser et al., 2017).

Previous research on the role of motion patterns in the visual displays of anoles demonstrated that motion is fundamental to attract the attention of lizards, particularly when a specific motion pattern is exhibited (Fleishman, 1992). The response to motion patterns is used by many territorial species to signal their presence to intraspecific opponents. These lizards use dewlap extension to communicate with rivals and partners and this display is often accompanied by a stereotypical head-bobbing movement. A detailed study on signal efficacy showed that motion patterns that combined high acceleration with high velocity were particularly effective. Nonetheless, at a short distance, even a small-amplitude motion in the visual periphery can be perceived by a lizard, causing a shift of gaze so that the image falls on a high-resolution portion of the retina (Fleishman, 1992). Of course, lacertids and anoles do not share the same evolutionary history (Pyron et al., 2013), so a detailed study on visual acuity and efficacy of the formers would be hoped. Simple stimulus, such as shape, could be enough to trigger a territorial response in basal lizard clades, but not in modern lizards that use chemical stimuli as the main releaser in intraspecific communication. Previous studies on animal communication demonstrated that the sensory system of the receiver determines which signals can be detected and that, in majority of cases, sensory systems serve multiple purposes and must be capable of detecting many types of different stimuli, such as mates, food, habitat, and opponents (Fuller and Endler, 2018). Responding to all the stimuli could be extremely costly, so there is selection on sensory systems to efficiently capture relevant stimuli in the environment (Fleishman, 1992; Fuller and Endler, 2018). This could explain why motionless or slow-moving shapes do not elicit aggressive responses in the common wall lizard, whereas mirrored images do.

Further studies will be necessary to fully understand the aggressive behaviour of the common wall lizard and the underlying releasing mechanisms, as well as to support the relationship between phylogeny, territoriality, and the complexity of visual stimuli. Nevertheless, we demonstrated that mirrored images are able to activate the aggressive response also in a lacertid lizard, which sets the stage for a wider and comparative study using other species and stimuli.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < http://www.unipv.it/webshi/appendix > Manuscript number 24651.

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Using an in-situ infra-red camera system for sea turtle hatchling emergence monitoring

Fatima N. Oğul[1,#,*], Franziska Huber[2,#], Sinem Cihan[1], Kumsal Düzgün[3], Ahmet E. Kideyş[1], Korhan Özkan[1]

[1] *Institute of Marine Sciences, Middle East Technical University, Erdemli, Mersin, Turkey. *Corresponding author.* E-mail: fogulnua@gmail.com
[2] *Paris Lodron University of Salzburg, Faculty of Natural Sciences, Salzburg, Austria*
[3] *Faculty of Veterinary Medicine, Ankara University, Altındağ, Ankara, Turkey*
 # The two authors contributed equally

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Abstract. We tested for the first time the efficiency of the use of infra-red (IR) cameras for sea turtle hatchling monitoring. The cameras were installed on one green turtle (*Chelonia mydas*) and four loggerhead turtle (*Caretta caretta*) nests during 2014 and 2015 nesting season in the south-east Mediterranean, Turkey. The camera monitoring, even with the limited sample size, have successfully corroborated the previous observations and provided further insights on hatchling emergence behavior. The analysis of the camera recordings revealed that hatchlings emerged from the nests asynchronously in varying numbers of groups and different group sizes, while c. 60% hatchlings emerged during the first 5 days of emergence activity. 98.6% of hatchlings emerged at night with a peak activity between 21:00 and 00:00. The day of first emergence varied between 38 and 64 days since egg deposition, while the day of last emergence varied only between 60 and 65 days. Total emergence activity continued up to maximum of 22 days, which is longer than that of previous records. Overall, the present study showed that IR camera monitoring is a promising tool for sea turtle monitoring and can provide detailed insights on sea turtle hatchling behavior.

Keywords. Loggerhead turtle, green turtle, hatchling emergence duration, infra-red camera, continuous camera monitoring, sea turtle nest monitoring, sea turtle conservation.

INTRODUCTION

Breeding success has been an essential component of sea turtle conservation (Musick and Limpus, 1997; Hamann et al., 2010; Rees et al., 2016). Therefore, extensive monitoring of sea turtle breeding beaches has become an integral part of sea turtle breeding habitat management (Fowler, 1979, Hays et al., 2001; Taskin and Baran, 2001). These monitoring efforts have focused on various aspects of sea turtle breeding, such as, habitat quality, nest predation, anthropogenic effects and hatchling emergence patterns; leading to the identification of important pressures on the breeding habitats of these endangered species (Kasparek et al., 2001; Tomás et al., 2002), which has led to implementation of better conservation measures.

Sea turtle breeding beach monitoring have been almost exclusively based on regular beach patrols during the breeding period that may last for five months (Henson and Boettcher, 2006; MEDASSET, 2017). These direct visual observations have important limitations in temporal resolution, feasibility and man power (Garciía

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et al., 2003). Therefore, sea turtle conservation management may be benefitted from more efficient monitoring alternatives providing standardized survey data. Continuous camera recordings have been used in several organism groups, such as, wild boars (Huckschlag, 2008), deers (Scheibe et al., 2008) and birds (Pierce and Pobprasert, 2013); however, they have not previously been used for sea turtle hatchling monitoring. Only in Florida Keys beach, a live-streaming webcam was installed on a sea turtle nest in 2014 in order to raise awareness on sea turtle conservation (http://www.fla-keys.com/turtlecam/).

Continuous camera monitoring may provide opportunities for sea turtle breeding beach monitoring by improving monitoring efficiency as well as providing detailed insights on hatchling behavior. There are currently different monitoring and excavation protocols for different research teams and volunteer groups (Henson and Boettcher, 2006; MEDASSET, 2017). For example, there is no standardized nest excavation time, although many management groups prefer to conduct an excavation within the few days of the last detected emergence. Furthermore, beach patrolling during hatchling emergence period is mostly conducted in the mornings (Henson and Boettcher, 2006; MEDASSET, 2017), while knowing temporal emergence patterns could facilitate the researchers to better allocate labor if encountering hatchlings is required. Therefore, detailed understanding of hatchling emergence behavior specific to breeding beaches through novel technologies and standardized data may lead to a more efficient decision on excavation dates and beach patrolling schedules. Furthermore, temporal patterns and group formation of sea turtle hatchling emergence might also be an important determinant of the survival of hatchlings (Carr and Hirth's 1961) and therefore a more detailed understanding of hatchling behavior may also be instrumental in conservation management.

Overall, quantitative and standardized observations on hatchling behavior using technological monitoring tools may provide a more comprehensive understanding of sea turtle breeding ecology and conservation. The aim of this study is testing a novel method – IR camera monitoring – and assess its efficiency in sea turtle hatchling monitoring of the temporal patterns and group formations of hatchling emergence as well as hatchling behavior.

MATERIAL AND METHODS

Study site

The study was conducted at the beach of the Institute of Marine Sciences, Middle East Technical University (METU IMS), Turkey. The beach stretches along a 1.2 km long coast and is located in a heavily urbanized area of the eastern Mediterranean. The study site has restricted public access and the human activity is limited. The beach is mostly sandy and spans 15-25 m in width with insignificant tidal activity. It consists of natural sand dunes approximately 0.5-3.0 m above sea level, hosting natural coastal vegetation dominated by sand lily (*Pancratium maritimum*, L.; Cihan, 2015). The activity of breeding sea turtles from May to August and hatchlings from July to September have been monitored since 2013 using conventional beach patrols.

Camera monitoring system

Conventional infrared (IR) security cameras (BALITECH BL-6150) with 200 m range, 8 mm stable lens and 650 TVL resolution were installed on wooden poles placed approximately 1.5-3.0 m away and 1.0-1.5 m above the nests (Fig. 1). All the cameras were connected to a digital video recorder (SAMSUNG SRD-1650D) with 16 channels and 1 TB memory, placed in a cabinet that was installed c. 20 m away from the most distant nest. Recordings were transferred every second day to an external 1 TB hard drive. The recordings were commenced after 51 days of egg deposition and lasted for c. 30 days. Five cameras in total were installed on loggerhead (4) and green turtle (1) nests.

Analyses of hatchling behavior and emergence activity

The video recordings were analyzed to elucidate the patterns in hatchling group size, emergence date and time. We pooled and analyzed green and loggerhead nest data together for the present analyses, although these two species significantly differ for other aspects of their breeding biology.

All video recordings were analyzed with automatic screen captures at 30-second intervals. When emergence activity was detected in photos, the corresponding video clip was examined, for the exact emergence date and time, hatchling count, crawling duration, orientation, behavior as well as any predation event. Emergence activity was accepted to start with the earliest time of a hatchling observed on the nest surface, end with disappearance of the last hatchling from camera view.

Emergence groups were categorized by the number of individuals: 1-3 as small, 4-10 as intermediate, more than 11 as large groups. Individuals appeared at the same time or subsequently (less than one minute between individuals) were taken as one group even if there was a lag between their crawling activities. Total days until first emergence, peak activity and last emergence were calculated from the night of nest deposition. The day when the largest emergence occurred was designated as the nest's day of peak activity. An emergence events were designated as day or night activity according to the time of sunrise (05:50-06:23 h) and sunset (18:51-19:46 h) during study period. The emergences that occurred 10 min before sunset and 10 min after sunrise were considered as night activity to be able to account for local shading. In Nest E11R, 30 hatchlings waiting on the top of the nest chamber were accidentally dug by chil-

Fig. 1. A sample setup of camera system around a sea turtle nest. Photographer: Korhan Özkan.

dren just before the sunset. The surveyor immediately noticed the event, monitored the nest and the hatchlings subsequently released under monitoring. We accepted that emergence as night emergence, since the event occurred just before the sunset, and the hatchlings were ready to emerge immediately that evening.

