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*Front cover: Rattlesnake *Crotalus enyo* from Coronados Island. See article on page 167.
Photograph by Gustavo Arnaud.*

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Genetic reconstruction of the invasion history of *Anolis watsi* in Trinidad with a comment on the importance of ecological similarity to invasion success

Sozos N. Michaelides¹, Noah Gilbert¹, Brian E. Smith², Graham L. White³, Adrian Hailey⁴ & Jason J. Kolbe¹

¹ Department of Biological Sciences, University of Rhode Island, Kingston 02881, RI, USA

² Department of Biology, Black Hills State University, 1200 University Street, Spearfish, South Dakota, USA

³ Lot 14, Surrey Village, Lopinot Road, Arouca, Trinidad and Tobago, West Indies

⁴ Department of Life Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago, West Indies

Human activities have reshaped patterns of island biogeography for many groups of amphibians and reptiles. In Trinidad, an island in the West Indies with only one native *Anolis* lizard species, four additional anole species have been introduced since the 1800s. The most recent introduction, *Anolis watsi*, native to Antigua, has become established despite the presence of multiple species of resident anoles. We used genetic data (mitochondrial DNA) to infer the geographic origin and genetic structure of introduced *A. watsi* on Trinidad. We then gathered published data to compare eco-morphological traits of all anole species currently established on the island. We found three mtDNA haplotypes in Trinidad that clustered with two different clades from the northern part of the native range in Antigua, rejecting the hypothesis that the lizards originated in the pineapple-growing region of the south. However, a lack of fine-scale population structure precluded precise identification of the origin in Antigua of haplotypes introduced to Trinidad. Compared to the other anole species on Trinidad, *A. watsi* is smaller, perches lower, and has a higher field body temperature. Thus, the successful establishment and spread of *A. watsi* should not be surprising given the success of two previous introductions of anole species that were more similar eco-morphologically to the native species and each other, and the fact that Trinidad has relatively few anole species for its size.

Keywords: *Anolis* lizards; Caribbean islands; eco-morphological traits; invasions; mtDNA

INTRODUCTION

In the Anthropocene epoch, islands and mainland coastal regions are hotspots of established non-native species (Dawson et al., 2017) and human-aided introductions have reshaped species-area and species-isolation relationships (Sax et al., 2002; Helmus et al., 2014). Although successful invasions have certainly altered biogeographic patterns for *Anolis* lizards in the Caribbean (Helmus et al., 2014), we know relatively little about the process of transport, establishment and spread for these invading anole species. Genetic analyses can be used to better understand the pathways for introduction and spread of non-native anoles (e.g. Kolbe et al., 2004; 2007) and comparisons of ecological similarity to native species may be useful for predicting the outcome of these novel interactions (Losos et al., 1993) and their potential ecological impact on native communities.

Species co-existence in *Anolis* lizards is strongly influenced by their position on three ecological niche axes; structural microhabitat (e.g. perch diameter and

height), thermal microhabitat, and body size, which influences both diet and interspecific interactions (Losos, 2009). Greater ecological similarity on these niche axes should increase the strength of interspecific competition (Roughgarden, 1995) and may limit successful establishment of introduced species. Indeed, a previous study found that *Anolis* lizard introductions were more successful when resident and invading species were ecologically different; a designation based primarily on ecomorph (or habitat specialist) groups for Greater Antillean species and body-size differences for Lesser Antillean species (Losos et al., 1993). However, this type of categorical assessment of ecological similarity lacks the detail needed to identify which niche axes might limit coexistence and precludes evaluation of the role of ecological and evolutionary trait change during the invasion (Kolbe et al., 2012; Stuart et al., 2014).

Trinidad is by far the largest island in the Caribbean with only one native *Anolis* species, *A. planiceps* (formerly *A. chrysolepis planiceps*). Martinique, the next largest island with only one native anole species, is less than one-

Correspondence: Sozos N. Michaelides (msozos@gmail.com), Jason J. Kolbe (jjkolbe@uri.edu)

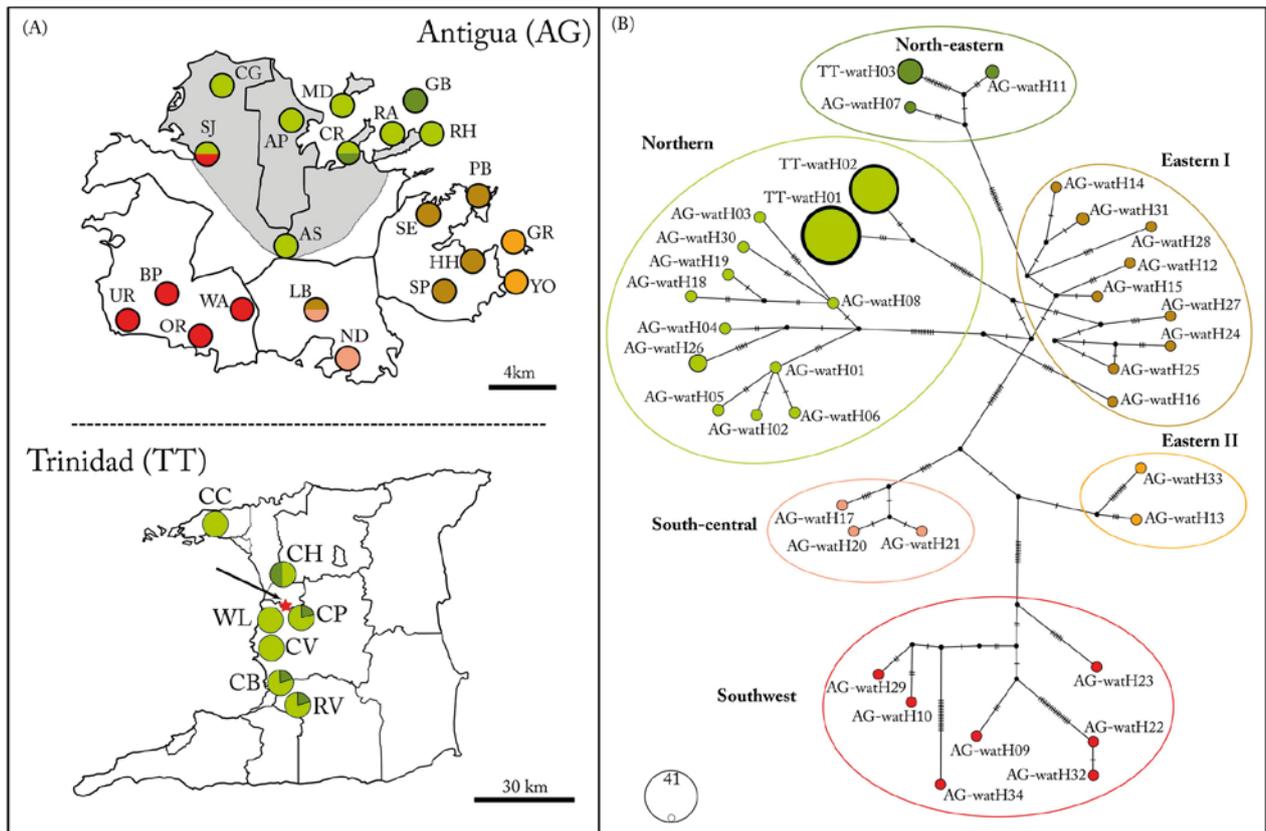


Figure 1. Sampling locations and mtDNA haplotype distribution. **(A)** Distribution of sampling locations in the native (Antigua) and introduced (Trinidad) ranges with pie charts indicating percentage of different haplogroups present. The arrow and red star show the location where lizards were first discovered at the Caroni Research Station in Trinidad. Grey-shaded area in Antigua indicates the most likely region of origin based on mtDNA haplotype divergence. **(B)** Median-joining network of 135 mtDNA ND2 sequences. The size of the pie indicates the number of individuals sharing that haplotype. The six haplogroups correspond to the mtDNA clades from the phylogenetic analysis (see Fig. S1). The three haplotypes found in Trinidad (TT-watH01, TT-watH02 and TT-watH03) group with the northern and north-eastern clades in Antigua.

quarter of its size. However, due to the high economic connectivity with other islands across the Caribbean, four exotic anoles have been introduced since the 1800s (Kraus, 2009), the highest number of introduced *Anolis* species for any island in the Caribbean. The first non-native anole to establish on Trinidad was *A. aeneus*, previously endemic to Grenada, and currently the most abundant anole in suburban gardens (White & Hailey, 2006). *Anolis trinitatis*, previously endemic to St Vincent, is another naturalised species in Trinidad, whereas *A. extremus* from Barbados is now considered extirpated despite multiple introduction attempts since the 1960s (White & Hailey, 2006). The most recent introduction was *A. watsi*, native to Antigua. The co-occurrence of ecologically similar anole species on Trinidad provides an opportunity to assess the predictive power of ecological similarity for the establishment, spread and ecological impact of invasive species.

Anolis watsi was first discovered in 1992 on the grounds of Caroni Research Station in Waterloo (Fig. 1, Boos, 1996; White & Hailey, 2006). Because the lizards were spotted on pineapple plants (cultivar “Black Antigua”), it has been suggested that planting material shipped from south-western Antigua was the likely source of the population (White & Hailey, 2006). However, a

thorough survey by White & Hailey (2006) found no direct evidence that the introduction was on pineapple plants. In addition, relatively early accounts of lizards at an import/export terminal of a cement factory in Claxton Bay, Trinidad (CB, Fig. 1), approximately 15 km south of Waterloo (WL, Fig. 1), could have been another site of introduction (White & Hailey, 2006). Consequently, the source and the timing of the introduction remain unresolved, which limits our ability to compare non-native populations to their native-range source. Furthermore, following its localised establishment in west-central Trinidad, the species is currently found in at least seven localities (White & Hailey, 2006). Their discontinuous distribution, separated by presumably unfavorable habitat (i.e., areas of sugar cane cultivation) suggests establishment through jump dispersal (A. Hailey, unpubl. data; White & Hailey, 2006), however, testing these hypotheses requires extensive sampling and analysis of genetic data.

In this study, we first used phylogenetic and population-genetic analyses of mitochondrial DNA (mtDNA) sequences to infer the geographic source (origin) and genetic structure of introduced *A. watsi* populations on Trinidad. We then gathered published data of eco-morphological traits to compare all resident

anole species in Trinidad and evaluate the importance of ecological similarity for invasion success.

MATERIALS AND METHODS

Genetic analyses

We sampled 95 adult *A. watsi* from seven locations on Trinidad (Fig. 1, Table 1) and 39 from the native range in Antigua (20 locations, Fig. 1, Table 1). We sequenced an approximately 1200 base pair (bp) region of mitochondrial DNA including the ND2 gene for all individuals and used Bayesian inference and median-joining network analysis to reconstruct phylogenetic relationships among haplotypes and assign non-native range haplotypes to their geographic origin in the native range (detailed protocol in Supplementary Material). We calculated nucleotide diversity (Π) per sampling location in DNAsp v.6 (Rozas et al., 2017) and compared Π in native versus non-native range with a Welch Two Sample *t*-test in R v3 (R Development Core Team 2017). For this comparison, we excluded two locations in the native range where only one sample was collected.

Eco-morphological similarity

We collected data from the literature to determine whether each introduced anole species in Trinidad was ecologically similar to the resident species preceding their introduction. We included data on body size (maximum male snout to vent length, SVL), hind-limb length (HLL), head length (HDL), and field active body temperature (Tb) as well as the mean, niche breadth (NB) and niche overlap (NO) for aspects of structural microhabitat use (i.e., substrate, perch height and perch diameter).

RESULTS

Our analyses of sequences from 134 individuals identified 36 haplotypes (GenBank accession numbers MH375645-MH375680), 33 in the native range of Antigua and three in the introduced range of Trinidad. No haplotypes were shared between Antigua and Trinidad, and introduced-range haplotypes showed considerable nucleotide divergence (1.2-1.4 %) from the closest native-range haplotypes. Two haplotypes (TT-watH01 and TT-watH02) represented 85 % of the individuals sampled in the

Table 1. Details on sampling locations with mtDNA haplotypes identified.

Island	Locality	Abbreviation	Date [§]	Ns	Haplotype*
Trinidad	Claxton Bay	CB	2003	10	TT-watH01(3), TT-watH02(6), TT-watH03(1)
Trinidad	Cocorite	CC	2005	10	TT-watH02(10)
Trinidad	Charlieville	CH	2008	20	TT-watH01(10), TT-watH03(10)
Trinidad	Carapichaima	CP	2004	15	TT-watH01(6), TT-watH02(7), TT-watH03(2)
Trinidad	Couva	CV	2004	10	TT-watH01(4), TT-watH02(6)
Trinidad	Reform Village	RV	2005	10	TT-watH02(9), TT-watH03(1)
Trinidad	Waterloo	WL	1992	20	TT-watH01(18), TT-watH02(2)
Antigua	Airport	AP	-	2	AG-watH01, AG-watH02
Antigua	All Saints	AS	-	2	AG-watH03, AG-watH04
Antigua	Cedar Grove	CG	-	2	AG-watH05, AG-watH06
Antigua	Crabbs Peninsula	CR	-	2	AG-watH07, AG-watH08
Antigua	Urlings	UR	-	2	AG-watH09, AG-watH10
Antigua	Great Bird Island	GB	-	2	AG-watH11(2)
Antigua	Green Island	GR	-	2	AG-watH12, AG-watH13
Antigua	Harmony Hall	HH	-	2	AG-watH14, AG-watH15
Antigua	Liberta	LB	-	2	AG-watH16, AG-watH17
Antigua	Maiden	MD	-	2	AG-watH18, AG-watH19
Antigua	Nelson's Dockyard	ND	-	2	AG-watH20, AG-watH21
Antigua	Old Road	OR	-	2	AG-watH22, AG-watH23
Antigua	Pineapple Beach	PB	-	2	AG-watH24, AG-watH25
Antigua	Rabbit Island	RA	-	2	AG-watH26(2)
Antigua	Red Head Island	RH	-	2	AG-watH26(2)
Antigua	Seatons	SE	-	2	AG-watH27, AG-watH28
Antigua	St John's	SJ	-	2	AG-watH29, AG-watH30
Antigua	St Philips	SP	-	2	AG-watH31(2)
Antigua	Wallings	WA	-	1	AG-watH32
Antigua	York	YO	-	2	AG-watH33(2)
Antigua	Boggy Peak	BP	-	1	AG-watH34 [#]

[§] Date first documented/observed * Number in parenthesis corresponds to the number of individuals sharing that haplotype.