The hatchling orientation and predation events were also recorded to understand the effectiveness of beach management and efficiency of nest cages. The one side opened pyramidal shaped metal nest cages were placed on the nests' surface as open side was directed toward sea (Fig. 1). Orientation of each hatchling movement relative to a seaward direction was recorded. We classified hatchling crawls within +70° of the seaward direction as seaward orientated. Mortality or predation events were also recorded.

A nest excavation was performed after the end of camera monitoring. The total number of eggs were estimated from the remains and compared with the counts from the video recordings. The emergence success of each nest was calculated as the ratio of hatchlings those reached to the sea and the total clutch size (Miller, 1999).

RESULTS

The values of all the analyzed parameters for the single green turtle nest varied always within the range that was observed for the loggerhead turtle nests (Table 1). Accordingly, we did not discard the single green turtle nest data, instead, green turtle and loggerhead turtle nests were pooled together for the analyses. Camera recordings revealed that a total of 357 hatchlings in 71 groups emerged from five nests with 42-94 hatchlings per nest. In total 62% of hatchlings emerged in large groups, 17 % of hatchlings emerged in intermediate groups and 21% of hatchlings emerged in small groups (Table 1). At least one large group emergence was observed for each nest (Table 1). Additionally, 69 of the 72 groups (95.8%) emerged at night accounting for 352 of 357 hatchlings (98.6%; Fig. 2, 3a, 3b). All of the large and intermediate groups as well as 49 of the 51 small group emergences occurred during night (Fig. 2). The highest emergence

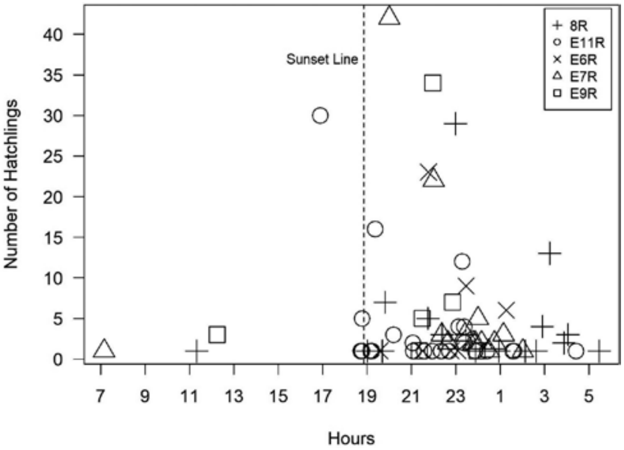

Fig. 2. The daily temporal patterns of hatchling emergence activity. Each symbol represents a different nest and the dashed line represents the approximate sunset time. The emergence activity was overwhelmingly nocturnal.

Table 1. Number of emergence groups and hatchlings for each nest with clutch size and incubation period. CM and CC denote for green and loggerhead turtle respectively. NG and NH denote for number of groups and total number of hatchlings respectively.

Nest	Species	Large emergences		Intermediate emergences		Small emergences		Incubation Duration	Clutch Size
		NG	NH	NG	NH	NG	NH		
8R	CM	2	42	3	16	13	20	53	97
E11R	CC	3	58	3	13	19	23	38	103
E6R	CC	1	23	2	15	4	4	64	50
E7R	CC	2	64	1	5	11	22	54	104
E9R	CC	1	34	2	12	4	6	54	71
	Total:	9	221	11	61	51	75		

Table 2. Surveyed nests' characteristics and nest excavation records.

Nest	8R	E6R	E7R	E9R	E11R
Species	*Chelonia mydas*	*Caretta caretta*	*Caretta caretta*	*Caretta caretta*	*Caretta caretta*
Egg deposition date	12/07/2014	6/06/2015	10/06/2015	16/06/2015	27/06/2015
Camera installation date	1/09/2014	12/07/2015	12/07/2015	12/07/2015	12/07/2015
First emergence date	3/09/2014	9/08/2015	3/08/2015	9/08/2015	4/08/2015
Last emergence date	14/09/2014	10/08/2015	14/08/2015	16/08/2015	26/08/2015
Incubation duration (from egg deposition to first emergence)	53	64	54	54	38
Excavation date	2/10/2014	23/08/2015	19/08/2015	21/08/2015	28/08/2015
Emergence duration	11	1	11	7	22
Hatched/Empty eggs (from excavation)	77	41	93	49	95
Camera hatchling count	78	42	91	52	94
Clutch Size	97	50	104	71	103
Hatchlings reaching the sea	76	42	91	50	94
Hatchlings predated	1	0	0	0	0
Dead hatchlings (after emergence)	1	0	0	3	0
Early stage embryos	9	4	2	12	2
Middle stage embryos	1	0	2	1	0
Late stage embryos	2	0	1	0	2
Unfertilized eggs	7	4	8	6	5

activity (60% of the group emergences) occurred between 21:00 and 00:00 (Fig. 3a and 3b). The total number of hatchlings captured with camera recordings and the number of empty eggs found in excavations were largely consistent with an error rate between 1% and 5.8% (only 1 to 3 differences have been found per nests, Table 2).

Total incubation period varied between 38-64 days since the egg depositions (mean = 52.6 days; Table 2). The day of the peak activity varied between 47 and 64 days (mean = 54.6 days; Fig. 4). The day of the last emergences was least variable among nests and varied between 60 and 65 days (mean = 63 days; Fig. 4). Total emergence duration between the first emergence and last emergence had a large variation, changed between 1 and 22 days (mean = 10.4 days).

Overall, 121 hatchlings out of 357 (33.9%) emerged during the first day (24.2 hatchlings on average per nest). 221 (61.9%) hatchlings emerged over the first 5 days following the first emergence (8.8 hatchlings per day for the

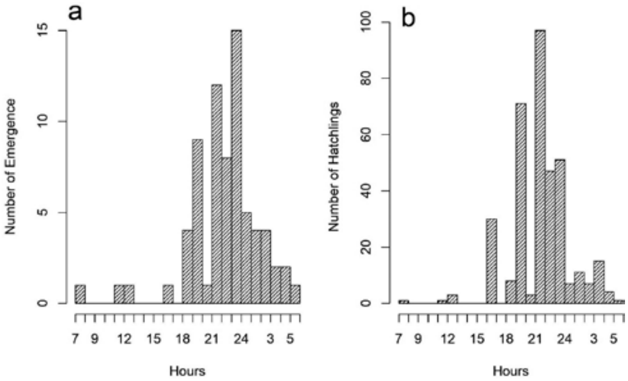

Fig. 3. The histogram of emergence activity by hour for number of emergence groups (a) and hatchlings (b).

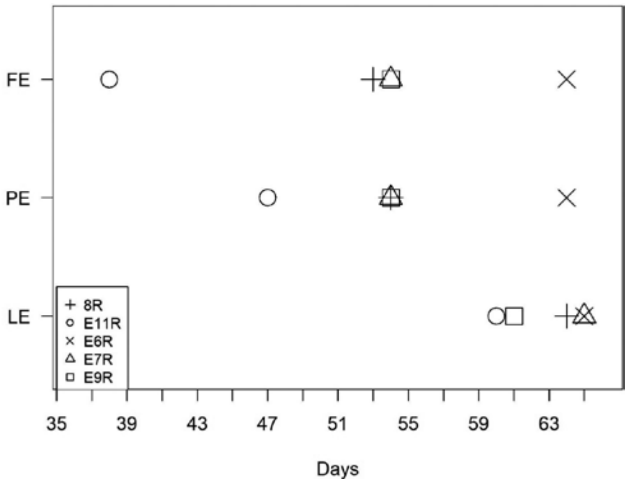

Fig. 4. The duration of the first emergence (FE), peak emergence (PE), last emergence (LE), calculated as the total number of days since egg deposition. The least variation was observed in the day of last emergence.

first five days). Three nests' peak activity occurred on the first day and one nest's peak activity occurred on the second day of emergence period.

The majority of the hatchlings (88.8%) oriented successfully towards the sea. Only three disorientated emergence groups (40 hatchlings) were recorded, but the predation cages re-directed them to the sea. The hatchling emergence success of the nests varied between 66% and 92% (Table 2) and no dead hatchlings were found within the nest chamber during excavations. A total of five dead hatchlings were observed due to overturning and subsequent heat shock (Table 2). Four of the deaths happened during the day, with only one happened during the night. Only one hatchling was predated by Hooded Crows during day.

DISCUSSION

The results of the present study showed that continuous camera monitoring can be an efficient tool, especially for hatchling behavior monitoring. Furthermore, the analyses of the recordings corroborated the previous findings on the patterns in emergence group sizes, timings and durations, as well as provided further insights on hatchling behavior. Continuous camera recordings with IR cameras provided data with very high temporal resolution on sea turtle hatchling behavior. Although the present study performed on a limited number of nests, the camera monitoring documented an exceptionally long emergence activity duration of 22 days. Previously, 18 days of emergence duration was reported as the longest emergence activity duration for loggerhead turtles in Japan (Moriya and Moriya, 2011). Our results suggest that longer emergence durations might be more frequent than expected and continuous video recordings may provide a more reliable estimation for the duration of hatchling emergence activity in comparison to conventional beach monitoring.

Continuous camera recordings also enabled us to study hatchling emergence behavior at temporal scales and with small sample sizes that are difficult to account for with conventional beach monitoring. For example, we observed a strong intra-nest asynchrony in hatchling emergences; i.e., hatchlings tended to emerge in several groups with different sizes in successive days. Synchronous emergence (emerging as one large group) has been proposed to reduce the probability of hatchling predation on land (Delm, 1990; Heithaus, 2013; Santos et al., 2016) and hatchlings might stimulate each other both during and after emergence (Carr and Hirth, 1961). However, several studies previously documented asynchronous emergence (Peters et al., 1994; Glen et al., 2005; Adam et al., 2007; Moriya and Moriya, 2011), similar to our findings. This might also be due to a decrease in predation intensity for the hatchlings emerging in small groups (Pilcher et al. 2000) or due to ambient temperature differences in the nest (Adam et al., 2007).

Moreover, the present study demonstrated that the natural emergence activity since the egg deposition lasted between 60 and 65 days with a very limited variability among nests. However, the total emergence activity since the first hatchling emergence lasted between 1 and 22 days with a large variation. Accordingly, if the natural incubation and emergence process is preferred by the local conservation managers, 65 days after nesting or 22 days after the first emergence may be waited until any excavation, if the nesting beaches are not under high predation pressures.

We observed in the present study that the in-situ camera systems had considerable advantages over direct visual observations on effort, consistency and repeatability especially if the proper equipment is selected. However, we have also observed some limitations on the use of in-situ camera systems. The field of view of the cameras only enabled us to monitor close vicinity of the nest and prevented us following the hatchlings to the sea, which only documented the immediate survival of the hatchlings on the nest. We also encountered hardware and recording failures mostly due to corrosion. Therefore, using durable technical equipment and backing up data frequently are essential for successful in-situ camera applications. METU IMS Campus has limited access to public and thus it is well protected from robbery or vandalism, which enabled us to install electronic equipment freely. However, using this method in larger or remote breeding beaches would require necessary security precautions.

The analyses of hatchling emergence data in the present study strongly corroborated previous observations on loggerhead and green turtle nests monitored using conventional methods. The majority (98.6%) of the hatchling events in the present study occurred nocturnally similar to previous findings (Mrosovsky, 1968; Witherington et al., 1990; Hays et al., 1992; Glen et al., 2005), probably to avoid diurnal predators and lethal daytime temperatures (Glen et al., 2005). The peak emergence activity occurred between 21:00 and 00:00 h, similar to the observations in Florida (23:00 and 00:00 h, Witherington et al. 1990) and in Greece (00:30 and 01:00 h, Adam et al., 2007). Accordingly, the night patrolling efforts aiming at monitoring hatchlings could be prioritized for early evening, when man power is limited. Furthermore, nocturnal emergences occurred mostly (~80%) in large groups, while all diurnal emergences were in small groups (including single emergences) in the present study, further corroborating previous studies (Glen et al., 2005).

The mean total incubation period (from egg deposition night to the first emergence) was 52.6 days (38-64 days) in the present study. This is in accord with previous observations for green turtles in Northern Cyprus (57.9 days; Ilgaz and Baran, 2001), for loggerhead turtles in Turkey, Greece and Northern Cyprus (varied between 49 and 55.2 days; Ilgaz and Baran, 2001; Taskin and Baran, 2001; Margaritoulis, 2005; Fuller et al., 2013). The difference between the incubation periods among different studies might be due to the differences in ambient temperature of the breeding beaches (Hays et al., 1992; Drake and Spotilla, 2002; Glen, 2005; Adam et al., 2007). Therefore, continuous camera recordings with temperature measurement devices might provide more accurate hatchling activity parameter estimates specific to different breeding beaches. It should also be noted that the number of the samples in the present study is limited and further studies with larger sample sizes is required for more accurate parameter estimations.

The great majority of the hatchlings (98.6%) reached to the sea successfully in the present study, indicating a very high survival rate in comparison to other observations in the region (49.9% in Ilgaz and Baran, 2001 and 43.5 % in Taskin and Baran, 2001). Only a single hatchling (in 357) was predated by Hooded Crows, which is very low predation rate (Carr and Hirth, 1961; Tomillo et al., 2010; Türkozan et al., 2011). The hatchling deaths occurred only in small group emergences in the present study. This is in accord with previous observations, where larger groups of hatchlings have been observed to be more motivated to reach the sea and more directional in their effort (Carr and Hirth, 1961; Burger and Gochfeld, 2014), while single emergences have had less chance to reach to sea than group emergences (Carr and Hirth, 1961). The high success rate probably reflected the efficiency of the conservation efforts at METU IMS beach (i.e., artificial light and human use management).

Overall, the present study showed that in-situ camera systems is an alternative or complementary tool to the conventional beach monitoring for sea turtle conservation with significant advantages on labor and efficiency. Furthermore, the high frequency data gathered through continuous camera monitoring even with small sample sizes provide important opportunities for studies on sea turtle hatchling behavior.

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Descriptive osteology of an imperiled amphibian, the Luristan newt (*Neurergus kaiseri,* Amphibia: Salamandridae)

Hadi Khoshnamvand[1], Mansoureh Malekian[1,*], Yazdan Keivany[1], Mazaher Zamani-Faradonbe[1], Mohsen Amiri[2]

[1] *Department of Natural Resources, Isfahan University of Technology, Isfahan, Iran. *Corresponding author.* Email: mmalekian@cc.iut.ac.ir
[2] *Department of Basic Sciences, Islamic Azad University of Hamedan, Hamedan, Iran*

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Abstract. Osteological structures are important biological features which provide valuable biological and ecological information. Luristan newt (*Neurergus kaiseri*), is an endemic salamander, inhabiting the southern part of Zagros Mountains of Iran. The current study was conducted to describe the osteological characteristics of the Luristan newt which might be important in understanding the evolutionary process of newt species. The skull of *N. kaiseri* has a dense structure, severely ossified elements and a low amount of cartilaginous elements, only in mobile facets. Vertebral number in the axial skeleton of the species equals 50. The cervical, abdominal and caudal parts of the vertebral column have two, 16 and 32 vertebrae, respectively. Each hand and foot consisted of four fingers, having three or four phalanxes. The metacarpal includes seven bones and the number of metatarsus bones is eight. Hands are connected to humur through ulnare and radius and then connected to scapulocoracoid. Each leg includes two bones (fibula and tibia) which are connected to femur. The head of the femur articulates with the acetabulum in the pelvic bone, while the distal part of the femur articulates with the tibia.

Keywords. Salamanderidae, newt, *Neurergus kaiseri*, skull, descriptive osteology.

INTRODUCTION

The vertebrate skeletal system and its elements are important in evolutionary biology. Vertebrate skeletons can be regarded as combinations of apparently discrete units (namely bones) which have attracted the interest of comparative anatomists (Simpson, 1944). Osteological data can be used to identify different taxa and phylogenetic relationships (e.g., Hill, 2005) and to understand biological features of animals such as feeding, respiration, swimming and movement (Eastman, 1980; Helfman et al., 2009). In addition, the skeletal structure contains biological information that can be used to distinguish species type, age, sex, size, and even environmental conditions of their habitats (Helfman et al., 2009).

In Iranian freshwater basins live three genera of salamanders including, *Triturus, Salamandra* and *Neurergus*, all belonged to Salamandridae. The latter genus has a relatively vast geographic distribution, ranging from Zagros Mountains (western Iran) to Iraq and southern Turkey (Baloutch and Kami, 1995). The Luristan newt (*Neurergus kaiseri*, Schmidt 1952) is endemic to the southern Zagros Mountains of Iran, with a distribution area of approximately 900 km^2 (Mobaraki et al., 2016). The species inhabits first order rivers and ponds in open woodlands dominated by oak trees (*Quercus brantii*). This newt is classified as vulnerable (VU) by the IUCN Red List because of its small range, illegal trading, habitat loss and climate induced drought (IUCN, 2018). It is also amended to the Appendix I of the convention to the international trade to endangered species (CITES, 2010).

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Current knowledge on *N. kaiseri* is restricted to some aspects of the ecology such as distribution, including reports of new localities for the species (Sharifi et al., 2013; Mobaraki et al., 2014), and demography (age structure, longevity and growth patterns) of a local population (Farasat and Sharifi, 2015). A genetic study reported the presence of two genetically distinct clades within the Luristan newt (Farasat et al., 2016). Sexual dimorphism in *N. kaiser* was evaluated using head- and body- related characters (Khoshnamvand et al., 2018). Skeleton structure of the species is yet to be described, which can provide additional data and a basis for understanding the remarkable and adaptive variation in bone-forms among newt species. Osteology also helps in understanding the important taxonomic characters for identification and classification of a species.

In Salamandridae, a number of studies include osteological characters in the description of a new taxon (Min et al., 2005; Wu et al., 2009; Wu et al., 2010; Wake et al., 2012) or provide comparative descriptions of closely related taxa (Wake and Özeti, 1969; Venczel, 2008; Wu et al., 2012). Amongst *Neurergus* species, little information is available on descriptive osteology. Akia et al., (2010) described the cranial osteology of a closely related species, *Neurergus microspilotus* and compared it with *Salamandra infraimmaculata semenovi*. The current study aims to describe skull characteristics of *N. kaiseri* and compare size, shape and connections of bones with other newts, where possible. Such data provide a basis for further investigations on the subject and help to understand the biological features of Iranian salamanders.

MATERIAL AND METHODS

Field surveys were conducted in late July 2015, when the Luristan newt breeding season was over to minimize disturbances to the species. The Luristan newt habitats are mainly streams and springs, located at high elevations (800 to 1500 m a.s.l), separating from each other by steep and rocky mountains. Further, the species is rare, occurring in low density in a limited number of sites, as many of the ponds and springs in the region have dried out due to the drought during the past few years (IUCN, 2018). The vulnerable (VU) status of the species also entails sampling restrictions for capturing live individuals. Therefore only deceased carcasses of *N. kaiseri* could be taken and used for osteological analyses.

Therefore, we accessed to the newt habitats by climbing the mountains and camping in the region for 10 days, with the assistance from the environmental guards. Due to the water flow, which could wash away the possible carcasses, 2 mm fish nets were installed in down streams. Fish nets were regularly checked during the day and removed from each site before the dark. Two dead individuals were captured by the fish net at two localities in Korki region, Lorestan province, at the elevation of 957 and 1100 m a.s.l. respectively.