[#] AG-watH34 corresponds to a published haplotype (Jackman et al., 1999) with GenBank accession number AF055931

Table 2. Summary statistics for mtDNA haplotype variation for the native and non-native ranges of *A. wattsi*. Number of sampling locations (NI), number of individuals sampled (Ns), number of haplotypes identified (Nh) and nucleotide diversity (percentage, Pi).

Range	Island	NI	Ns	Nh	Pi (%)
Native	Antigua	21	40	34	0.69
Non-native	Trinidad	7	95	3	0.42

introduced range and these haplotypes differed by five nucleotides (0.4 %) from each other and 1.8-2.1 % from TT-watH03 (Table 1).

The phylogenetic analysis supported a well-resolved topology with six geographically distributed clades in Antigua (two northern clades, two eastern clades, a south-central clade and a south-west clade, Fig. S1). The introduced haplotype TT-watH03 was nested within the Northern clade I and haplotypes TT-watH01 and TT-watH02 were nested within the Northern clade II (Fig. 1B). Four localities in Trinidad (CH, CP, CB and RV) contained haplotypes from both of these native-range clades (Fig. 1A). In addition, three locations in Antigua (SJ, CR and LB) contained haplotypes belonging to different combinations of two different clades (Fig. 1A). Nucleotide diversity (Pi) was lower in the introduced range compared to the native range (Table 2), albeit not significantly ($t = -1.19$, $df = 21.75$, $P = 0.25$).

Eco-morphological traits varied in similarity between pairs of introduced species in Trinidad (Table 3). In all instances, morphological and structural habitat values were greater in the older-established anoles, *A. aeneus* and *A. trinitatis*. Structural niche breadth (in all three dimensions; substrate, perch height, perch diameter) was generally greater in *A. aeneus* and *A. trinitatis* compared to *A. wattsi*, and both of these anole species perched on higher and broader vegetation compared to *A. wattsi*. Niche overlap for substrate (NO-S) was lower between *A. aeneus* and *A. wattsi* (NO-S = 0.666, Table 4) compared to *A. aeneus* and *A. trinitatis* (NO-S = 0.990, Table 4). Perch diameter overlap was high between both pairs; *A. aeneus* and *A. trinitatis* (NO-PD = 0.995) and *A. aeneus* and *A. wattsi* (NO-PD = 0.97). Perch height overlap was lower between *A. aeneus* and *A. wattsi* (NO-PD = 0.839) compared to *A. aeneus* and *A. trinitatis* (NO-PD = 0.997). Finally, body size ratio was 1.43 or less for all pairs (Table 4), well below the ratio of 1.65 for co-existing native Lesser Antilles anoles (Losos 2009).

DISCUSSION

In this study, we first used mtDNA sequences to infer the geographic origin and genetic structure of introduced *A. wattsi* and investigate their patchy distribution on Trinidad. We found three haplotypes in the non-native range that are closely related to native-range haplotypes from the northern part of Antigua around Crabbs peninsula. Presence of haplotypes from two different native-range clades in four out of seven non-native populations suggests the possibility of multiple

introductions and admixture. However, this did not lead to increased genetic diversity in terms of the numbers of haplotypes and nucleotide diversity within non-native populations as found in some other *Anolis* invasions (Kolbe et al., 2004, 2007; Michaelides et al., 2018). We discuss these results and the importance of ecological similarity to invasion success.

The phylogeographic history of a species in its native range determines the distribution of genetic diversity available from which colonists are drawn and only strong phylogeographic structure in the native range can support precise identification of the source locations (Taylor & Keller, 2007). In Antigua, the native range of *A. wattsi*, there is weak to moderate geographic structure. Given our geographically widespread sampling, it is somewhat surprising that we did not find the same or very similar haplotypes to those in Trinidad, which could suggest an origin from a location in Antigua that was not sampled. The closest native-range haplotypes are from the northern part of the island including several offshore islands: the airport, Crabbs peninsula, St John's, and Cedar Grove, which has a large nursery. Interestingly, Crabbs peninsula contains haplotypes from both northern clades similar to four out of seven locations in Trinidad with 1.2-1.4 % sequence divergence from all introduced haplotypes. The first location of *A. wattsi* in Trinidad at an agricultural research station suggests importation on live plant material, particularly pineapples. However, our genetic data reject the earlier hypothesis regarding the source being a location in the south-western part of Antigua (where a major pineapple farm is based), and support a more likely source somewhere in the vicinity of Crabbs peninsula, which is near a shipping port in the northern part of Antigua.

Lizards were first discovered in Trinidad at the Caroni Research Station, in Waterloo, which was also suspected to be the initial point of introduction. However, only two out of the three haplotypes were found there (TT-watH01 in higher frequency than TT-watH02), whereas in Carapichaima, which is less than a kilometer to the east, all three haplotypes are present (but only two out of 15 individuals have the third haplotype TT-watH03). The absence of TT-watH03 from Waterloo, and the low frequency overall across Trinidad, could be a result of a founder effect during the initial introduction. Another possibility would be that a second introduction occurred to Carapichaima either directly from Antigua or more likely from another location within Trinidad where the third haplotype also exists. For instance, Claxton Bay where all three haplotypes are also found could have been another point of introduction. This location was discovered in 2003 (White & Hailey, 2006) and could have served as another source of *A. wattsi* at other sites in Trinidad through human transport of building material. In Charleville, about 10 km north of Waterloo, *A. wattsi* was first discovered in 2008 by The University of the West Indies technicians collecting mosquitoes (A. Hailey, unpubl. data). Interestingly, samples collected (10 individuals) on each side of the major highway there, were fixed for two different haplotypes, TT-watH01 to the east and TT-watH03 to the west (highest frequency across

Table 3. Summary of eco-morphological characteristics of native and non-native anoles in Trinidad. Body size (maximum male SVL), hind-limb length (HLL), head length (HDL), active body temperature (Tb), mean perch height (PH), mean perch diameter (PD) and niche breadth (NB) for each dimension of the structural habitat niche: substrate (NB-S), perch height (NB-PH), and perch diameter (NB-PD).

Species	Date	Native Isl.	SVL ²	HLL ²	HDL ²	Tb ³	PH (cm) ⁴	PD (cm) ⁴	NB-S ⁴	NBPH ⁴	NB-PD ⁴
<i>A. planiceps</i>	-	Trinidad	76 ⁵	-	-	-	-	-	-	-	-
<i>A. aeneus</i>	1900	Grenada	77	16.4	17.0	31.0	83	12	4.85	4.74	3.39
<i>A. trinitatis</i>	1900	St Vincent	74	17.0	16.6	30.3 ⁶	105	14.3	3.67	4.14	4.02
<i>A. extremus</i>	1960	Barbados	83	-	-	-	-	-	-	-	-
<i>A. watsi</i>	1992	Antigua	58	13.9	14.3	33.3	46	11	4.46	2.65	2.87

¹ Approximate date of introduction

² Data from Poe (2014)

³ Data from Hertz et al. (2013)

⁴ Data from Hailey et al. (2009) and White & Hailey (2006)

⁵ Data for *A. planiceps* [*A. chrysolepis planiceps* from D'Angiolella et al. (2011)]

⁶ Data from Gorman et al. (1971)

Table 4. Ecological similarity and current status in Trinidad. The numbers below the diagonal represent niche overlap for substrate (NO-S), and the ones above the diagonal represent body size ratio. The diagonal represents the outcome of each introduction and the current distribution in the case of the native *A. planiceps*.

Species	<i>A. planiceps</i>	<i>A. aeneus</i>	<i>A. trinitatis</i>	<i>A. extremus</i>	<i>A. watsi</i>
<i>A. planiceps</i>	Forested areas	1.01	1.02	1.09	1.31
<i>A. aeneus</i>	-	Widespread ¹	1.04	1.07	1.32
<i>A. trinitatis</i>	-	0.990	Localised ²	1.12	1.28
<i>A. extremus</i>	-	-	-	Extinct ²	1.43
<i>A. watsi</i>	-	0.666	-	-	Widespread ¹

¹ From White & Hailey (2006)

² From Hailey et al. (2009)

all sites in Trinidad) suggesting separate introductions or different histories of establishment. Jump-dispersal within Trinidad is the most likely explanation for the patchy distribution and the variation in the frequency of haplotypes across localities. For example, the northernmost sampling location in Cocorite (CC, Fig. 1) had only a single haplotype and was probably established through human-assisted transportation of individuals from a southern location. Secondary introductions within the non-native range are common in invasions to remote locations (Kolbe et al., 2004; Floerl et al., 2009; Lombaert et al., 2010); for example, the green anole, *A. carolinensis* (native to south-eastern USA), has established several populations in Pacific islands from older-established populations in the region (Michaelides et al., 2018). Interestingly, *A. watsi* has also been introduced to another island in the southern Lesser Antilles, St Lucia in 1956 (Kraus, 2009). Given the high level of agricultural and horticultural trade among Caribbean islands (Helmus et al., 2014), the established population of *A. watsi* in St Lucia could have served as a secondary source of the lizards in Trinidad. Nevertheless, further sampling and genetic data are needed to test this alternative hypothesis.

Introduced populations of *A. watsi* on Trinidad had lower genetic diversity than native-range populations, however, intraspecific admixture (presence of haplotypes from two different clades was evident in 57 % of the sampling locations) may have counteracted the severity

of founder effects. Indeed, multiple introductions with admixture are a common feature for many introduced *Anolis* species (Kolbe et al., 2007). For example, *A. sagrei* populations in Florida are the result of at least eight introductions from different source populations across its native range in Cuba (Kolbe et al., 2004). These independent introductions, followed by admixture in the introduced range have led to higher genetic variation in the introduced range than their native counterparts, and probably assisted in their establishment and further spread (Kolbe et al., 2004).

Whereas most anole introductions fail where there is already an ecologically similar species established (Losos et al., 1993), there were four anole species in Trinidad at the time of this study, one native and three introduced (*A. extremus* is considered extirpated). Two additional *Anolis* species have been recently detected on Trinidad, *A. cristatellus* (Auguste et al., 2018) and *A. sagrei* (Auguste 2018). In the Lesser Antilles, ecological co-existence for anoles is primarily a function of body size, which generally correlates with prey size, where pairs of anole taxa usually differ in body size by a ratio of 1.65 (Losos, 2009). In Trinidad, the body size ratio is well below this threshold for all pairs, and this greater similarity in body size is predicted to lead to stronger interspecific competition. Indeed, the first two anole introductions, *A. aeneus* and *A. trinitatis*, were very ecologically similar in all three niche dimensions (i.e., body size, structural microhabitat, and thermal microhabitat). Hybridisation

along with intense competition has been suggested as the likely cause of the decline in *A. trinitatis* and its localised occurrence (Gorman et al., 1971; Hailey et al., 2009). However, the two species might have different habitat requirements (well-vegetated areas for *A. trinitatis* versus a wider range of habitats including open areas for *A. aeneus*) and thus differ in thermoregulatory ability (that of *A. aeneus* being greater) rather than body temperatures as such (Hailey et al., 2009).

The third anole introduction, *A. extremus*, is of similar size to the older-established *A. aeneus* and *A. trinitatis*, but the failure to become established was likely due to small population size (Hailey et al., 2009). The recent addition, *A. watsi*, has become established despite the presence of *A. aeneus* in the same area and habitat. Body size difference between these two species is the greatest among all extant anole pairs in Trinidad and could have facilitated their co-existence. Differences along other niche dimensions, for example thermal biology, substrate type and perch height might be sufficient to reduce competition (White & Hailey, 2006). For example, when *A. watsi* co-occurs with *A. leachii* (a larger anole) in its native range, it uses lower perches compared to when in allopatry (Kolbe et al., 2008). The same pattern of perching lower is found in Trinidad when in sympatry with *A. aeneus* (White & Hailey, 2006). A habitat shift in sympatric populations of anoles has been shown elsewhere; for example, between the native *A. oculatus* (used higher perches) and the invasive *A. cristatellus* (used lower perches) compared to allopatric populations of both species in Dominica (Dufour et al., 2018). Such shifts in habitat imposed by strong interspecific interactions can also drive rapid evolutionary change in native species (e.g. Stuart et al., 2014).

The native anole on Trinidad, *A. planiceps*, is the most terrestrial of the four species currently on the island as it forages on the ground but is restricted to forested areas (White & Hailey, 2006). Introduced *Anolis* have not yet moved into undisturbed areas. However, the lower perching *A. watsi* is more likely to compete with the native *A. planiceps* than are other introduced species due to the ground-dwelling habits of the latter (Murphy, 1997; White & Hailey, 2006). Yet, higher active body temperatures for *A. watsi* may restrict its ability to invade forested areas inhabited by *A. planiceps*. Given its depauperate native anole fauna for an island the size of Trinidad, it should not be surprising that several non-native anole species are now well established on the island. This has occurred despite some eco-morphological similarity among the native species (*A. planiceps*) and early invaders (*A. aeneus* and *A. trinitatis*). Whether the successful invasion of *A. watsi* was facilitated by it being more eco-morphologically distinct from resident species on Trinidad will require experimental assessment or observations of the success or failure of future invasion attempts by anole species based on their ecological similarity to the current anole fauna on the island.