Both specimens were adult male with the body length of 132 and 130 mm respectively. Samples were preserved in 96% ethanol in the field prior to the lab experiment. We followed the standard protocols of cleaning and staining bones (Taylor and Van Dyke, 1985; Torres and Ramos, 2016). The carcasses were fixed in 500 ml of 10% neutral formalin for 4 days. Before proceeding with the staining procedure, the specimens were washed thoroughly under running tap water for at least one hour to remove excess of formalin. The specimens were then placed in 1% alizarin red solution, added drop by drop to the freshly prepared 5% KOH solution. About 15 ml of the staining solution was utilized, holding the specimens for three days or until the medium (KOH) showed pinkish violet color. The specimen were then transferred into alkaline blue stain solution for two days. To clean bones, specimens were transferred into trypsin solution at ~37 °C, changing enzyme solution and washing the specimens in distilled water every 3 days to avoid bacterial digestion (repeated three times). The stained specimens were examined using a stereomicroscope (HP SNP 120), and different skeletal elements were dissected and scanned in lateral, dorsal and ventral view, using a scanner (HP Scanjet G4050). For each specimen or view, a series of images were taken to produce a single image with maximum depth of field. The final image was drawn using CorelDrawX7 software. The terminology of the skeletal elements follows Vassilieva et al. (2015).

RESULTS

The skull of *N. kaiseri* has a dense structure, severely ossified elements and a low amount of cartilaginous elements, only in mobile facets. The premaxillae are unpaired and nearly arched dorsally. The pars dorsalis of premaxillae are distinctly and widely separated by a midline fontanelle. A groove that starts in the top of nasal opening is separating the pars dorsalis of premaxillae and continues forward to pars dentalis. This groove further goes toward the antroventral part of the palate and form a pore in the vomer bone. The premaxilla contacts the prefrontal and nasal posteriorly, and contribute to the nasal cavity laterally. The maxilla are paired and complete the arch of the upper jaw that borders the nostril at the anterior part and meet the nasal bones medially (Fig. 1). The paired nasal bones are nearly subrectangular and bordered anteriorly by the premaxillae. Anteriorly, they border the nostril and laterally connected to the prefrontals. The prefrontals are relatively triangular, anteriorly connected to the pars dorsalis, anterolaterally to the nasals and posteromedially to the frontal (Fig. 1). The lacrimal is a small triangular bone enclosed by maxilla, prefrontal, and nasal bones. This bone participates in forming the orbit cavity. In the right half of the head, it has a distinct boundary on both sides, but in the left half

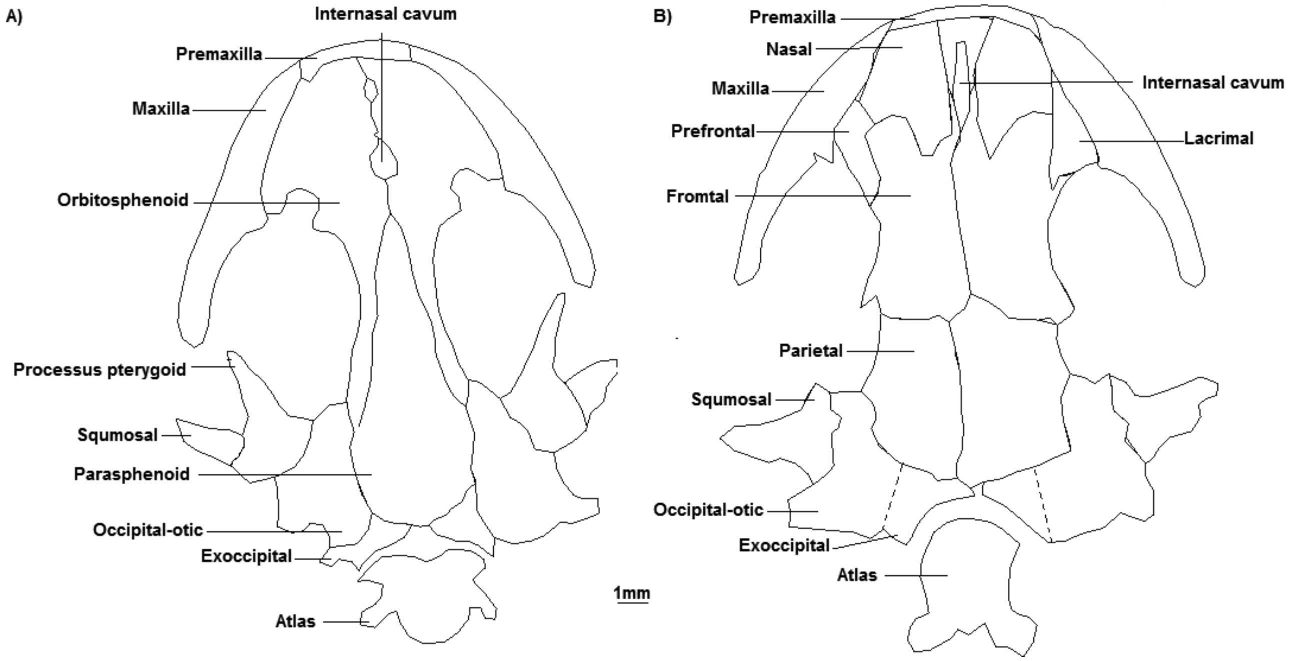

Fig. 1. Explanatory drawings of Dorsal (A) and ventral (B) view of *N. kaiseri* skull. Scale bar: 1 mm

of the head, this bone is fused to frontal with no boundary (Fig. 1). The frontals are flattest paired bones in the skull and trapezoid that makes a posterolateral projection. A very tiny cartilage binds this projection to squamosal to form a ring. The frontals meets the prefrontal anterolaterally, premaxillae anteriorly and parietal posteriorly. The parietals are relatively flat with small curvature in the posterolateral region towards to the orbit cavity. The parietals are rather smaller than the frontals and together complete the roof of the neurocranium. Two parts of parietal, having an overlap toward the foramen magnum. The squamosal is almost triangular that lying on the lateral side of exoccipital, becoming closely fixed with it to form a lateral projection. The base of the skull is ossified.

The orbitosphenoid articulates with the premaxillae (pars dentalis1) and maxilla anteriorly and laterally, respectively. The orbitosphenoid is located in the orbital region and makes an optic foramen posteriorly to pass off the optic nerves into the cranial cavity. Near the premaxilla, the orbitosphenoid has a medial foramen that opens into the braincase. Pterygoids bear relatively triangular depression on ventral surface from basicranial to orbital foramen. The unpaired and oval-shaped parasphenoid is the largest bone in middle part of ventral view of the skull. The quadrate is a relatively small bone located in anterior side of squamosal. The exoccipital forms the posteriormost part of the skull base, encircling the foramen magnum and articulating via the occipital condyles with the first vertebra. Exoccipitals have trapezoid shape ventrally and paired but never meet together at midline. Exoccipitals are anteriorly in contact with the parietal and laterally with squamosal (Fig 1). Further, exoccipital can be seen at the top of the skull. Squamosal is connected to the pterygoids bone and both pterygoids and squamosal are connected to the occipital.

Mandibles are paired and include mentomandibulars, dentaries and pre-articulars. The mandible is separated and symmetrical on both sides. The shape of the mandible (lower jaw) is a simple solid bony arch and dentary is the main element of the mandible (Fig. 2).

Phalanxes are well developed in all fingers, showing a tendency toward bifurcation, and are consistently seen to have small cartilaginous tips (Fig. 3). Further, each hand has four fingers. The biggest finger has four phalanxes, while the other fingers have only three phalanxes. The metacarpal includes seven bones. One of them is larger than the others and is connected to the ulnare and radius. The hands connected to humur through ulnare and radius and then connected to scapulocoracoid (Fig. 3).

Feet have five fingers and each finger has three phalanges (Fig. 3). The number of metatarsus bones is eight and four of them are bigger than the others. Each leg includes two bones (fibula and tibia) which are connect-

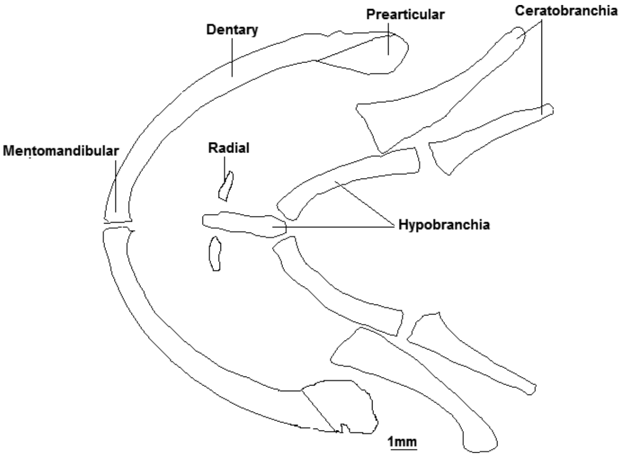

Fig. 2. Explanatory drawing of the lower jaw and hyobranchial apparatus of *N. kaiseri*. Scale bar: 1 mm

ed to femur. The head of the femur articulates with the acetabulum in the pelvic bone, while the distal part of the femur articulates with the tibia (Fig. 3).

Vertebral number in the axial skeleton equals 50. The cervical, abdominal and caudal parts of the vertebral column have two, 16 and 32 vertebrae, respectively. Also hands are connected to the body at the vertebrae number 2-3 and legs are connected at vertebrae number 16-17.

DISCUSSION

We described here, for the first time, the skeletal structure of *N. kaiseri*, an endemic and threatened newt in the southern Zagros Mountains of Iran. The species inhabits mountainous streams at high altitudes, which makes its habitat hard to access Due to the rarity and vulnerability of the species, only two deceased individuals of *N. kaiseri* could be obtained and used for osteological analyses. The two specimens examined here were similar (adult females with the total length of ~130 mm), which minimizes differences in ossification due to age. Body length is linearly correlated with age in many salamandrid species until maximum length is reached (Lima et al., 2000; Üzüm, 2009).

The skull of *N. kaiseri* is very compact and mineralized with low amounts of cartilaginous elements. Within the *Neurergus* genus, skull characteristics has only studied in *N. microspilotus* (Akia et al., 2010). The cranial characters and the number of characters of *N. kaiseri* and *N. micropilotus* show a general similarity. Posterior part of the skull in both species is wide and the snout being short and the entire skull parts are almost fully mineralized in the adults. The frontal and premaxillae of both *N. kaiseri* and *N. microspilotus* is relatively the same. However, it seems that the skull of *N. kaiseri* being smaller compared to *N. micropilotus*. Other noteworthy differences between the skulls of *N. kaiseri* and *N. microspilotus* include differences in the shape and size of

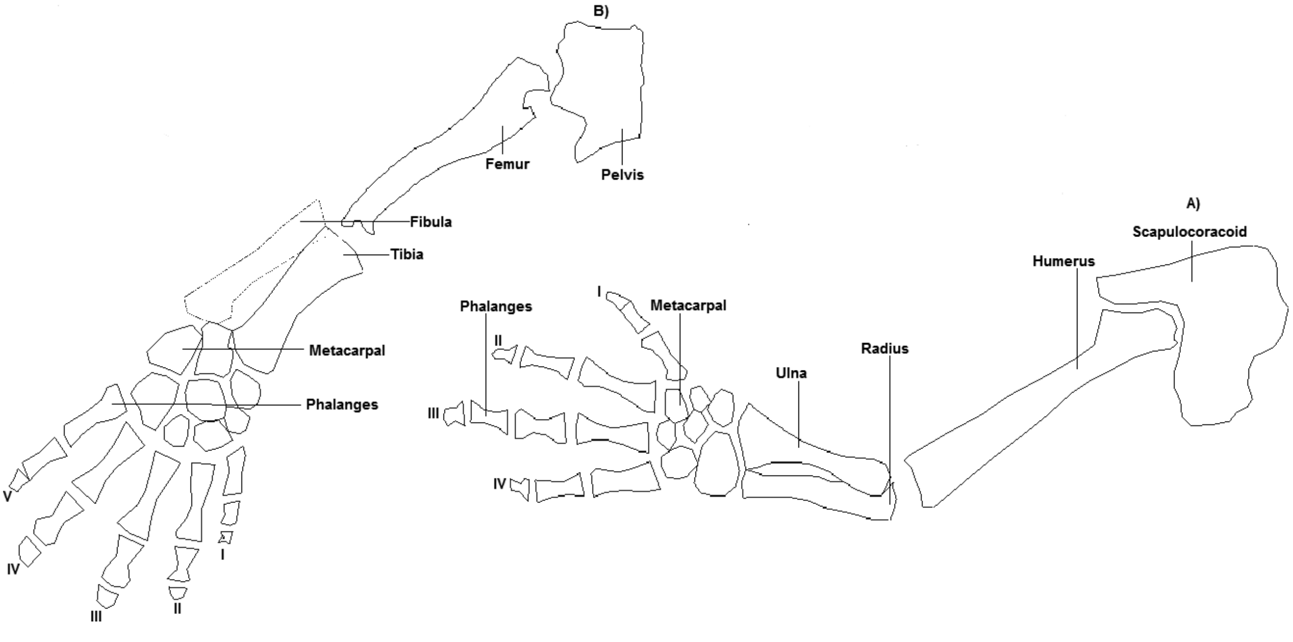

Fig. 3. Hand (A) and leg (B) of *N. kaiseri*. I-IV: digits I-IV. Scale bar: 1 mm.

maxilla, squamosal, exooccipital and cavum internasale. The pterygoids in *N. kaiseri* is shorter and weakly cartilaginous, whereas in *N. microspilotus* is larger, narrower with a great cartilaginous part. Squamosal is connected to the pterygoids bone and both pterygoids and squamosal are connected to the occipital. The premaxillae in *N. kaiseri* is distinct and well ossified. Less ossification, however, has been observed in other salamandrids (e.g., *Salamandra infraimmaculata semenovi* (Akia et al., 2010), which may agree with the primitive position of this taxon (Steinfartz et al., 2007) compared to more derived taxa such as *Neurergus* species. Skull morphology of *N. kaiseri* may be an adaptation for living in mountainous streams. Skulls in plethodontine salamanders that live in the mountain habitats (usually at altitudes above 900 m) tend to be harder and stronger (Buckley et al., 2010) compared to those inhabiting the flatter areas. Maxillary bones in *N. kaiseri* are more flattened and thicker than *N. micropilotus* and nasal bones are long and elongated in the length of skull roof. Naylor (1978) has suggested that the squamosal arch of newts is an anti-predator adaptation designed to add structural support to the skull and protect retracted eyes. Ehmcke and Clemen (2006) showed that the skull of the plethodontid salamanders is membranous and flexible.

Similarities are observed between the osteological structures of the hands, legs, lower jaw and hyobranchial apparatus of *N. kaiseri* with other newt species (Ghosh et al., 1994). Metacarpal establishes a connection between fingers and forearm bones which facilitate the animal movement (Fabrezi and Barg, 2001).

In general, similarities are observed between the skeleton structure of *N. kaiseri* and *N. micropilotus*. Both species are mountains species, however, there are some interspecific differences which could be related to the different total size of the adult individuals. However, there are other differences, which apparently only depend on the phylogenetic position of a species. Further research with a larger sample size and including other *Neurergus* species is required to investigate the remarkable and adaptive variation in bone-forms among these newt species.

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Does color polymorphism affect the predation risk on *Phalotris lemniscatus* (Duméril, Bibron and Duméril, 1854) (Serpentes, Dipsadidae)?

Fernanda R. de Avila[1,2,*], Juliano M. Oliveira[3], Mateus de Oliveira[1], Marcio Borges-Martins[4], Victor Hugo Valiati[2], Alexandro M. Tozetti[1]

[1] *Laboratório de Ecologia de Vertebrados Terrestres, Programa de Pós-Graduação em Biologia, Universidade do Vale do Rio dos Sinos, Avenida Unisinos, 950, Cristo Rei, CEP 93022-970, São Leopoldo, RS, Brasil. *Corresponding author.* Email: fernandar.avila@gmail.com
[2] *Laboratório de Genética e Biologia Molecular, Programa de Pós-Graduação em Biologia, Universidade do Vale do Rio dos Sinos, Avenida Unisinos, 950, Cristo Rei, CEP 93022-970, São Leopoldo, RS, Brasil*
[3] *Laboratório de Ecologia Vegetal, Programa de Pós-Graduação em Biologia, Universidade do Vale do Rio dos Sinos, Avenida Unisinos, 950, Cristo Rei, CEP 93022-970, São Leopoldo, RS, Brasil*
[4] *Laboratório de Herpetologia, Departamento de Zoologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, Agronomia, CEP 91501-970, Porto Alegre, RS, Brasil*

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Abstract. The snake *Phalotris lemniscatus* is a polymorphic species regarding color, which varies between light shades with a yellow predominance (pale yellow-brown) to darker shades with a red predominance (red-dark). Pale yellow-brown individuals are more frequent in coastal populations while there is a tendency of increasing the frequency of red-dark morphs in inland areas. Considering the variation in substrate color along the species distribution (light/sandy on the coast to reddish and dark/argillaceous in inland areas), we raise the hypothesis that the predation rate of each morph would be lower in sites were its crypsis potential is higher. If correct, this hypothesis would reinforce the idea that the predation risk is one of the factors influencing the spatial structuring in morph frequency distributions in populations of *P. lemniscatus*. To test this hypothesis, we performed a field experiment using plasticine *P. lemniscatus* artificial models that represent two morphs: red-dark and pale yellow-brown. The models were distributed in three localities where the following substrate types predominate: light (Coastal Site), intermediary (Lowland Site) and reddish dark (Highland Site). Our predictions were corroborated only at the coastal site, where the less cryptic morph was the most preyed one. We verified that there is a regional variation in the predation risk on different morphs. Thus, the possibility that the selective pressure by predators is a relevant element in the structuring of the frequencies of different morph populations of this species cannot be completely excluded.

Keywords. Behavior, Brazil, coral-pattern, mimetism, phenotype, snake.

INTRODUCTION

Polymorphism is characterized by the presence of different phenotypes in a population (Mayr, 1963) and does not only include the morphological features, but also those related to the life history and behavior of the organisms (Huxley, 1955; Hedrick, 2006). There are many processes behind the maintenance of different morphs in a population, and these processes are not easily identified (Calsbeek and Cox, 2012; Deitloff et al., 2013; Karpestam et al., 2016, Barnett et al., 2018).

Questions such as "how do morphs vary their

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appearance and abundance in a spatial scale" and "what are the habitat components that favor the existence and sympatry of two or more morphs" would be better answered from experiments performed under natural conditions (Hoffman and Blouin, 2000; Roulin, 2004; Gray and McKinnon, 2006).

In squamates, particularly in snakes, some of the most well-documented types of polymorphism are the multiple color forms (color polymorphism). From an ecological view, morphs can be considered cryptic (when they maximize the animal's camouflage; Clarke, 1962; King and Lawson, 1995; Eizirik et al., 2003; Hoffman et al., 2006), aposematic (when they highlight a warning signal; Brodie and Brodie, 2004; Noonan and Comeault, 2009) or a combination of both (Brodie and Brodie, 1980; Wang and Shaffer, 2008; Barnett et al., 2018). Variations in the color are recorded in other taxa and are associated with an improvement in the performance of intraspecific communication, thermoregulation or as anti-predation mechanisms (Endler, 1978; Pérez et al., 2017). Snakes have many different and complex patterns of intraspecific color polymorphism, from systems with bright and contrasting colors to those with cryptic color sets or disruptive patterns (Cox and Rabosky, 2013; Holmes et al., 2017; Martínez-Freiría et al., 2017; Santos et al., 2017).