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The effect of thermal gradient design on the evaluation of thermoregulation in snakes

Ricardo Figueroa-Huitrón¹, Hibraim Adán Pérez-Mendoza¹, Aníbal Helios Díaz de la Vega-Pérez², Fausto Roberto Méndez-De la Cruz³ & Leticia Margarita Ochoa-Ochoa⁴

¹Módulo de Ecología y Conservación, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Avenida de los Barrios 1, Los Reyes Iztacala 54090, Tlalnepantla, Estado de México, México

²Consejo Nacional de Ciencia y Tecnología, Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Carretera Tlaxcala-Puebla km. 1.5, C.P. 90062, Tlaxcala de Xicohténcalt, Tlaxcala, México

³Laboratorio de Herpetología, Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, A. P. 70515, C.P. 04510. CDMX, México

⁴Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autónoma de México, Circuito Exterior sn, Ciudad Universitaria, A. P. 70-515, C. P. 04510. CDMX, México

Hertz et al. (1993) designed what is now the most widely used protocol to analyse the thermal strategies and efficiency of small squamates. Preferred temperature range (T_p) is one of the most important variables required for determining the thermal efficiency index, and is calculated by monitoring the body temperature of the individuals in an enclosure containing a thermal gradient. Although thermoregulation studies of lizards have traditionally employed thermal gradients under laboratory conditions, this approach is not suitable for snakes given that such thermal gradients do not accurately represent their natural thermal environment and thus may result in snakes selecting suboptimal temperatures. Here, we compare the results of this thermal efficiency protocol using a laboratory thermal gradient (LG) and a semi-captivity thermal gradient (SCG) in the rattlesnake *Crotalus polystictus*. We found traces of seasonal variation in the SCG T_p , but this could not be assessed in the LG. T_p from the LG was much higher (29 – 34.3 °C) than from the SCG (22.5–30.9 °C). Values for the accuracy of thermoregulation (d_b) and thermal quality of the environment (d_e) indices from the LG were consistently higher than from the SCG. However, the efficiency of thermoregulation (E) was higher when calculated from the SCG. T_p estimates were wider than most that have been obtained from other snake species, suggesting that *C. polystictus* is eurythermic. The Blowin Demers and Weatherhead index was nearly identical in both gradients. Results from the LG indicated that *C. polystictus* is an inaccurate and inefficient thermoregulator, due to the higher temperatures chosen in this environment. In contrast, results from the SCG suggested that it is a highly accurate and active thermoregulator. We suggest that the LG could represent a stressful environment for snakes, and, as a consequence, they might select higher temperatures to increase anti-predatory performance at the expense of less efficient thermoregulation. Generally, a thermal gradient that more accurately replicates the natural habitat of snake species should reduce stress and result in more robust estimates of thermoregulatory variables.

Keywords: behaviour, *Crotalus polystictus*, preferred temperatures, snakes, refuges, thermal efficiency

INTRODUCTION

In non-avian reptiles, behaviour and physiological processes depend mainly on temperature (Webb & Whiting, 2005). Thus, it is critically important for reptiles to maintain their body temperature (T_b) at or near their physiological optimum (Blouin-Demers & Weatherhead, 2001; Brown & Weatherhead, 2000; Harvey & Weatherhead, 2011). To achieve this, reptiles use a variety of thermal strategies like heliothermy, tigmothermy, and behavioural or passive thermoregulation (Fitzgerald et al., 2003; Garrick, 2008; Molina & Leynaud, 2017; Shine & Madsen, 1996). Behavioural thermoregulation, wherein individuals modify their position in the environment to maximise heat gain or loss, is the most frequent thermoregulatory strategy used by reptiles to regulate their T_b (Navarro-García et al., 2008; Vitt & Caldwell,

2014; Woolrich-Piña et al., 2006).

Hertz et al. (1993) designed what is now the most widely used protocol to analyse the thermal strategies of small and active ectotherms and how efficient they are in maintaining their temperature within their optimal range (Lara-Reséndiz et al., 2015). This protocol requires three basic sets of data: operative environmental temperatures (T_e), the organisms' preferred temperature range (T_p) and in situ active T_b . By comparing the T_p range with both T_b and T_e , researchers can calculate indices of accuracy of thermoregulation (d_b), thermal quality of the environment (d_e) and efficiency of thermoregulation (E) (Hertz et al., 1993).

T_p is the range of body temperatures that ectotherms prefer for optimal behavioural and physiological performance, and it is calculated by monitoring the body temperature of individuals in an enclosure containing a thermal gradient. The assumption is that, without

Correspondence: Hibraim Pérez-Mendoza (hibraimperez@ciencias.unam.mx)

biological interactions and environmental constraints, individuals will select temperatures most suitable for them. It is important to note that the Hertz et al. (1993) protocol states that T_p is calculated under laboratory conditions, where no factor could limit thermoregulation. However, most habitats possess a variety of features that could limit the ability of individuals to thermoregulate effectively, including habitat structure (e.g. rocks, logs, and vegetation), the thermal quality of the environment (Weatherhead et al., 2012), and biological interactions (Vitt & Caldwell, 2014). As Hertz et al. (1993) mention, a laboratory setting for the thermal gradient will never perfectly replicate the natural environment of a species. Researchers should be cautious when interpreting the biological significance of T_p estimates obtained using a laboratory thermal gradient, as individuals might behave unnaturally in such artificial settings. This problem is even more salient in snakes, as they commonly experience prolonged bouts of stress in captivity (Sparkman et al., 2014) and, in their natural habitats, can often spend substantial lengths of time within refuges or retreat sites (Fitzgerald et al., 2003). When basking, some rattlesnakes use ground cover to avoid detection from predators or potential threats (Shoemaker & Gibbs, 2010). Additionally, reptiles often have variable basking patterns that are difficult to replicate under laboratory conditions. Reptiles can bask in open areas to increase exposure to UV radiation and stimulate vitamin D3 production (Seebacher & Franklin, 2005). However, they must be careful to avoid overexposure to UV radiation, which can cause eye and skin damage (Ferguson et al., 2010).

For these reasons, we propose that researchers should carefully consider the ecological and behavioural tendencies of snakes when designing thermal gradients to obtain more robust estimates of their T_p . Here, we describe and test a new approach to estimate the thermal efficiency of snakes using a semi-captivity thermal gradient (SCG) and compare its results with those obtained using a traditional laboratory thermal gradient (LG). We estimated thermal efficiency in the rattlesnake *Crotalus polystictus*, a species that is commonly associated with rocky areas and uses mammal burrows as shelters (Campbell & Lamar, 2004; Meik et al., 2012). As these microhabitat associations likely affect the species thermoregulatory behaviour, we expect estimates of thermal efficiency to differ depending on the type of thermal gradient used.

METHODS

Species and study site

Crotalus polystictus is a medium sized viperid endemic to central Mexico (Mocifio-Deloya et al., 2009; Setser et al., 2010). Individuals are commonly between 50 and 60 cm in total length, but some large individuals can reach 80 cm. Their dark spotted colour pattern over a pale brown or yellow base colour is unique among rattlesnakes (Campbell & Lamar, 2004). It inhabits dry and humid grasslands, pine-oak forests and scrublands of the Mexican Plateau between 1450 and 2739 m (Meik et al.,

2012). They can be active by day or at night (Campbell & Lamar, 2004), but individuals are primarily diurnal at the highest extent of their altitudinal distribution, such as at our study site. However, the activity patterns of this species have not yet been formally described. Setser et al. (2010) report that parturition in this species is synchronised with seasonal rains in a locality of central Mexico, near our study site.

The study was conducted in San Bartolo Morelos, located in the municipality of Morelos, north of Estado de Mexico (19.77 N, 99.65 W). This area is composed of croplands surrounded by natural and induced grasslands and oak forest patches at 2660 meters above sea level. The weather is temperate sub-humid with a rainy season that ranges from June to October (INEGI, 2009). Rocky areas between croplands provide suitable and numerous shelters for rattlesnakes.

Fieldwork

We sampled the locality monthly in March – October 2017 and March – July 2018. Each visit was performed by 3-4 people during three days. Active searches for rattlesnakes ran from 0900 to 1900. Within the first 30 seconds after capture, we measured T_b with a digital thermometer (Fluke model 52-II, ± 0.1 °C) whose sensor was inserted 1 cm into the cloaca. We also measured substrate temperature (T_s) and air temperature (T_a) 10 cm above the substrate of the capture site. We recorded time of capture, mass (g), snout-vent length (SVL, mm), sex and geographic coordinates (Garmin Etrex GPS).

Within each sampling occasion, the snakes were kept in cloth bags and released at the end of the visit. We performed temperature preference trials in the SCG on the last day of the visits made in May ($n = 12$ ♀, 3 ♂), June ($n = 8$ ♀, 5 ♂), August ($n = 5$ ♀, 3 ♂), and September 2017 ($n = 11$ ♀, 3 ♂), and April 2018 ($n = 13$ ♀, 4 ♂). Twelve snakes captured between April 20-22, 2018 (9 ♀, 3 ♂) were transported to a laboratory in Mexico City. We kept the snakes on crystal terrariums where water was provided ad libitum. We conducted five daily temperature preference trials in the LG from May 1-5, 2018.

Operative environmental temperatures (T_o)

We used biophysical models to determine the range of potential body temperatures available to snakes in the absence of thermoregulatory behaviour (Bakken, 1992; Hertz et al., 1993; Peterson et al., 1993). We made the models with green-coloured, polyvinylchloride (PVC) pipes, filled with tile adhesive and sealed with tape. The models were similar in shape and size (50 cm long x 2 cm wide) to *C. polystictus*, and were previously calibrated under field conditions during the activity period of the snake following the methods of Adolph (1990), Díaz de la Vega-Pérez et al. (2013), Lara-Reséndiz et al. (2014) and Sinervo et al. (2010). Our models accurately mimic the gain and loss of heat by *C. polystictus* at our study site ($r^2 > 0.95$, $P < 0.01$). We deployed two biophysical models at the study site, one under refuge cover and one in sunlit conditions. The models were connected to a data-logger with two sensors (HOBO® Pro V2-U23-003)

which was set to record T_e every 15 minutes from June 2017 to April 2018. For the thermoregulation analyses we selected only the T_e data corresponding to the activity period of the snakes based on our in-situ observations (09:00 – 18:30).

Preferred temperature range (T_p)

The LG was built in three adjacent 120 x 40 x 40 cm crystal enclosures, each containing a layer of approximately 2 cm of potting soil (Fig. 1A). Using cardboard barriers, we divided each tank into four racks to prevent individuals from interacting or competing for basking sites. Each rack was 10 cm wide, which allowed the snakes to rest in a coiled position. In each enclosure, we hung two heat lamps, at 55 cm above the soil and placed one heat mat under the tank. Thus, the gradient consisted on a total of six lamps and three mats. At the other end of the tanks we placed 15 frozen refrigerant gel packs, five per enclosure, on the outside. These refrigerant packs were replaced every four hours during the experiments (Fig. 1A). This design generated a thermal gradient from ~ 15 °C to 45 °C.

The gradient was set up at 0730, and all organisms were maintained in it for 1.5 hours to adjust to gradient temperatures. We began the measurements at 0900 and, from then on, we recorded the preferred body temperatures every two hours until 1900. We used an infrared thermometer (Fluke 561, ± 0.1 °C) to take temperature recordings to minimise time, handling and stress of the snakes during the experiment. Unpublished data from a similar study with *C. triseriatus* showed no significant difference between temperatures measured with the Fluke 52-II and Fluke 561 thermometers. Every measurement was made in the middle of the body, under shade, and at a distance of 15 cm away from the snake. Each day of the LG test, we moved the snakes to a different rack and cleaned the tanks superficially to minimize the possible effect of olfactory signals.

To make the SCG, we built a 3 m diameter circular enclosure bounded by polycarbonate plates (Fig. 1B). The enclosure was placed approximately 4 km away from the study site on a flat, uncovered area. The enclosure was prepared with multiple rocks of similar size and shape as those found in the natural habitat of *C. polystictus*. The rocks functioned as refuges in which the rattlesnakes could thermoregulate as normally as possible, without most of the stress associated with captivity in a LG. We placed six heat lamps at one end of the gradient, approximately 100 cm from the ground, which also was uncovered from sunlight (Fig. 1B). We covered the other end of the gradient with a canvas to provide shade during the whole experiment. We tied up the canvas to two poles at 2-3 m from the ground and diagonally to the gradient, so that sunlight was blocked but the snakes had no direct cover from the canvas. The conditions described above generated a relatively smooth thermal gradient from ~ 23 °C to ~ 45 °C. The temperature transition was smoother from the cold area to the middle (9 °C in 150 cm) than from the middle to the hot area (13 °C in 150 cm). The locality can experiment considerable drops of temperature during night, specially towards the end of

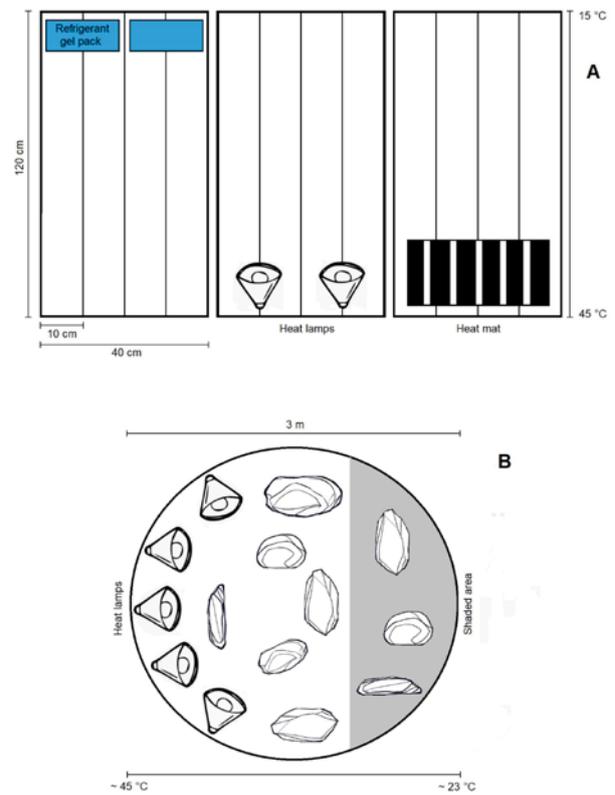


Figure 1. Diagram of the laboratory thermal gradient (A) and the semi-captivity thermal gradient (B) used to test the T_p range of *C. polystictus*. Heat lamps, heat mats and refrigerant gel packs in the laboratory gradient were used on all three enclosures.

the year. On a particular cold morning, we worked with a colder thermal gradient (from ~ 18 °C to ~ 42 °C) during the first measurements in the T_p trial of September 2017. The SCG stabilised around noon to the normal range. As in the LG, rattlesnakes were subjected to an acclimation period prior to experimental testing. T_p was recorded using the same procedure as described for the LG. If the snake was under a rock, we carefully moved the rock, took the measurement and repositioned the rock. We also repositioned the snake, if needed.