Undoubtedly, crypsis is an important factor that might bring higher survival chances to the morphotype, since the animal's color matches the color of the substrate, making it difficult to be detected by vision-oriented predators (Johannesson and Ekendahl, 2002; Venesky and Anthony, 2007). These predators are expected to find and attack more easily the more conspicuous morphs in the population, according to their crypsis (Stimson and Berman, 1990).

In southern Brazil, there are consistent records of polymorphic variations spatially structured for the Dumeril's Diadem Snake (*Phalotris lemniscatus*) (Duméril, Bibron and Duméril, 1854) (Fig. 1A and 1C). This species has different morphs with shades going from red-dark to pale yellow-brown (Ferrarezzi, 1993; Esteves, 2011; Noronha, 2012). The variations seem to be restricted to these shades, with only one record of albinism (Abegg, 2015) and no records of a melanistic form. The distribution of *P. lemniscatus* morphs is spatially structured in the following way: individuals of predominantly pale yellow-brown color occur more frequently in populations from regions of sandy substrate of the southern Brazil and Uruguay coasts, while the predominantly red-dark individuals occur more frequently in more continental regions of Brazil and Argentina (Noronha, 2012). In these more continental localities, the substrates are darker due to the predominance of organic matter and clay in the soil (Lema, 2002; Esteves, 2011), allowing differential crypsis between the morphs.

Many snake predators in the extreme South of Brazil are visually oriented (e.g., birds: Dell' Aglio et al., 2012; Santos et al., 2013), and probably respond to variations in the level of contrast between their prey and the substrate. With this premise, we expect that predation is an important selective factor for the definition of the rare (more predated) and more frequent (less predated) morphs in each population. Thus, the predation rate of the morphs of *P. lemniscatus* should vary between regions with different substrate colors, being higher on artificial models of the red-dark type than on those of the yellow-brown type in the coastal region, and the opposite in continental regions.

Because predation events are generally difficult to observe in the wild, they have been largely studied using experimental approaches as a manner to observe the interactions between predator and prey (Brodie, 1993; Guimarães and Sawaya, 2011; Purger et al., 2017). The use of artificial plasticine models has been employed successfully in predation experiments with invertebrates (Koh and Menge, 2006), amphibians (Kuchta, 2006) and reptiles (Stuart-Fox et al., 2002; Valkonen et al., 2011; Dell´Aglio et al., 2012), with highlight on snakes (Brodie, 1993; Dell'Aglio et al., 2012; Farallo and Forstner, 2012; Santos et al., 2013; Akcali et al., 2019).

In the present study, we used models of *P. lemniscatus* to test the hypothesis that the predation rate of each morph would be lower in sites were its crypsis potential is higher. If correct, this hypothesis would reinforce the idea that predation risk is one of the factors for the spatial structuring in the distribution of morph frequencies in populations of *P. lemniscatus*.

MATERIAL AND METHODS

We distributed, in the wild, two types of artificial models of the snake: red-dark and yellow-brown (Fig. 1B and 1D). Their color was based on live specimens captured in the study area in order to accurately represent the color of the morphs that have the extremes of variation between the lighter and darker shades (Noronha, 2012; Fig. 1A and 1C). These models were manufactured with non-toxic plasticine that allowed the record and quantification of marks left by predator attacks (Brodie, 1993). The models measured 30 cm in length and 1 cm in diameter, which represents the mean size of adult animals (Carreira et al., 2012).

The samplings were performed in localities with different natural proportions in the abundance of the morphs of *P. lemniscatus*, these being: 1 – Coastal Site (32°43'2.49"S; 52°28'29.87"W) in the municipality of Rio Grande (annual mean temperature of 17.5 °C, Rossato, 2014), where the pale yellow-brown morph is more abundant; 2 – Highland Site (29°39'23.04"S; 51°23'7.40"W) in the municipality of São Francisco de Paula, where the red-

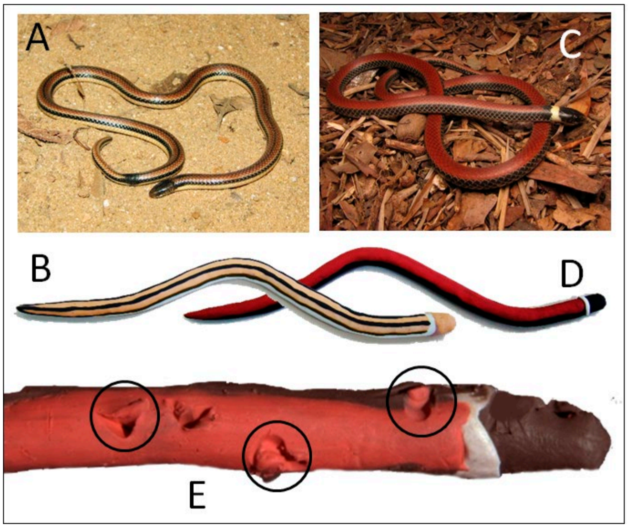

Fig. 1. General aspect of the specimens of *Phalotris lemniscatus* used as a reference to the pale yellow-brown (A) and red-dark (C) patterns and their models manufactured in plasticine (B and D, respectively). The image E show some marks considered as attacks from predators.

Fig. 2. Geographic location of the sampling sites.

dark morph is more abundant (annual mean temperature of 14.5 °C; Rossato, 2014; minimum temperatures frequently close to 0 °C in winter; Maluf, 2000); 3 – Lowland Site (29°27'0.20"S; 50°34'59.83"W) in the municipality of Capela de Santana (annual mean temperature of 17.0 °C; Rossato, 2014), where both morphs are observed in similar proportions (Noronha, 2012). The sampled localities extend in extreme points of an area of approximately 12660 km^2 located at least at 404 km from each other (Fig. 2). The prevailing substrates in each locality are light and sandy (Coastal Site) and dark and argillaceous (Highland and Lowland Sites), allowing the evaluation of different contrast levels between model and background.

We distributed 200 models in each locality (100 red-dark and 100 pale yellow-brown), along five transects, each 400 m long. The transects were at least 1000 m from each other and each received 40 snakes (one every 10 meters). In each transect, the two morphs were interspersed so that they had 20 red-dark and 20 pale yellow-brown models, a protocol similar to that of Dell'Aglio et al. (2012) and Farallo and Forstner (2012). We used this 1:1 ratio to avoid a possible frequency-dependent predation effect. Each model received an identification code and its position was marked with a GPS device to facilitate their monitoring. We also took the care to arrange all the transects in an area of similar vegetation cover (low and scarce vegetation with the prevalence of exposed soil) that would not provide any visual barrier to the predators. Thus, the contrast between the models and the background happened due to the color of the artificial snake and the soil.

The artificial models remained exposed in the field for 48 hours. During this time, they were inspected twice, after 24 and 48 hours since the installation. During the inspections, we recorded the presence of attack marks on the models. Each artificial model that clearly showed marks of bird attack (e.g., pecking) was considered a predation event (Brodie, 1993; Dell'Aglio et al., 2012). The models showing attack marks during the first inspection were replaced by new models. It is worth highlighting that the three localities have a similar fauna of predatory birds (Fontana et al., 2008; Petry and Scherer, 2008; Accordi and Hartz, 2006), mainly of birds of prey (e.g., *Caracara plancus*, *Milvago chimango*), egrets (e.g., *Ardea alba*, *Syrigma sibilatrix*) and even some other generalist foragers (e.g., *Guira guira*).

Data analysis

Just clearly identifiable pecking marks were considered as an attack (or predation event). We quantified only the presence and not the number of marks. Thus, a model with one or more marks corresponded to one predation event. To evaluate the predation intensity of each morph, we calculated the predation rate. To do so, we divided the number of models of each morph with predation evidence (number of events) by the number of exposure hours of these models. The number of exposure hours corresponds to the total number of hours between the installation of the model and its final inspection. This calculation was done for each transect. To test the differences in predation rates of the morphs between the localities we performed an Analysis of Variance with randomization test (Pillar and Orlóci, 1996). In these analyses, we used Euclidian distance matrices between the morphs, restricting the permutations within the transects (blocks) to control possible discrepancies in factors related to the predation that were not directly verified (e.g. number and species of predators). The Analyses of Variance were performed by means of the software MULTIV v.3.34b (Pillar, 1997).

RESULTS

We recorded altogether 162 predation events, which corresponds to an overall predation rate of 1.06 events per hour. At the Lowland Site, 90 models were attacked (23%). This locality had a larger number of attacks directed to the

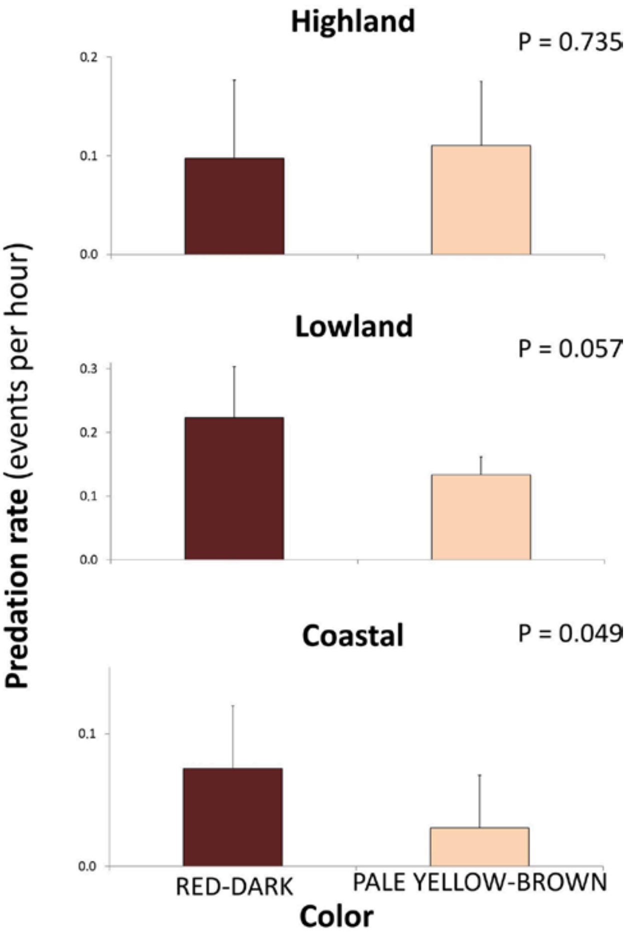

Fig. 3. Mean predation rate (events per hour) on the different morphs (red-dark and pale yellow-brown) in the three areas (Highland, Lowland and Coastal).

red-dark morph (57 models; 0.22 events per hour) than to the pale yellow-brown morph (33 models; 0.13 events per hour), this difference being marginally significant (SQE = 0.02; $R^2 = 41\%$; P = 0.057; n = 10). At the Coastal Site, we recorded 22 predation events (11%). As for the Lowland Site, the Coastal Site also showed more attacks on the red-dark models (16 models; 0.07 events per hour) than on the pale yellow-brown models (6 models; 0.03 events per hour) (SQE = 0.005; $R^2 = 25\%$; P = 0.049; n = 10). At the Highland Site, there was no significant variation in the number of attacks between the different morphs (SQE = 0.0004; $R^2 < 1\%$; P = 0.735; n = 10) (Fig. 3).

DISCUSSION

Our results suggest that the morphs of *Phalotris lemniscatus* have different levels of predation and the predation rate varies between areas. Our predictions regarding the importance of crypsis were corroborated only at the Coastal Site, where the predation rate was higher on the red-dark models that have higher contrast (less cryptic) in relation to the substrate of the region. Similar results were also observed for the morphs of the Mottled Rock Rattlesnake (*Crotalus lepidus lepidus*; Farallo and Forstner, 2012) and the sand hills mice (Linnen et al., 2013), both in the United States. Experiments with artificial models showed that differential crypsis and predation are the main forces of the spatial structuring of the western rattlesnake's morphs (Farallo and Forstner, 2012). Similarly, Linnen et al. (2013) pointed out that the color of the soil offers differential camouflage opportunity to sandhill mice against owls and other raptors and is determinant to the spatial structuring of their colored morphs.

However, our data show that predation does not seem to be the main factor acting on the spatial structuring of the morphs of *P. lemniscatus* since the naturally more uncommon morph in the Lowland Site was the one that suffered less predation (pale-yellow). In other words, the low frequency of the pale-yellow morph in the Lowland Site population does not seem to be the result of predation.

It is worth highlighting that all the sampled localities have a similar predator composition. However, there is a possibility of existing regional variations in the ability of these predators in detecting prey, which would have influenced the local number of attacks on each model type. Experiments showed that the capacity of predators to detect the different morphs based on motionless prey is variable and depends on their ability to generate a specific searching image according to the form, size and color of the prey (e.g., Brodie, 1993; Olsson, 1993; Gotmark, 1994).

It is reasonable to imagine that the search image established by a predator is compatible with a certain type of prey that has a higher probability of being found. Thus, the naturally rare morphs in each locality may not be part of the searching image of local predators (Dukas, 1998). In this case, they might not be easily detectable, even if their color shows more contrast with the background. Therefore, their predation rate would remain relatively low, even if their population was experimentally increased with the introduction of artificial models (Wennersten and Forsman, 2009; Karpestam et al., 2014). In addition, animals that attack potentially dangerous organisms (e.g., snakes) commonly reduce their exploratory behavior toward new and different prey morphs (Greenberg and Mettke-Hofmann, 2001), thus exhibiting a neophobic behavior (avoiding a new environmental aspect) (Greggor et al., 2016). Although possible, this hypothesis would be applicable to the lowland and highland sites, but not to the coastal site, where the less common and less cryptical models were the most preyed ones.

Our results point toward the possibility that other factors besides crypsis interfere on the predation rate. Among those, we can mention the mimetic aposematic potential of the red morph since their bright colors are associated with poisonous or nonpalatable animals (Cuthill et al., 2005; Tarvin et al., 2017). The establishment of a parallel between our data and such studies seems convenient due to the slightly reddish pattern of one of the morphs of *P. lemniscatus*. Yet, the effectiveness of the aposematism based on coral-like coloration is questionable regarding canids (Tozetti et al., 2004) and birds (Smith, 1969). Besides the passive strategies such as crypsis and immobility (Venesky and Anthony, 2007), snakes show active defense strategies or behavioral displays such as escape (Forsman and Appelqvist, 1998; Creer, 2005; Allen et al, 2013). Additionally, *P. lemniscatus* has the antipredatory behavior called "erratic movements" (Tozetti et al., 2009), which would reduce the predation effectiveness after being detected by a predator (Forsman and Appelqvist, 1998). These behaviors may act in combination with the crypsis for the maintenance of each morph in each population.

Our study failed to obtain answers to the proposed questions. One of the reasons might be that the frequency of different morphs in each population may be related to other environmental factors such as thermoregulation (Trullas et al., 2007). In general, ectotherms from cold environments tend to have darker colors, which favor the absorption of solar radiation (Gibson and Falls, 1979; Clusella-Trullas et al., 2008; Allen et al., 2013). Considering that the Highland Site has the lowest temperatures in the distribution area of *P. lemniscatus*, there would be a positive selective pressure favorable to red-dark individuals in the Highland Site and to pale yellow-brown individuals in the Coastal Site (see Bittner et al., 2002).

Despite the weakness of our hypothesis based on our results, we verify a regional variation on the predation risk of the different morphs of this species, which does not completely exclude the possibility that the selective pressure by predators is a relevant element in the structuring of the frequencies of different morphs in populations of this species.

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Age structure of a population of *Discoglossus scovazzi* Camerano, 1878 (Anura - Discoglossidae) in extreme environmental conditions (High Atlas, Morocco)

Mohamed Amine Samlali, Abderrahim S'khifa, Tahar Slimani*

*Cadi Ayyad University, Faculty of Sciences Semlalia, Biodiversity and Ecosystems Dynamic Laboratory, PO Box 2390, Marrakech 40 000, Morocco. *Corresponding author.* Email: slimani@uca.ac.ma

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Abstract. The age structure and growth of the Moroccan Painted Frog (*Discoglossus scovazzi* Camerano, 1878) in a population living in the High Atlas at the upper altitudinal limit of the species' range was estimated for the first time by skeletochronology. Individual age was determined by counting the lines of arrested growth (LAGs) from cross-sections of the phalanges. Both males and females reached sexual maturity at 3-4 years of age at which point the speed of osteogenesis and body growth slow down**.** Males and females have maximum lifespans of six and five years, and average sizes of 47. 50 mm (n = 21, SD = 1.40) and 39.70 mm (n = 53, SD = 0.90) respectively. We detected a positive relationship between age and size, suggesting that the oldest individuals are always bigger and heavier. Sizes corresponding to the same age class are very heterogeneous reflecting divergent conditions and growth strategies.

Keywords. Endemic species, skeletochronology, life-history traits, extreme environment, Morocco.

Degradation and fragmentation of natural habitats due to human activities have harmful effects on amphibian populations (Collins and Crump, 2009; Hamer and Mcdonnell, 2008, 2010; Collins and Fahrig, 2017). The central plateau of the Oukaimeden massif in the High Atlas region of Morocco has been heavily impacted by overgrazing and the banks of its two main rivers are seriously degraded by the bulk removal of sand for construction (Ait Babahmad, 2012; Ait Zidan, 2018). Both human activities have degraded water habitats and altered the richness and abundance of their amphibian communities. Morphological abnormalities have been recorded in adults and tadpoles (Ait Babahmad, 2012; Lansari, 2018), especially in the frog *Pelophylax saharicus*, which is widespread in the massif. This habitat degradation could cause a demographic collapse leading to local disappearances (Mckinney, 2002; Löfvenhaft et al., 2004; Bionda et al., 2013; Babini et al., 2015; Green and Bailey, 2015; Zhelev et al., 2017), especially in species that are relatively sensitive to human activities due to habitat specialization or specific life history (Rubbo and Kiesecker, 2005; Hamer and Mcdonnell, 2008, 2010).

The Moroccan Painted Frog (*Discoglossus scovazzi* Camerano 1878, family Discoglossidae) is listed as Least Concern in the IUCN Red List of Threatened Species (Salvador et al., 2009; Başkale et al., 2018). Endemic to Morocco, the species is generally common, especially in sub-humid and humid areas. They are quite cryptic outside the breeding season, with adults preferring the proximity of small bodies of water such as temporary ponds, low-flow streams and sources. Although the species may tolerate a slight modification of its habitat, its sensitivity to the particular conditions and the quality of its aquatic and terrestrial environment remains largely unknown (Reques et al., 2013). Because the demographic changes are in response to environmental conditions (Sinsch et al., 2004;

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Sinsch et al., 2007; Spear et al., 2009), the purpose of this study is to estimate the age structure, age at first reproduction and longevity of a high mountain population of *Discoglossus scovazzi*, by using skeletochronology.

Discoglossus scovazzi is an anuran of small body size known from a large number of aquatic biotopes throughout Morocco from sea level up to the Atlas Mountains, excluding the Saharan areas. It reaches its upper altitudinal limit in the High Atlas at Oukaimeden, over 2600 m a.s.l. (Bons and Geniez, 1996).