Thermoregulation indices and statistical analyses.

We calculated indices for thermal quality of the environment (d_e) and accuracy of thermoregulation (d_b) (Hertz et al., 1993). d_e and d_b are the mean of all individual deviations between T_e and T_b and the interquartile range of T_p , respectively. Deviations were calculated as follows: if T_e or $T_b > T_p$ interquartile range, then $d_b = T_b - T_p$ and $d_e = T_e - T_p$. If T_e or $T_b < T_p$, then $d_b = T_p - T_b$ and $d_e = T_p - T_e$. If T_e or T_b lie within the interquartile range of T_p , then both d_b and d_e are equal to zero. d_b and d_e values equal to or similar to zero represent high thermoregulatory accuracy and ideal thermal environments for the organisms. High values of the indices indicate low thermoregulatory accuracy and environments with low thermal quality.

We then calculated the Hertz et al. (1993) index for efficiency of thermoregulation: $E = 1 - (\bar{d}_b/\bar{d}_e)$. An E value close to one indicates that environmental temperatures available for the individuals do not match

their physiological requirements, and therefore active thermoregulation is necessary to achieve optimal temperatures. In contrast, an E value close to zero indicates that the organisms find themselves in an ideal thermal environment and therefore must resort to thermoconformity. We also calculated the index developed by Blouin-Demers and Weatherhead (2001), which represents the effectiveness of thermoregulation and the deviation from thermoconformity. This index is expressed directly in $^{\circ}\text{C}$ and is calculated simply by subtracting d_b from d_e .

We used Spearman correlations to test whether T_s and T_a are correlated with T_b . We tested for a difference between male and female T_b using a student's t -test. We used a Mann-Whitney's test to analyse differences between male and female T_p within each gradient and differences in overall T_p between gradients. We used Welch's ANOVA and a Games-Howell post hoc test to analyse differences between T_p of the different trials from the SCG. We tested normality and homoscedasticity with Shapiro-Wilk and Bartlett tests, respectively. All analyses were done in R, version 3.3.2 (R Core Team, 2018).

RESULTS

During the sampling period, we recorded 93 female and 36 male T_b recordings. Most of the individuals were average-sized adults. Despite the high number of females captured, we only detected seven pregnant individuals, which were captured unevenly between March and July 2018. Due to logistical reasons, in that period we only made gradient experiments in April, so in the end only one pregnant snake was used in each gradient. Therefore, the effect of reproductive status on thermoregulation could not be assessed. The activity period of *C. polystictus* was between 0900 and 1830; the highest peak of activity was between 1000 and 1200. We detected a second lower activity peak from 1600 to 1800.

Mean T_b , T_a and T_s are presented in Table 1. We found no significant differences between male and female T_b 's ($t = -1.446$, $P = 0.152$). T_b was significantly related with T_a ($\rho = 0.218$, $P < 0.05$) and T_s ($\rho = 0.584$, $P < 0.05$). T_p did not differ significantly between sexes in the LG ($W = 11266$, $P = 0.192$) nor in the SCG ($W = 9848$, $P = 0.265$). T_p was not equal through the five SCG trials ($F = 4.798$, $P < 0.01$). T_p from May ($\bar{x} = 27.9$ $^{\circ}\text{C}$), June ($\bar{x} = 26.1$ $^{\circ}\text{C}$) and August ($\bar{x} = 27.2$ $^{\circ}\text{C}$) were higher than T_p measured in April ($\bar{x} = 24.1$ $^{\circ}\text{C}$) and September ($\bar{x} = 24.1$ $^{\circ}\text{C}$). However, differences were only significant between May and September ($t = 3.02$, $P < 0.05$) and May and April ($t = 3.02$, $P < 0.05$). Overall T_p differed significantly between gradients ($W = 77830$, $P < 0.001$). Below we present the results from each thermal gradient type separately.

T_p and thermoregulatory indices derived from the semi-captivity gradient

Mean T_p and the interquartile range of T_p are presented in Table 1. A total of 52 % of field body temperatures fell within this range, 43 % below it and only 5 % above it (Fig. 2A). Only 17 % of T_e fell within the T_p interquartile range, while 19 % fell above it and 64 % below it. (Fig.

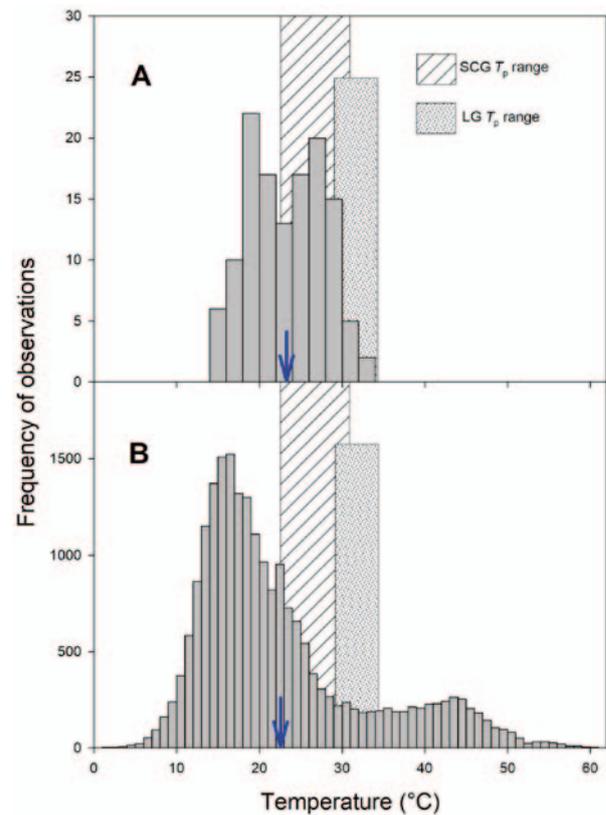


Figure 2. Distribution of field body temperatures (**A**) and operative temperatures (**B**) of *C. polystictus* at San Bartolo Morelos, Estado de México. The arrows point to each temperature distribution's mean. The interquartile of the preferred temperature range obtained from the semi-captivity gradient (22.5 – 30.9 $^{\circ}\text{C}$) is represented by diagonal lines, whereas the one obtained from the laboratory gradient (29 – 34.3 $^{\circ}\text{C}$) is represented by a dotted area.

Table 1. Descriptive thermoregulatory data and thermoregulation indices for *C. polystictus* in San Bartolo Morelos, Estado de México. Means and SD are shown. *shows the parameters that are significantly different between the gradients.

Variables	Temperature	
Field body temperature (T_b)	23.2 \pm 4.5	
Air temperature (T_a)	20.4 \pm 4.1	
Substrate temperature (T_s)	20.2 \pm 3.6	
Operative environmental temperatures (T_e)	22.5 \pm 10.3	
	Thermal gradients	
	Laboratory gradients (LG)	Semi-captivity gradient (SCG)
Mean preferred temperature range (T_p) *	30.9 \pm 4.9	26.5 \pm 6.1
T_p interquartile range ($T_{p25}-T_{p75}$) *	29 – 34.3	22.5 – 30.9
Accuracy of thermoregulation (d_b) *	5.84 \pm 4.3	1.63 \pm 2.3
Thermal quality of the environment (d_e) *	10.08 \pm 5.3	5.88 \pm 4.9
Thermoregulatory efficiency (d_e-d_b)	4.24	4.25
Thermoregulatory efficiency (E)	0.42	0.72

2B). The d_b indicates relatively accurate thermoregulation, while the d_e suggests that the habitat is of low thermal quality, but considerably more benevolent in comparison with the estimation from the LG (Table 1). E indicated a preference for active thermoregulation, which is consistent with the value of the Blouin-Demers and Weatherhead index.

Most individuals chose the refuges on the warm part of the gradient. However, they did not directly move to the warmest spot; most snakes actively changed refuges during the experiments. Generally, snakes selected the lowest temperatures at the beginning of the experimental period, higher temperatures in the middle and lower temperatures at the end of the trials. The highest temperatures were selected between 1200 and 1400.

T_p and thermoregulatory indices derived from the laboratory gradient

Mean T_p and the interquartile range of T_p are presented in Table 1. Only 13 % of field T_b fell within this range, while 87 % were below it and none above it (Fig. 2A). The overlap of T_e with the T_p interquartile range was minimal (5 %), while 79 % of T_e fell below it and 16 % above it (Fig. 2B). Both d_b and d_e were high values, indicating a low accuracy of thermoregulation and a habitat of thermal quality (Table 1). The efficiency index from Blouin-Demers and Weatherhead indicates that individuals are active thermoregulators. The index E , on the other hand, suggests that individuals have a tendency towards thermoconformism.

Snakes in the LG consistently chose higher temperatures. Despite this, snakes were more static, often choosing a spot near the lamps early in the day and staying there during the whole experiment. One snake, on a couple of occasions, climbed the cardboard barrier and accessed the next rack. We considered this to be an escape attempt and, therefore, did not use that period's temperature reading in the analyses.

DISCUSSION

Due to the manifold biological implications of T_b for reptiles, robust estimates of this physiological trait are critically important for ecological and evolutionary studies. Although T_a and T_s have been widely proposed to provide possible predictors of T_b , some researchers have found them of little use (Seigel & Collins, 1993). We found a positive relationship between T_b and both T_a and T_s , but the relationship was stronger with T_s , suggesting that temperature regulation is tigmothermal.

We found traces of seasonal variation in the preferred temperatures. Within the SCG, snakes chose the highest T_p in May. This preference could be related to female reproduction. It is well known that reproductively active snakes prefer higher and less variable temperatures during pregnancy (Charland & Gregory, 1990; Setser et al., 2010; Shine, 2003). Parturition in this species begins in June (Setser et al., 2010), so it makes sense that snakes chose higher temperatures in May prior to giving birth. We did not detect pregnant females during this period, though;

but females might generally follow this pattern to some degree. Alternatively, we might have failed to diagnose some individuals as pregnant during fieldwork. We were not able to analyse T_p seasonality from the LG since we only obtained data from April 2018. It is possible that the seasonal pattern found in the SCG is also present in the LG. However, we suspect that seasonality could not have a large impact on the differences between the T_p from both gradients. Since the conditions of the SCG are closer to the natural conditions, we would expect this seasonal pattern to be the regular one for the population, and therefore a potential pattern in the LG could be similar. Among the SCG trials, some of the lowest temperatures were recorded in April. Nevertheless, the LG T_p in April was much higher than any of the T_p from the SCG. If the same seasonal pattern from the SCG happens in the LG, we would expect even higher LG T_p in subsequent months, so it is possible that the overall LG T_p would still be much higher than the SCG T_p even with seasonal variation.

Overall, *C. polystictus* chose a lower T_p interquartile range in the SCG than in the LG. Indeed, the upper limit of the SCG's range (30.9 °C) barely exceeded the lower limit of LG's range (29 °C). The range obtained from the SCG was 8.4 °C, the widest interquartile range yet to be reported among thermal studies of snakes (Blouin-Demers & Weatherhead, 2001; Brown & Weatherhead, 2000; Fitzgerald et al., 2003; Harvey & Weatherhead, 2011). The T_p range calculated from the LG was considerably lower (5.6 °C) but was still wider than most T_p 's estimated from other snake species. These findings indicate that *C. polystictus* is eurythermic at our study site. Eurythermy is commonly favoured when the energetic costs of thermoregulation are high and individuals live in thermally variable climates (Powell & Russell, 1985).

The operative environmental temperatures obtained from the biophysical models are indeed highly variable compared to T_b at our study site. Our estimates of the thermal quality of the environment are low, especially when estimated from the LG (Table 1). A more in-depth study in which more models are deployed in different microhabitats should be done in the future, but the evidence found suggests that San Bartolo Morelos does not provide a thermally ideal habitat for *C. polystictus*. Nevertheless, according to the data obtained from the SCG, *C. polystictus* is considered a highly accurate and active thermoregulator. This finding is consistent with studies by Blouin-Demers & Weatherhead (2001) and Row & Blouin-Demers (2006), which have found that ectotherms thermoregulate more precisely within environments with low thermal quality.

Interestingly, estimates of the Blouin-Demers and Weatherhead index are nearly identical based on data obtained from both gradients, where the snakes have to compensate 4.2 °C to reach T_p . The scale of the d_b and the d_e differs between gradients, despite the actual difference between both indexes within each gradient being minimal. This finding indicates that there is a preference for active thermoregulation over thermoconformity by individuals in this population.

The important differences found in E are directly caused by the higher temperatures the snakes chose in

the LG. However, stress might explain why individuals selected high temperatures. Generally, reptiles often exhibit stress responses to handling and captivity (Franklin et al., 2003; Moore et al., 1991; Schuett et al., 2004). Many studies have confirmed that blood levels of corticosterone increase after capture and confinement in several snake species (Bailey et al., 2009; Dayger et al., 2013; Herr et al., 2017; Mathies et al., 2001; Schuett et al., 2004; Sykes & Klukowski, 2009).

Behavioural and anti-predator responses in reptiles depend on temperature (Mori & Burghardt, 2001). Snakes generally prefer escaping and showing more defensive behaviours such as striking when the temperatures are higher and motor abilities are optimal (Citadini & Navas, 2013; Llewelyn et al., 2010; Mori & Burghardt, 2001; Passek & Gillingham, 1997; Prior & Weatherhead, 1994; Schieffelin & De Queiroz, 1991). When cold, snakes rely more on crypsis given that motor capability in ectotherms decreases with lower temperatures (Brodie III & Russell, 1999; Keogh & DeSerto, 1994; Prior & Weatherhead, 1994). It is likely that, due to increased stress, snakes chose higher temperatures in the LG to enhance their ability to perform anti-predatory displays (Currin & Alexander, 1999), and also the addition of refuges to the SCG might have helped to reduce such stress. Further studies that evaluate the level of corticosterone in snakes during T_p experiments should be made.