This study is based on mark-recapture tracking of 74 adult individuals (21 males and 53 females) between 2016 and 2018. The study took place at the population's breeding site, which consists of small pools of water fed by a permanent watercourse. Solitary and discreet during most of the year, *Discoglossus scovazzi* begins reproduction in early spring (Ait Babahmad, 2012; Beukema et al., 2013; Samlali, 2016). Egg-laying is spread over 8 to 10 days from early April to mid-June. During this period, the water temperature is about 13 °C and the outside temperature is about 10 °C.

The population is located at 2660 m of altitude in the Oukaimeden massif (31°11'N, 07°50'W) about 75 km South-East of Marrakech. The climate is typically Mediterranean with cold winters and an average annual temperature of 9.5 °C. The average maximum temperature of the hottest month (July) is 22.2 °C and the average minimum temperature of the coldest month (January) is -3.3 °C. The average annual precipitation is circa 518 mm and snow fall mainly between November and March. The dry period lasts about four months (June-September). The vegetation consists of spiny xerophytes and a herbaceous layer, essentially graminaceous, relatively rich during rainy periods but heavily overgrazed. The population breeds in pits (diameter 6-7 m and at least one meter deep) that resulted from sand extraction and used as waterers in the bed of a permanent river (Assif-N-Irène). Intensive use for livestock watering negatively impacts water quality and accelerates drying (Ait Babahmad, 2012; Samlali, 2016).

Sampling by hand occurred from February to June. We measured the frogs from snout to cloaca with calipers and weighed them using a field balance (accuracy: 0.1 g). We determined sex by the presence of nuptial callosities and calling in males. After measurements, we collected the third toe of the left hind leg and we immediately released the frog at the place of its capture. The samples are kept in absolute alcohol.

We sectioned phalanges from the toe to count lines of arrested growth (LAGs) following the method by Castanet and Smirina (1990), which is based on the detection of LAGs generated by the cold season (each LAG is interpreted as one year of age). In order to avoid the errors of the estimation of the age induced by the medullary resorption (Francillon-Vieillot, 1987; Fretey and Le Garff, 1992; Tsiora and Kyriakopoulou-Sklavounou, 2002; Guarino et al., 2003; 2008; Liao and Lu, 2010; Liao, 2011; Huang et al., 2013; Bionda et al., 2015; Jin et al., 2017), we selected, for each individual, diaphysis sections in which the size of the medullar cavity was at its minimum and that of the periosteal bone at its maximum (Oromi et al., 2016). On the other hand, bone remodeling and the close rapprochement of the outer most lines, especially in the oldest individuals (Wagner et al., 2011) could also compromise the age estimation. In these cases, at the insertion site of the phalangeal ligament allows to discern the peripheral LAGs and to reliably count them (Bionda et al., 2018). The number of lines of arrested growth (LAGs) in each section is counted by three authors (M.A.S., A.S. and T.S.). Since all the individuals studied were collected during the breeding season, the age at sexual maturity was revealed by the presence of LAGs suddenly becoming very closely adjacent, reflecting the slowing of growth after sexual maturity.

All variables were first tested for normality (Kolmogorov-Smirnov test) and the difference between sexes was tested using ANOVA. In addition, the relationship between size and age was analyzed by Spearman correlation test. We used the significance level of alpha = 0.05 in all tests.

A total of 74 reproductive adults (21 males and 53 female) were studied. Individuals were collected at breeding sites during the reproduction periods in 2016, 2017 and 2018. The cross-sections show the presence of strongly stained growth lines in periosteal bone in most individuals (Fig. 1). Since the frogs were collected in March and April, we considered the perimeter of the bone as corresponding to an indicator LAG of last winter. We clearly detected a decrease in the distance that

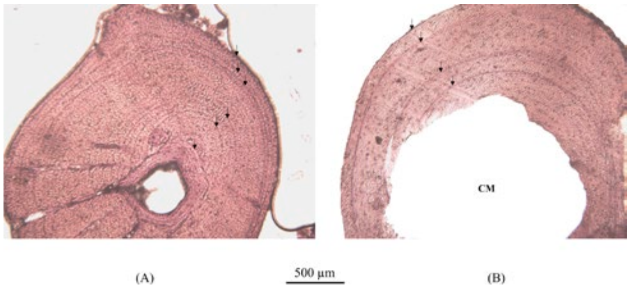

Fig. 1. Transverse sections at diaphysal region of phalanx bones of *Discoglossus scovazzi*. (**A**) Male with 6 visible LAGs. (**B**) Female with 4 visible LAGs (CM: medullary cavity.

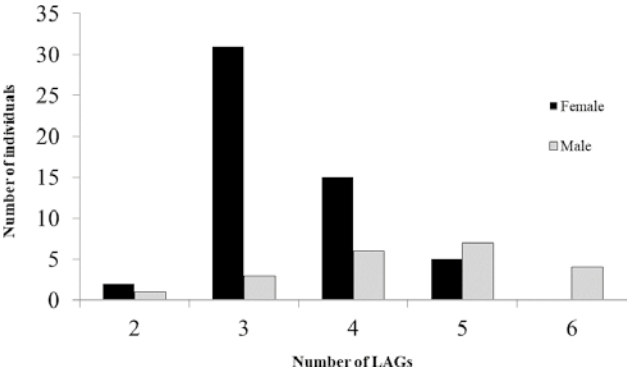

Fig. 2. Age distribution of the reproductive population of *Discoglossus scovazzi* at Oukaimeden in 2016-2018.

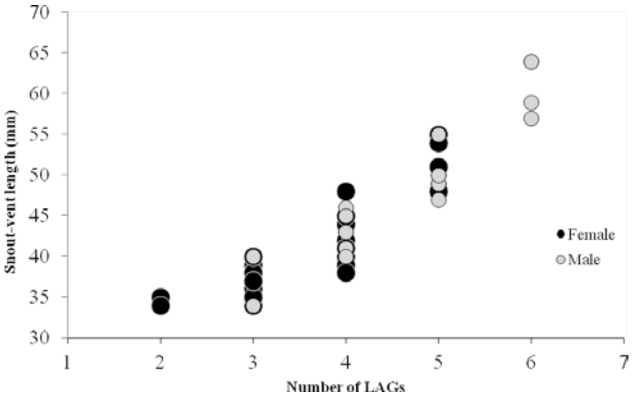

Fig. 3. Relationship between estimated age (X-axis, in year) and body size (Y-axis, snout-vent length in mm) in the studied population.

separating the LAGs suggesting that sexual maturity is reached at 3-4 years in both males and females (Fig.1 A, B). At the breeding site, we sampled some females younger than three years, although three-year individuals were the most frequently observed (Fig. 2). Highest estimated age is six years in males and five years in females.

Body size varied from 34.0 to 64.0 mm in males (mean ± SD = 47.5 ± 1.4 mm, n = 21) and from 33.0 to 56.1 mm in females (39.7 ± 0.9 mm, n = 53), with a mass of 14.5 ± 5.17 g in males and 8.05 ± 3.45 g in females. The observed pattern of sexual dimorphism in body size was significant ($F_{1, 72} = 21.4$; $P < 0.0001$). Because we did detect a significant relationship between age and body size in males (r = 0.786, P = 0.018, n = 21) and in females (r = 0.847, P = 0.003, n = 53) (Fig. 3), the oldest individuals were always the largest ones. Indeed, anurans often exhibit continuous growth, particularly females because of the strong relationship between fecundity and body size (Lengagne et al., 2007).

In most anuran species, males are smaller than females (Shine, 1979). For *D. scovazzi,* the sexual size dimorphism is inversed (García-Paris et al., 2004; Oromi et al., 2016). The variation in size dimorphism results from differences in the age structure between sexes (Monnet and Cherry, 2002; Liao et al., 2013a, b). In fact, in species where females exhibit smaller sizes (*Rhinella achalensis*, *Rana cascadae* and *Hylarana nigrovittata*), males mature at later ages (Monnet and Cherry, 2002), suggesting that delayed maturity in males produces size dimorphism as stated in the Rensch's rule (Liao et al., 2015). However, as in *D. pictus* (Oromi et al., 2016), we did not find differences in the age structure between the sexes in *D. scovazzi* which suggests that these two species failed to support Rensch's rule. In some anuran species, increased male body size is an important determinant of male mating success (Wells, 2007; Liao and Lu, 2011). Furthermore, large males are more likely to breed than smaller ones if competition for females is intense (Oromi et al., 2016). However, little information is available on the mating tactics of *D. scovazzi* and other factors might explain the sexual size dimorphism in this species.

Our study provides original data on the structure of a population at the upper altitudinal limit of this endemic species of Morocco. The earliest age at which sexual maturity was attained in most individuals of both sexes is about 3 years. However, the three-year-old frogs, theoretically fewer numerous than those aged two as a result of annual mortality, are more frequent at breeding sites than younger females. As observed in Brongersma's Toad in arid environments (Fattah et al., 2014), this asymmetrical age structure in females of *Discoglossus scovazzi* in the High Atlas suggests that this difference in young females is due to the fact that not all the females reach sexual maturity before three years, or that some females reach sexual maturity at the age of two years.

Compared to its Mediterranean congener, *D. pictus*, studied by Oromi et al. (2016) in North Africa, the late age at maturity in our population suggests a low growth rate in juveniles, strongly constrained by the harsh climatic conditions of this alpine environment (low temperature, snowfall, strong wind), or the modification of aquatic environments (due to excessive sand removal) and the deterioration of their physico-chemical quality (due to livestock).

The low values in terms of longevity (5-6 years) and reproductive potential (about two years) recorded in this population are related to climatic conditions and ongoing changes in habitat quality. Several other hypotheses could, however, be formulated, including moving larger adults /older to other sites, the low survival of larger individuals /older, or the case of an unstable population maintained by smaller / younger adults. This highlights a need monitoring.

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Bottom/left: Boaedon fuliginosus (photo by John Lyakurwa)

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