Aside from the effect that refuges have in avoiding detection by predators, the use of refuges also has thermoregulatory considerations (Todd et al., 2016). Some have suggested that many squamates choose specific retreat sites that provide thermal variation and could be used for thermoregulatory purposes (Amo et al., 2004; Cox et al., 2018). For example, Cox et al. (2018) found that solitary *Diadophis punctatus* individuals select rocks that allow them to maintain a stable range of T_b within their preferred range. Webb and Shine (1998) found that *Hoplocephalus bungaroides* selects thin rocks during the spring, but changes to thicker rocks during the summer as the temperatures under the refuges increase. Similarly, Huey et al., (1989) showed that *Thamnophis elegans* chooses rocks of intermediate thickness that improve energy gain and temperature regulation.

For our study population of *Crotalus polystictus*, refuges appear to provide an important microhabitat for thermoregulation. Most of the individuals that we captured were found under rocks instead of basking out in the open, regardless of the time of day. Traditionally, snakes located under shelters would be considered inactive (Whitaker & Shine, 2002). However, this characterisation fails to consider that some snakes could prioritise the use of refuges for thermoregulatory reasons. *Crotalus polystictus*, in particular, is known to prefer burrowing rodents as prey (Meik et al., 2012), which might explain its high preference for dwelling under rocks as sit-and-wait predators. However, a trade-off between selecting refuges for foraging and thermoregulatory purposes can exist in some species (Huey et al., 1989). Thus, additional study of the effects that refuges have on the thermoregulation of *C. polystictus* is needed.

Our SCG also has some limitations. For instance,

adapting an outdoors thermal gradient with considerably low temperatures at the cold end is difficult, since materials like refrigerant gel packs or ice are not suitable for this method. Making the thermal preference trials including colder parts of the day or on particularly cold days, like in our September 2017 trial, can help to mitigate this limitation. However, a more consistent method would be desired, since the lower limit of the gradient should be more stable. If a thermal gradient is not wide enough, results drawn from it could be misleading.

As we stated, a SCG design can help to reduce stress in the T_p trials. However, some interaction between researchers and snakes still had to occur during the measurements. The two-hour periods between measurements can mitigate this issue, giving the snakes plenty of time to adapt and resume their thermoregulatory behaviour if they felt too much disturbed. Other type of devices, like ultra-thin thermocouples or ibuttons, could help even further to reduce stress in the SCG gradient. Other issues like background noise and inadequate weather conditions can also affect the snakes' behaviour or even impede the SCG trials. All these considerations need to be addressed when designing this type of gradients. We consider that, with meticulous experimental design and careful planification, the possible limitations of these type of gradients can be overcome and adapted to many reptile species.

Overall, we suggest that a LG like the one used in most thermoregulatory studies of reptiles is not suitable for snakes. A thermal gradient in semi-captivity that generally resembles the original habitat can reduce the stress associated with captivity while also providing more thermal variability than a laboratory gradient. The addition of refuges also allows snakes to use the same substrates that they would naturally thermoregulate on. Most thermal gradients in snake studies have been made with wood and covered with various substrates (Aubret & Shine, 2010; Blouin-Demers & Weatherhead, 2001; Fitzgerald et al., 2003; Harvey & Weatherhead, 2011; Whitaker & Shine, 2002). However, different materials conduct heat at different rates; thus, using substrates from the natural habitat of a species is ideal for estimating their T_p . Providing a more naturalistic thermal gradient allows snakes, and potentially vertebrate ectotherms in general, to thermoregulate as accurately as they would in their natural habitat, which, in turn, enhances the accuracy of preferred temperature range estimates.

We can conclude, therefore, that *C. polystictus* is an accurate thermoregulator despite living in a low thermal quality habitat. Although it is an active thermoregulator, it has a wider set of preferred temperatures than most other snakes that have been studied to date (Blouin-Demers & Weatherhead, 2001; Brown & Weatherhead, 2000; Fitzgerald et al., 2003; Harvey & Weatherhead, 2011). Our results also emphasise the importance of tailoring the design of thermal enclosures to each study species. In sum, our study shows that the Hertz et al. (1993) protocol can be robustly adapted to more species, thereby increasing the quality of data on thermoregulation obtained from a variety of non-avian reptiles.

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Effects of environmental factors and conservation measures on a sand-dune population of the natterjack toad (*Epidalea calamita*) in north-west England: a 31-year study

Philip H. Smith¹ & Graeme Skelcher²

¹ 9 Hayward Court, Watchyard Lane, Formby, Liverpool L37 3QP, UK

² 8 Coach Road, Warton, Carnforth, Lancashire LA5 9PP, UK

A 31-year study monitored Britain's largest natterjack toad population on the Sefton Coast sand-dune system in north-west England. Key objectives were to describe changes in numbers and breeding success, relate these to environmental variables and explore whether conservation efforts have been well targeted. Considerable variation was found in the number of water bodies used for spawning, the number of spawn strings laid and breeding success based on estimated toadlet production. There was a declining trend in annual totals of spawn strings and toadlets. Positive correlations were established between spring and April rainfall and both spawn count and toadlet production. April rainfall for the study area declined between 2000 and 2017. Premature desiccation of water bodies and associated poor breeding success were frequently observed, there being a positive relationship between water-table height and toadlet numbers. A long-term declining trend in the height of the water-table was established. The mean adult population of Sefton natterjacks was estimated at about 1200 individuals, with a peak around 3150. Successful breeding is increasingly reliant on management to excavate appropriately designed and managed "scrapes" that hold water long enough for metamorphosis to occur. Overgrowth of vegetation and loss of dynamism in the dune system threaten both the natural production of new breeding slacks and the natterjack's open terrestrial habitat. Encouragement of dune dynamics by removing trees and scrub, increasing areas subject to livestock grazing and mechanical rejuvenation may offer the best hope of conserving this species in the future.

Keywords: Amphibian; climate-change; rainfall; sand-dunes; vegetation-overgrowth; water-table

INTRODUCTION

The natterjack toad (*Epidalea calamita*) is native to 22 countries in western and northern Europe, as far east as western Russia. Although still locally abundant, especially in southern Europe, it is declining in much of its northern range and is consequently listed as a European Protected Species under Annex IV of the EU Habitats Directive. At the north-western edge of its global distribution, this is a rare amphibian in Britain, having disappeared from 70-80 % of its range between the late 19th century and 1970 (Beebee, 1977). Declines have been attributed to habitat loss through afforestation, urbanisation and agricultural improvement, changes in habitat structure leading to seral succession and displacement by common toad (*Bufo bufo*) and/or common frog (*Rana temporaria*) and, in some cases, acidification of breeding pools (Baker et al., 2011; Beebee, 1983; Beebee et al., 1990; Denton & Beebee, 1994; Denton et al., 1997). By the early 1970s, the natterjack was restricted to around 50 British sites and was thought to be in danger of declining to extinction (Buckley & Beebee, 2004). Populations persisted in

coastal north-west England and south-west Scotland and in a few scattered localities in southern and eastern England. Being protected by UK statute since 1975, this species has been the subject of considerable research and much conservation effort (Buckley & Beebee, 2004). The natterjack toad is a good coloniser of shallow, ephemeral ponds with a relatively high pH (6.0-8.0), mainly in sand-dunes, heathlands and on high-level saltmarshes, taking advantage of higher water-temperatures, lower predator numbers and reduced competition from other amphibians. An open, unshaded terrestrial habitat is required with areas of minimally vegetated ground for foraging and burrowing (Baker et al., 2011; Beebee, 1979; Banks & Beebee, 1987a, b; 1988).

The largest remaining natterjack population in Britain occurs on the calcareous sand-dunes of the Sefton Coast in north-west England (Beebee & Buckley, 2014a), where it has been known since the 1830s (Smith, 2009). Populations were evidently large in the late 19th and early 20th centuries but housing and other developments, extensive planting of conifers and introduction of invasive shrubs, such as Sea Buckthorn (*Hippophae rhamnoides*), led to losses of suitable habitat (Smith,

Correspondence: Philip. H. Smith (philsmith1941@tiscali.co.uk)

2009). Following a prolonged drought in the 1970s which caused breeding failures (Smith & Payne, 1980), about 50 “scrapes” were excavated in existing slack basins to create new breeding sites. However, as the water-table rose again in the late 1970s and early 1980s, many of the scrapes became more-or-less permanent ponds and were invaded by large numbers of common toads and invertebrate predators, thereby becoming less suitable for the natterjack (Simpson, 2002; Smith, 2009).

Subsequent surveys highlighted large variations in breeding success, seemingly linked to changes in the water-table, a shortage of suitable wetlands in some parts of the dune system and the growth of coarse vegetation and scrub which adversely affected the natterjack’s terrestrial habitat. A Sefton Coast Natterjack Conservation Strategy (Herpetological Conservation Trust, 2003; Simpson, 1992; Smith, 1984) recommended additional breeding pools to reduce population isolation and co-ordinated monitoring of distribution and breeding activity. From 1987 onwards, an annual monitoring report was produced, these data being summarised by Skelcher (2010). His report (updated to 2017) forms the basis for the current study whose main aims were to:

1. Use annual reports and other relevant sources to summarise how natterjack distribution, abundance and breeding success have changed over time.
2. Link these changes, where possible, to environmental factors.
3. Determine whether conservation efforts have been well targeted and make recommendations for future management.

METHODS

Population measures

The 2100 ha dune system was divided up into nine units (Fig. 1) that could reasonably be covered by individuals or small teams of surveyors made up of trained volunteers and site managers. Using methodology developed during earlier surveys (e.g. Smith & Payne, 1980; Buckley & Beebee, 2004) visits were made at roughly weekly intervals from the start of breeding activity. There was an emphasis on counting natterjack spawn strings and estimating toadlet production at each water-body (site) used for breeding. Female natterjacks usually lay only one pair of spawn strings per year, although a second, smaller pair of strings may be produced on rare occasions (Denton & Beebee, 1996). Therefore, string counts can be used as an index of the breeding female population. The appearance of freshly laid natterjack spawn changes markedly over a few days, making it possible to avoid double-counting strings recorded on earlier visits.

Toadlet numbers give an indication of breeding success but it is usually impossible to obtain accurate counts, so estimates for each breeding site were made on a logarithmic scale (Buckley & Beebee, 2004). Toadlets were often difficult to find and their numbers were probably underestimated in some years.

Supporting information included counts of assembling adults, estimates of tadpole numbers, presence of other amphibians, evidence of mortality, changes in water

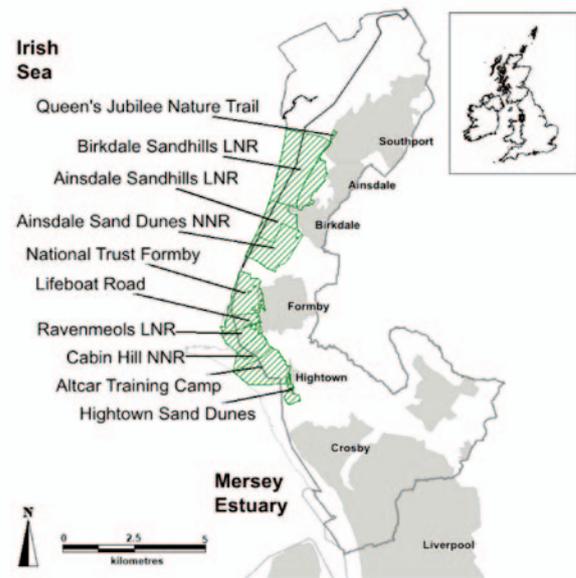


Figure 1. Map of Sefton Coast, north-west England, showing locations of survey units

depth, date at which the site dried up and details of rescue operations.

Monitoring visits commenced at the start of the breeding season, usually late March to mid April, continuing until all metamorphosis had taken place or sites had dried up, often in late June to mid-July. Survey effort was standardised as far as possible by ensuring all potential breeding sites were covered in each year using the same methodology. Several of the same recorders were involved for a large part of the study.

Environmental correlates

To investigate possible relationships between breeding success and environmental variables, monthly rainfall and temperature data were obtained from the Ainsdale Sand Dunes National Nature Reserve (NNR) weather station, situated near the centre of the dune coast. Monthly water-table variations for the dune system have been measured since 1972 using a series of tube-wells. The most representative of overall dune water-table is considered to be tube-well no. 11 in the open dunes of Ainsdale NNR (D. Clarke in litt., 2015), so data from this well were used in the analysis. Based on a Shapiro-Wilk test, the dependent variables (spawn and estimated toadlet counts and their log vales) were considered to have normal distributions, while most of the independent variables (rainfall, temperature and water-table height) were not normal. Therefore a non-parametric test, Spearman’s Rank (SR), was chosen to investigate correlations involving the latter variables, while Pearson’s correlation coefficient was used for the former.

RESULTS

Adult assembly and spawning

Between 33 and 125 sites potentially suitable for

natterjack breeding (mean 84) were monitored annually, the total depending on water-table conditions and whether or not the sites were flooded. Adult natterjacks assembled to between 7 and 96 sites (mean 48), the maximum adult peak count being 2442 in 1987 while the lowest was 15 in 2017 (mean 656). However, the counting effort for adults was inconsistent, so these data should be viewed with caution.

Most spawning took place during April or early May but, exceptionally, it occurred earlier or much later. Ideal conditions for assembly and spawning seemed to be relatively windless, damp and mild weather with a night-time temperature of at least 8 °C (personal observations). The number of sites used for spawning varied from a maximum of 78 in 2000 to a low of 7 in 2017 during a prolonged drought, the mean being 42. Annual totals of spawn strings ranged from 1576 (1988) to 77 in 1996 (mean 606). Figure 2 shows great variability in annual spawn counts but with a declining trend over the period of the study of about 70 %.

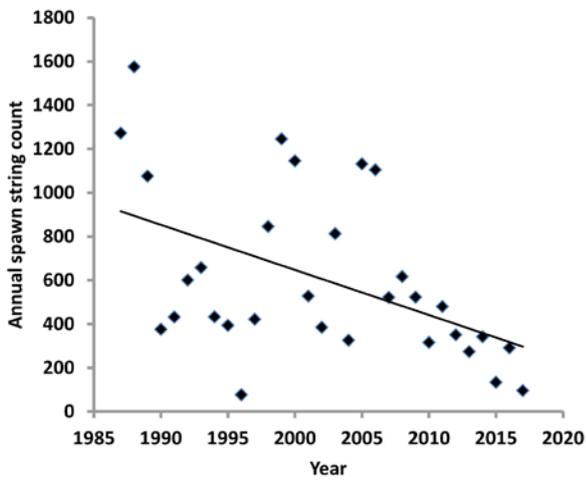


Figure 2. Number of spawn strings counted in each year of the study. ($r = -0.485$; $p = 0.0057$)

Contributions of different units to the annual totals of spawn strings varied considerably both within years and over longer time scales. Overall, Ainsdale NNR and the adjacent Ainsdale Sandhills Local Nature Reserve (LNR) provided 30 % and 28 % of total strings respectively. Annual totals at Ainsdale NNR tended to increase from 1987 to 1999 (Simpson, 2002) but then, despite high spawn counts in 2005 and 2006, showed significant declines from 2000 (Fig. 3a), especially so from 2007. Spawn counts for Birkdale Sandhills LNR fluctuated in line with coastwide trends until 2005, when numbers increased to a peak in 2008 before declining (Fig. 3b). This was mainly attributable to colonisation of pioneer slack and embryo dune habitat on the so-called “Birkdale Green Beach” from 1999 (Smith, 2007), while the more mature slacks to the east were progressively abandoned. After 2008, Green Beach spawn counts also fell. Further south, Cabin Hill NNR, together with the adjacent Altcar Training Camp, achieved a peak spawn count of 456 in 1985, just before the current study began (Smith, 2012).

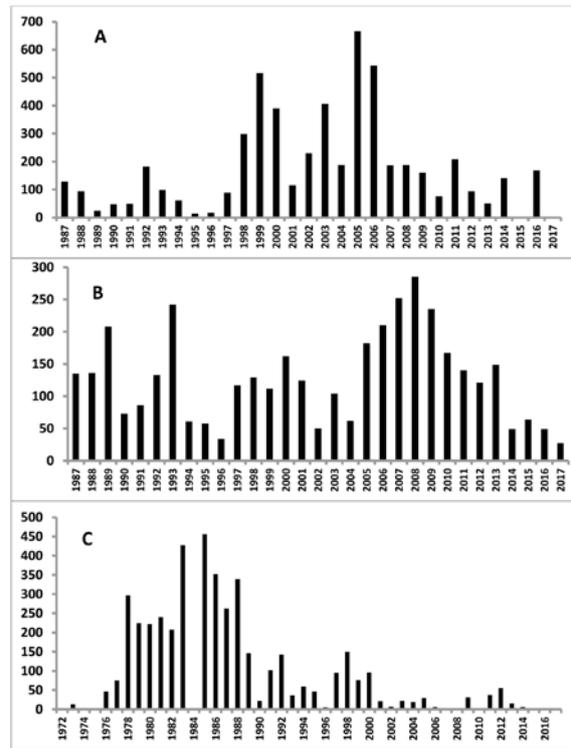


Figure 3. Annual spawn counts for Ainsdale Sand Dunes NNR, 1987-2017 (A), Birkdale Green Beach 2005-2017 (B) and Cabin Hill/Altcar, 1972-2017 (C). Ainsdale NNR shows a declining trend from 2000 ($r = -0.58$, $p = 0.012$)

There was then a significant decline in annual totals over the next three decades (Fig. 3c), though Altcar showed increased spawning activity in 2011-2013, probably due to the provision of new scrapes. Spawn counts at other units were generally low and trends unclear.

Metamorphosis

Breeding sites often dried up in spring or early summer, so successful metamorphosis occurred at only about half of the sites in which spawn was laid. A mean of 18.4 sites per annum produced toadlets, with a wide range from 48 sites in 2000 to none in 2011.

The use of a log scale to record numbers of toadlets at individual sites makes it difficult to compare annual coastal totals. In an attempt to overcome this problem, it was decided to use the midpoint of log values for each site, so that $\log 1 = 50$ toadlets, $\log 2 = 500$, $\log 3 = 5000$, etc. From these approximations, annual toadlet production ranged from 0 to 526,000 per annum (mean 45,000). Peaks in toadlet numbers occurred in 1987, 1988, 1994, 2000 and 2006. Particularly poor metamorphosis was recorded in 1997, 2009, 2010, 2011 and 2017. After the 2006 peak, a series of five years of falling metamorphic success culminated in no toadlets being found in 2011. Overall, there was a declining trend in toadlet production (Fig. 4). Years with high spawn counts also tended to produce more toadlets than in years with lower spawn totals (Fig. 5).

The importance of excavated scrapes for successful breeding was evident, especially in drier years. Thus,

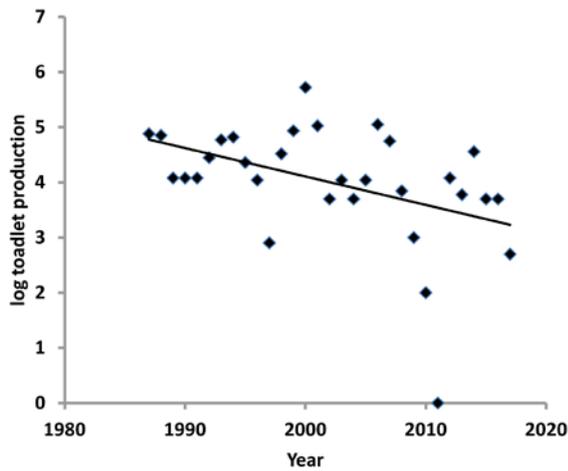


Figure 4. Trend of annual toadlet production. The y-axis is transformed to \log_{10} ($r = -0.436$, $p = 0.014$)

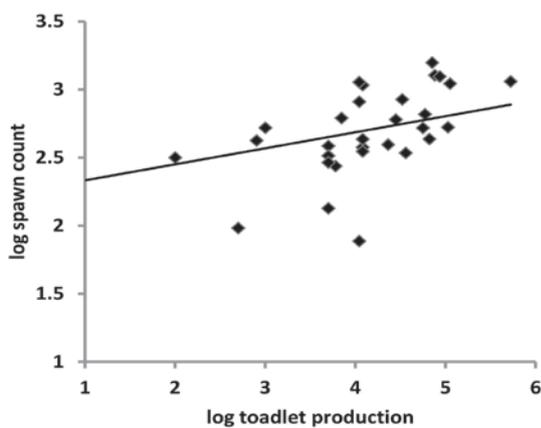


Figure 5. Relationship between \log_{10} annual toadlet production and \log_{10} spawn count ($r = 0.401$, $p = 0.025$)

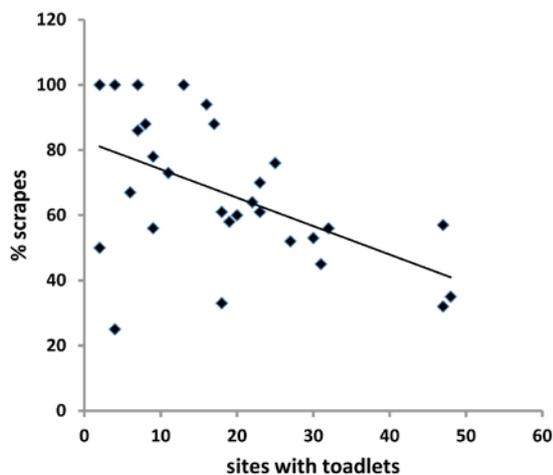


Figure 6. The relationship between the proportion of successful breeding sites that are artificial scrapes and the number of sites producing toadlets in each year ($SR = -0.405$, $p = 0.027$)

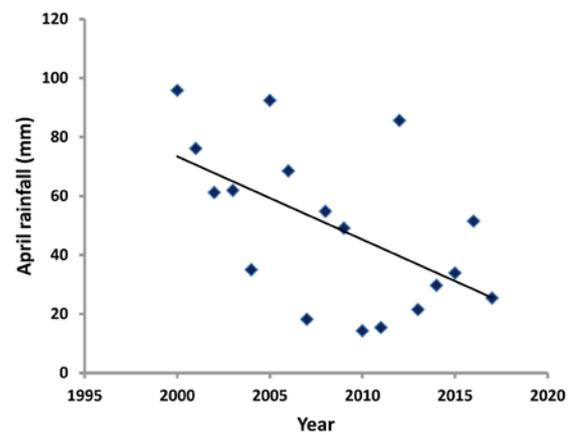


Figure 7. April rainfall trend at Ainsdale, 2000–2017 ($SR = -0.567$, $p = 0.016$)

a mean of 10.5 scrapes per year produced toadlets, representing 57 % of successful sites, the range being from 25 % scrapes in 2004 to 100 % in 1996, 1997 and 2017. Figure 6 shows that, in years when fewer sites had successful breeding, the proportion of them that were scrapes was significantly higher.

Environmental variables

With an average annual rainfall of about 820 mm, Sefton is one of the driest places in north-west England. The coast is also relatively windy, resulting in rapid evapotranspiration. Therefore any reduction in precipitation can have an adverse and cumulative impact on the water-table with potential impacts on natterjack breeding success. Monthly rainfall data from the Ainsdale NNR weather station for the study period were combined to provide totals for autumn (September–November), winter (December–February) and spring (March–May). Summer rainfall (June–August) was not included in the analysis as this usually has little impact on the water-table, most summer precipitation being lost to evapotranspiration (Clarke & Sanitwong Na Ayutthaya, 2010). The autumn mean rainfall (268 mm) was considerably higher than the winter mean (220 mm), spring having the lowest mean value (154 mm). There was a highly significant difference between the three sets of rainfall data (Kruskal-Wallis = 32.11, $p = 0.0000001$).

Autumn rainfall showed great variability, with a slight increasing trend that was not significant. There was no particular trend over time for winter rainfall, while spring totals suggested a decline over time which was not significant. However, rainfall for April, the month in which most spawning takes place, showed a steeper declining trend, this being significant from 2000 (Fig. 7). Since that year, there have been eight particularly dry Aprils with mean rainfall about 50 % of the long-term average. These eight seasons had mean spawn counts of 311, compared with the overall mean annual count of 606, this difference being significant (Man Whitney = 144, $p = 0.018$). Positive Spearman Rank correlations were found between log toadlet production and spring rainfall ($SR = 0.509$, $p = 0.0041$) and April rainfall ($SR = 0.45$, $p = 0.011$)

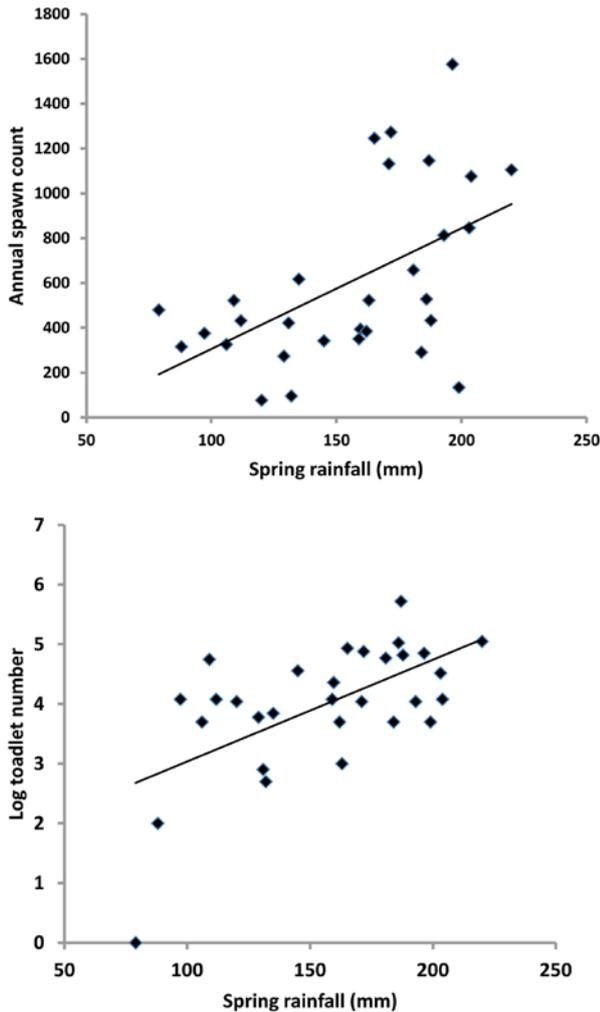


Figure 8. The relationships between annual spawn count (above) and \log_{10} toadlet number (below) and spring rainfall (SR toadlets = 0.509, $p = 0.0041$; SR spawn = 0.533, $p = 0.0028$)

and also between annual spawn count and both spring rainfall and April rainfall (Fig. 8). Correlations were not established between either toadlet numbers or spawn counts and annual rainfall.

During the study period, spring mean maximum temperature increased (SR = 0.526, $p = 0.0024$), while spring minimum mean temperature declined (SR = -0.44, $p = 0.013$). We had thought that April temperatures would impact on spawning; but correlations were not found between mean minimum or mean maximum April temperatures and spawn counts and between spawn counts and spring mean minimum or maximum temperatures.

Figure 9 shows a declining trend of monthly water-table during the study period for tube-well no. 11 in Ainsdale NNR. The time-line shows the expected seasonal oscillations in water-table, maxima occurring in early spring and minima in summer. In most years, the difference between annual high and low points was about 85 cm, reflecting high rates of evapotranspiration in spring and summer. There was also great variation in annual high and low points, with winter peaks in 1988,

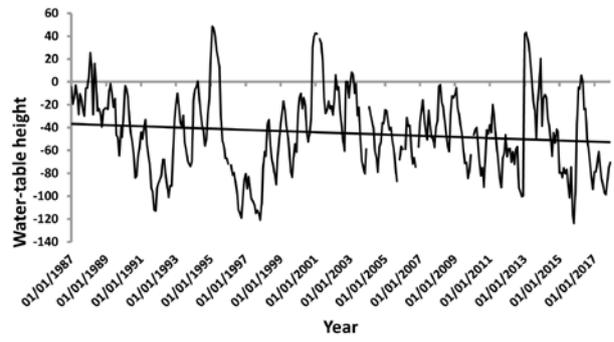


Figure 9. Monthly water-table in well 11 Ainsdale NNR from 1987 to 2014. Zero on the y axis denotes the ground surface; positive values indicate depth of standing water; negative values depth below ground surface

1995, 2001 and 2013, while particularly low values occurred in the summers of 1991, 1997, 1998, 2006 and 2011. Changes over short time-scales could be strikingly large. For example, the water-table declined by 167.5 cm in the 20 months between February 1995 and October 1997. This meant that deeply flooded dune slacks in late winter could be completely dry within a few weeks and remain so for many months.

Surprisingly, no Spearman Rank correlations were found between spawn counts and water-table data expressed either as April water-table (SR = 0.180, $p = 0.331$) or mean annual water-table (SR = 0.311, $p = 0.088$). However, there was a correlation between mean annual water-table and log toadlet number (SR = 0.394, $p = 0.028$) but not between mean April water-table and log toadlet number (SR = 0.29, $p = 0.114$). There was no relationship between mean March-June water-table and both spawn count and log toadlet number (SR = 0.268, $p = 0.144$ for spawn; SR = 0.326, $p = 0.074$ for toadlets).

DISCUSSION

Population dynamics

Continuous survey over many years is the most reliable way of quantifying changes in natterjack populations (Buckley & Beebee, 2004). The 31-year timescale of this study provides a unique opportunity to analyse trends for a large population in a sand-dune habitat, the only other published long-term investigation being a 37 year data-set for a much smaller population of natterjacks on lowland heath in southern England (Beebee, 2011; Di Minin & Griffiths, 2011).

During the current study, annual spawn counts varied greatly, the pattern of peaks being similar to that reported in the UK as a whole by Buckley et al. (2014). Between 1990 and 2009, peak national spawn counts were in 1993, 2000, 2003, 2005 and 2009, while those on the Sefton Coast occurred in 1993, 1999, 2000, 2005, 2006 and 2008. Annual Sefton spawn counts, roughly equivalent to numbers of females breeding in that year, ranged from 1576 to 77 (mean 606). A small proportion of females do not spawn every year, as some may

require resources from more than one year to mature a batch of eggs. Also, they assess pond conditions or the presence of competitor larvae before spawning (Beebee & Buckley, 2014b). Spawn counts are likely therefore to underestimate the numbers of females in particular years. Bearing in mind this proviso and assuming a 1:1 sex ratio (Beebee & Denton, 1996), the mean Sefton adult population may have been about 1200 individuals, with a peak around 3150. Some strings were probably missed, so the actual population was probably somewhat larger. Smith & Payne (1980) estimated an adult female population of about 2000 in their 1978 Sefton Coast study, while Davis's (1985) data suggest that just the Cabin Hill adult population averaged about 1400 between 1981 and 1983.

Peak counts of adults (mostly males) at breeding sites were sometimes larger than the number of spawn strings recorded, the largest counts being 2442 in 1987 and 2354 in 1998. However computed, these data represent a high proportion of the UK natterjack toad population, estimated "very approximately" at an average of 4000 adults between 1990 and 2009 (Beebee & Buckley, 2009).

Beebee (2009) examined the relationship between "apparent" breeding population size (from spawn counts) and "effective" breeding population size estimated from genetic (microsatellite) data. The latter is usually much smaller than census size because many adults fail to leave progeny. In a mid-1990s sample year, Sefton had a census size of 896 and an effective size of 97 adults (ratio 9.2:1). An effective breeding size of more than 50, as here, is important because a smaller number promotes genetic drift which is likely to erode genetic diversity and, ultimately, population viability.

Estimated metamorphic success was extremely variable (from 0 to 526,000 toadlets per annum), there being a positive relationship between spawn count and toadlet numbers. Beebee & Buckley (2014b) also reported a positive correlation between spawn counts and \log_{10} toadlet production in a 20-year analysis of UK natterjack populations.

Climatic factors

The great variation and decline in spawn counts and breeding success recorded in Sefton was probably due to many interacting factors, some of which were investigated during this study. Most convincing was the positive relationship between spring (March-May) and April rainfall and both spawn count and toadlet production. Declining April rainfall since 2000, with associated reduction in spawning and metamorphic success, seems to accord with national trends and, according to Hanna et al. (2016), is linked to changes in high pressure blocking over Greenland. This has resulted from a warming trend in that part of the Arctic and has affected the strength of the North Atlantic Jet-stream.

McGrath & Lorenzen (2010) investigated the effect of 25 climatic, site and management variables on British natterjack populations using general linear models. They found that rainfall but not temperature was a predictor of population trends. Surprisingly, however, these trends declined with increasing rainfall, this being mainly due to

the dominant influence of three sites in Cumbria, UK, that were subject to high rainfall. No clear relationship was apparent for other sites that experienced lower rainfall. The authors concluded that, while rainfall is needed to trigger natterjack activity, having just enough to maintain shallow breeding ponds is preferable to high rainfall. However, a drier climate coupled with large fluctuations in water-table, as on the Sefton Coast, risks an increasing frequency of breeding pond desiccation. Thus, Banks et al. (1994) showed that from 1970 and 1990 there was a greater risk of natterjack breeding failure for years in which any month between March and May had less than 25 mm of rainfall. In Sefton, the eight driest Aprils since 2000 had a mean rainfall total of 24.2 mm, these springs also having fewer spawn strings. Similarly, Banks & Beebee (1988) found that slack desiccation was a key mortality factor in some years, especially affecting tadpoles at their Drigg dunes (Cumbria, UK) study area. However, desiccation was much less important at their Woolmer (Hampshire, UK) heathland study area, being replaced by tadpole loss to predation as the key mortality factor. Davis (1985) found that invertebrate predation was responsible for 87-97% of tadpole mortality at Cabin Hill, Sefton, from 1981 to 1983, while pool desiccation accounted for only 2.9 to 12.8% of tadpole loss. However, his study took place during a period of high water-levels and involved "mature" water-bodies over ten years old, in which invertebrate predator populations were likely to be high. Davis also pointed out that losses due to desiccation often occur late in the season, affecting mature tadpoles that have escaped other forms of mortality.

Unusual climatic conditions were shown by Buckley & Beebee (2004) to coincide with low spawn counts at many British natterjack colonies, thus the exceptionally high water-levels in spring 1995, followed by low levels the following year corresponded with low numbers of spawn strings nationally, as well as in the Sefton dunes. Beebee (2011) used models to test a series of hypotheses on the effects of conservation management and climatic factors on natterjack population dynamics. His results supported those of McGrath & Lorenzen (2010), inferring positive influences of management and negative effects of rainfall. To account in part for the latter, Beebee (2011) suggested that high winter rainfall causes mortality of juveniles by flooding hibernation sites.

A link was established between annual water-table height and toadlet production, it being clear that premature desiccation of Sefton breeding sites in spring and early summer, especially after 2000, caused catastrophic mortality of tadpoles. In some years, water-levels were so low that few breeding sites were available; for example, only seven were used for spawning in 2017. The major fall in water-table between February 1995 and October 1997 coincided with the lowest spawn count of 77 in 1996. Hydrological studies of other British dune systems suggest such variations are not confined to the Sefton Coast. Thus, while the mean annual fluctuation of the Sefton dune water-table is about 85 cm, Kenfig in South Wales averages 81 cm (Jones & Etherington, 1989), Whiteford Burrows 115 cm (Stratford et al., 2012),

Braunton Burrows 95 cm (Clarke & Stratford, 2010) and Newborough Warren between 82 cm and 133 cm (Clarke & Stratford, 2010). However, of these localities, only Sefton supports natterjack toads. The fact that there has been an overall decline in the height of the water-table may have increased the likelihood of breeding sites drying out prematurely.

Our study found significant increases in mean maximum temperatures and decreases in mean minimum temperatures during spring over the survey period. Warmer daytime temperatures may increase rates of desiccation of breeding sites, while cooler temperatures at night are less favourable for natterjack breeding activity (McGrath & Lorenzen, 2010).

Conservation

It is well established that a viable natterjack population is dependent on two key habitat resources:

- The availability in spring of suitably shallow, sparsely vegetated water bodies with appropriate water chemistry and few aquatic predators or competitors
- Suitable terrestrial habitat comprising short turf with large open sandy areas for foraging and burrowing (Baker et al., 2011; Beebee & Denton, 1996).

In the past, Sefton natterjacks mainly bred in embryonic slacks created either by wind-erosion or accretion of new dunes (Smith, 2009). However, due to dune-sealing, the rate of slack formation in the Sefton dunes has been low for several decades (Houston, 2008; Smith, 2009), necessitating the excavation of scrapes to mimic this habitat. According to Beebee & Denton (1996), natterjack population size is usually limited by the number of suitable breeding pools. A high density of pools also increases the chance of some having suitable conditions for the immature stages, irrespective of rainfall or other factors that affect temporary water bodies. Between 2010 and 2015, 24 new scrapes were created in the Sefton dune system. Nearly all young scrapes were used successfully by natterjacks, their importance for toadlet production, especially in dry years, having been shown during this study. Indeed, without them, little or no successful breeding could have occurred in most recent years. Ideally, several scrapes of different depths are required at each site to ensure that at least one supports successful metamorphosis each year (Baker et al., 2011; Buckley et al., 2014).

Also important is the distance between breeding sites. Using radio-telemetry, Sinsch et al. (2012) estimated that a maximum distance of 2250 m between natterjack breeding ponds in central Europe was needed to maintain connectivity. In Sefton, as far as possible, suitable breeding sites have been provided at intervals of less than 2000 m.

Previous studies have demonstrated the importance of climatic variables on natterjack populations, especially the deleterious consequences of changes in rainfall (Banks et al., 1994; McGrath & Lorenzen, 2010). In the longer term, climate change could also have an adverse impact on slack habitat. Thus, using a conceptual water-

balance model, Clarke & Sanitwong Na Ayutthaya (2010) estimated that the Sefton dune water-table may fall by as much as 1.5 m by the end of the 21st century. Natural England (2014) found a 36 % and 44 % reduction in wetland area at Ainsdale and Birkdale, respectively, between 1989 and 2012 due to slack habitats becoming drier. Older slacks are gradually infilled by blown sand and organic matter (Simpson, 2002; Smith & Payne, 1980), and undergo succession to a densely vegetated condition, rendering them less suitable for natterjack breeding. Many of the older scrapes dug in the 1970s and 1980s have been colonised by emergent aquatic plants, the competitive common toad and aquatic predators such as dragonflies (*Odonata*), to the exclusion of the rarer amphibian (Simpson, 2002). Beebee & Buckley's (2014b) national study showed that a majority of natterjack breeding ponds remained productive for up to 25 years, though many required active management to maintain favourable conditions. Some of the earlier scrapes on Ainsdale NNR were re-profiled in the early 1990s and continued to be used by natterjacks (Simpson, 2002).

As in other West European dune systems, the natterjack's terrestrial habitat has been affected by increasing stability, vegetation overgrowth and loss of dynamism (Houston, 2008; Smith, 2009; 2012). Lower rabbit (*Oryctolagus cuniculus*) numbers in some areas (Simpson, 2002) and aerial deposition of nitrogen (Jones et al., 2004), is likely to have played a role in the development of coarse vegetation and scrub on the Sefton dunes. Over-stabilisation is disadvantageous to natterjacks as it restricts foraging opportunities and encourages colonisation by competitive amphibia (Beebee & Denton, 1996). These factors seem to have led to reductions in previously large populations, especially at Ainsdale NNR, Birkdale LNR and Cabin Hill/Altcar. Arntzen et al. (2017) reported a significant decline of the natterjack in northern France between 1974 and 2011, coastal dune populations being affected by re-growth of vegetation over open areas that had been created by military activities during World War II. Similarly, Stevens & Bagueette (2008) drew attention to the natterjack's susceptibility to habitat succession in Belgium.

In an attempt to hold back vegetation succession, domestic livestock have been re-introduced to several Sefton duneland holdings, as recommended for the management of natterjack terrestrial habitat by Baker et al. (2011). However, many of the natterjack breeding sites near the shore lie outside the grazing enclosures. In a review of UK breeding colonies, Beebee & Buckley (2009) found populations on grazed sites were stable or increased slightly between 1990 and 2009, while those on ungrazed sites declined. Simpson (2002) described the beneficial impact that tree and scrub removal followed by livestock grazing had on natterjack terrestrial habitat at Ainsdale NNR. Although it retarded succession at Ainsdale, grazing did not entirely prevent it, leading Millett & Edmondson (2013) to suggest that re-establishment of disturbance to create new slack habitat, followed by grazing, might be a more successful strategy. Evidently, active management is needed to maintain

and enhance natterjack populations (Arntzen et al., 2017; McGrath & Lorenzen, 2010). Stevens & Baguette (2008) point out that protection without management leads to habitat deterioration and natterjack extinction. While the considerable (and expensive) management efforts in Sefton seem to have been well-targeted, they have been insufficient to prevent an apparent recent reduction in the population. It is especially concerning that natterjacks appear to be declining on Ainsdale NNR (Fig. 3a), despite the fact that large parts of the reserve are managed in a way that should benefit this species, including provision of scrapes, scrub control and winter-grazing by livestock. Further action is desirable, including creating more scrapes, restoring older ones, increasing livestock grazing, removing invasive scrub and woodland and mowing coarse vegetation in breeding sites. It is hoped that additional resources for management will be allocated through Dynamic Dunescape, a joint Heritage Lottery Fund and EU LIFE project covering seven coastal dune systems in England and Wales, including the Sefton Coast. The delivery phase, from 2019 to 2023, proposes capital works, such as re-mobilisation, turf-stripping, mowing and scrub control which, if appropriately targeted, could greatly benefit natterjack toad conservation in Sefton.

CONCLUSIONS

The results of this long-term study suggest that the size of the natterjack toad population on the Sefton Coast remains significant in a national context but has declined in recent years and is increasingly dependent on active management to create and maintain suitable breeding sites. These are susceptible both to desiccation, due to low spring rainfall, and invasion by competitive amphibians. Large fluctuations in the water-table often resulted in the premature drying out of dune-slacks and reduced breeding success. Climate change is likely to exacerbate this trend in the future. Many former breeding sites have become overgrown, while vegetation maturation and the development of scrub and coarse grassland have reduced the suitability of terrestrial habitat. Encouraging dune dynamics by removing trees and scrub, increasing areas subject to livestock grazing and mechanical rejuvenation may offer the best hope of conserving this species in the future.

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Dealing with hot rocky environments: Critical thermal maxima and locomotor performance in *Leptodactylus lithonaetes* (Anura: Leptodactylidae)

Iván Beltrán^{1,2*}, Valeria Ramírez-Castañeda^{*}, Camilo Rodríguez-López^{3*}, Eloisa Lasso & Adolfo Amézquita

¹ Department of Biological Sciences, Universidad de Los Andes. Carrera 1 No 18A-12. A.A. 4976. Bogotá D.C., Colombia

² Current address: Department of Biological Sciences, Macquarie University, NSW, Sydney 2109, Australia.

³ Department of Cognitive Biology, University of Vienna, A-1090, Vienna, Austria

* these authors contributed equally to this work

Environmental temperature has fitness consequences on ectotherm development, ecology and behaviour. Amphibians are especially vulnerable because thermoregulation often trades with appropriate water balance. Although substantial research has evaluated the effect of temperature in amphibian locomotion and physiological limits, there is little information about amphibians living under extreme temperature conditions. *Leptodactylus lithonaetes* is a frog allegedly specialised to forage and breed on dark granitic outcrops and associated puddles, which reach environmental temperatures well above 40 °C. Adults can select thermally favourable microhabitats during the day while tadpoles are constrained to rock puddles and associated temperature fluctuations; we thus established microhabitat temperatures and tested whether the critical thermal maximum (CT_{max}) of *L. lithonaetes* is higher in tadpoles compared to adults. In addition, we evaluated the effect of water temperature on locomotor performance of tadpoles. Contrary to our expectations, puddle temperatures were comparable and even lower than those temperatures measured in the microhabitats used by adults in the daytime. Nonetheless, the CT_{max} was 42.3 °C for tadpoles and 39.7 °C for adults. Regarding locomotor performance, maximum speed and maximum distance travelled by tadpoles peaked around 34 °C, approximately 1 °C below the maximum puddle temperatures registered in the puddles. In conclusion, *L. lithonaetes* tadpoles have a higher CT_{max} compared to adults, suggesting a longer exposure to extreme temperatures that lead to maintain their physiological performance at high temperatures. We suggest that these conditions are adaptations to face the strong selection forces driven by this granitic habitat.

Keywords: CT_{max}; thermal tolerance; tadpoles; granitic rocks; hot environment

INTRODUCTION

From all environmental conditions, temperature is probably the most influential variable that controls animal performance (Hutchison, 1961). In ectothermic animals, environmental temperature profoundly affects protein tertiary structure and thereby physiology, which further constrains habitat preferences and ecological performance (Hutchison, 1961; Viña, 2002; Gunderson & Stillman, 2015). Individuals exposed to thermal stress often die because of cardiac dysfunction (Viña, 2002). In many amphibians, however, heat stress causes rapid desiccation due to their wet and permeable skins, which are in turn necessary to facilitate cutaneous gas exchange (Hutchison, 1961; Tracy, 1976; Duarte et al., 2012). Extreme temperatures may also affect locomotor performance and associated foraging and anti-predator behaviour, which eventually cause a decrease in fitness (Wilson & Franklin, 1999; Wilson et al., 2000; Herrel & Bonneaud, 2012).

Previous studies have addressed the existence of

an optimal range of temperatures for frog activity (e.g. Noland & Ultsch, 1981) and its relationship with the mean environmental temperature and the lethal temperature range (Wilson & Franklin, 1999; Wilson et al., 2000; Herrel & Bonneaud, 2012). Also, optimal temperature is known to vary between species and development stages (Navas et al., 2010; Katzenberger, 2014; Turriago et al., 2015). Although most studies regarding amphibians in extreme heat conditions have been addressed to the palearctic regions (Hillman et al., 2009), recent information about subtropical and tropical regions has been published (Duarte et al., 2012; Katzenberger, 2014; Gutierrez-Pesquera et al., 2016). However, studies about tropical species have been focused primarily on tadpoles (Simon et al., 2015; Gutierrez-Pesquera et al., 2016). Increasing information about extreme environments in tropical regions is highly important because mean optimal values are closer to the limit values of the optimal distribution range in most animals (Lillywhite & Navas, 2006). To thrive under extreme thermal conditions, frogs are expected to show behavioural and physiological adaptations,

Correspondence: Iván Beltrán (ic.beltran196@gmail.com)

such as protein polymorphisms or phenotypic plasticity (Hoffman, 2003; Somero, 2004; Denver, 1997; Somero, 2010; Brown, 2013 Gunderson & Stillman, 2015).

Leptodactylus lithonaetes (Anura: Leptodactylidae) is found on granitic outcrops in northern South America (Heyer, 1995). These habitats essentially lack any shadow, except for a few shrubs and grasses in rock puddles (see Fig. 1) thus, temperatures are considerably higher compared to the surrounding forest (IDEAM, 2018). However, adults appear to have better opportunities for thermoregulation by microhabitat selection compared to tadpoles: adults forage, call and mate at night, but during the daytime, they are hidden most likely in rock crevices. Tadpoles, on the other hand, are constrained to the small and shallow puddles and exposed to midday temperatures. Due to the positive correlation between maximum habitat temperature and maximum critical temperatures (Miller & Packard, 1977; Compton et al., 2007); if tadpoles are exposed to higher temperatures than adults, they should present higher values of critical thermal maximum (CT_{max}). Moreover, because temperature changes affect the power output, contractile and relaxation rates of muscles (Rome, 1983; Hirano & Rome, 1984; Fleming et al., 1990; Franklin & Johnston, 1997), locomotor performance of tadpoles might be adapted to their thermal environment and should be



Figure 1. Image of a representative puddle inhabited by *L. lithonaetes* tadpoles. These puddles are formed after rain fills the hollows dispersed over the granite rock outcrops.

higher at more suitable temperatures.

The aims of this study were thus (1) to establish whether puddle temperatures are indeed higher than the microhabitats allegedly chosen by adults, (2) to estimate CT_{max} of adult and larvae *L. lithonaetes* and compare it with other anuran species, (3) to test the prediction that tadpoles have higher CT_{max} values than adults and (4) to estimate the effect of temperature on locomotor performance of tadpoles and compare it with environmental temperatures experienced in the field.

MATERIALS AND METHODS

Study species

Leptodactylus lithonaetes (Heyer, 1995) is a moderate sized and cryptic frog. Females (snout-vent length (SVL): 55-78 mm) are slightly bigger than males (SVL: 45-71 mm) (Heyer, 1995). Adults forage, call and mate during the night; breeding pairs lay eggs in foam nests built near small puddles, on granitic or sandstone rocks, where tadpoles complete their development. Presumably, the breeding season takes place between May-August, which corresponds to the rainy season in the area (IDEAM, 2018). However, detailed information about the breeding behaviour and the ecology and development of the larvae of *L. lithonaetes* still needs to be assessed. The species occurs below elevations of 1250 m, more often on outcrops surrounded by savannah and gallery forests, in south-west Venezuela and eastern Colombia (Heyer, 1995; Heyer & Heyer, 2001).

Study sites

Our study was conducted in November 2015 at the Bojonawi Nature Reserve (6° 06' N, 67° 29' W, 50 m.a.s.l.), about 9.5 km south of Puerto Carreño (Vichada), Colombia. *Leptodactylus lithonaetes* is abundant on a granitic outcrop, about 0.27 km² in area, just at the edge of the Orinoco River. During three previous field trips, we spotted adults foraging at night on the exposed rock and calling from the puddles or from within the associated vegetation (Fig. 1). In the puddles, tadpoles of *L. lithonaetes* were observed foraging in small groups of about 8-10 individuals. To estimate maximum values and diurnal variation in environmental temperature, we registered water temperatures every hour during three consecutive days. Measurements were taken with an analogue thermometer (± 0.5 °C) in the centre of the puddles (n=12) at a depth of approximately 10 cm. Puddles differed in area but not in depth, and we only included puddles with tadpoles present. To estimate maximum values and diurnal variation in rock temperatures, we obtained thermal images by pointing an Infrared Camera (FLUKE® Ti32. Fluke Corporation. Everett, WA, USA) within 1 m of the surface. We took thermal images every hour from 0730 to 1700 h during four days and registered the temperature of 135 haphazardly chosen spots.

As a post hoc analysis, we included grass cushions temperatures due to the possibility that adults occupy this microhabitat during the day. However, because these measurements were not considered in our initial hypothesis, we obtained the data from thermal images of rocks in which the grass cushions were also visible.

In total, we obtained 60 measurements from grass cushions temperatures. These measurements were taken approximately every hour from 0800 h to 1200 h during one day.

CTmax and locomotor performance

We collected seven adult frogs by hand and 16 tadpoles with a net. Tadpoles were collected from three different puddles to reduce the probability of close relatedness between them. All adults and six randomly selected tadpoles were used to estimate Critical Thermal Maxima (CTmax) whereas the remaining ten tadpoles were used to estimate the thermal dependence of locomotor performance. All experiments were conducted in a research station within 600 m of the site where the animals were captured. For CTmax experiments, animals were captured between 0900–1200 h and measured between 1400–1900 h, giving them at least 2 hours of acclimation to the environmental temperature (about 28 °C). Locomotor performance experiments were conducted between 0845–0930 h.

CTmax is operationally defined as the mean temperature at which an animal loses its locomotor performance and the ability to escape from conditions that otherwise would cause death (Hutchison, 1961). Following Stebbins (1954) and Hutchinson (1961), we placed each individual (tadpole or adult) in a small plastic jar containing 50 mL of tap water, which itself was placed in a water bath heated at a rate of approximately 1 °C/min. Every 2 minutes we stimulated the tadpole and the adults with a soft push. In addition, when the adults halt their reaction to the stimuli, they were placed face up to be sure that they lost completely their locomotor abilities. Then we measured the water temperature with an analogue thermometer (± 0.5 °C). We then registered the water temperature at which animals lost their ability to swim (adults and tadpoles) and recover their body posture (adults). At the end of each experiment, the plastic jar containing the individual was removed from the setup and slowly cooled to avoid injuries or death due to thermal stress. All animals survived the experiments, looked healthy after some hours and were thus released at their capture sites.

The locomotor performance of tadpoles was measured by placing each individual in an 18 x 13 x 6.5 cm (H x W x D) plastic aquarium with a length scale at the bottom. To induce tadpoles to swim, we dropped a small pebble close to their tail from a height of 33 cm. For each tadpole, we conducted three trials in ascendant order at each of the experimental temperatures (25, 28, 31, 34 and 37 °C). The temperature values were chosen randomly according to the natural range of variation of the temperatures reached in the puddles (31.01 ± 2.46 ; see Fig. 2) plus two “extreme” temperatures (25 and 37 °C).

Two consecutive trials were separated by at least 10 min to avoid potential fatigue or habituation effects. All trials were recorded from the top of the aquarium with a Sony HDR-HC71080i video camera fixed to a supporting structure to avoid camera movement. Tadpole movements in the recordings were video tracked with the software Tracker® (<http://www.opensourcephysics.org>),

Open Source Physics, Boston MA, USA) to estimate the maximum speed (m/s), maximum acceleration (m/s²) and maximum distance (m) travelled during escape responses. Our three proxies for locomotor performance allegedly relate to tadpoles ability to escape from potential predators (Watkins, 1996; Bursik, 2000; Wilson et al., 2005).

Statistical analysis

Difference in CTmax values between tadpoles and adults of *L. lithonaetes* were analysed using an Exact Mann-Whitney-Wilcoxon Test available in R package “coin” (Hothorn et al., 2008) due to the non-parametric distribution of the dependence variable (ordinal data). To test the effect of temperature on each estimate of locomotor performance, we conducted Linear Mixed Models (LMM). Given that the effect of temperature on physiological performance is typically non-linear and generally resembles an asymmetrical bell-shaped curve, we smoothed the models using LOESS smoothing function. All statistical analyses and graphs were conducted on R 3.2.3 software (R Development Team 2016 Vienna, Austria. <https://www.r-project.org/>) and conducted at a significance level of $\alpha = 0.05$.

RESULTS

Mean water temperature in the puddles varied throughout the day between 27.5–35.75 °C, peaking at 12:00 h ($35.75 \text{ °C} \pm 0.25$; mean \pm standard deviation (SD)). Mean grass cushion temperatures varied throughout the day between 27.1–40.5 °C, peaking at 11:00 h ($33.7 \text{ °C} \pm 2.3$). Mean rock temperatures exhibited a much wider range of daily variation, between 36–52.54 °C, and peaked at 10:45 h with $52.54 \text{ °C} \pm 6.59$ (see Fig. 2). The maximum temperature we registered on the rock was 68.3 °C. The critical thermal maximum differed significantly between tadpoles and adults ($U = 0$, $Z = -3.25$, $P < 0.01$). The CTmax was about 2.6 °C higher for tadpoles ($42.33 \text{ °C} \pm 0.81$, mean \pm SD, $n = 6$) compared to adults ($39.71 \text{ °C} \pm 0.75$, $n = 7$). Two out of three proxies for locomotor performance in tadpoles were significantly affected by temperature: they swam faster and longer distances at higher temperatures between 31 °C (LMM_{distance}: $\beta = 0.04$, SE = 0.01, $P < 0.01$) and 34 °C (LMM_{speed}: $\beta = 0.09$, SE = 0.03, $p = 0.01$; LMM_{distance}: $\beta = 0.055$, SE = 0.01, $P < 0.01$); above this peak value, performance dropped with increasing temperatures (Fig. 3).

DISCUSSION

The CTmax of tadpoles was generally very high in *L. lithonaetes* in comparison to other species larvae from extreme heat environments in tropical regions, such as *Physalaemus* ssp. ($37.9 \text{ °C} \pm 0.91$), *P. diplolistris* ($40.7 \text{ °C} \pm 0.45$) and species from several tropical habitats ($40.9 \text{ °C} \pm 0.22$) (Simon et al., 2015; Gutierrez-Pesquera et al., 2016). Noland and Ultsh (1981) interpreted high CTmax in *Anaxyrus (Bufo) terrestris* as an adaptation to living in shallow ephemeral puddles, in contrast to *Lithobates (Rana) pipiens*, which inhabits permanent ponds and exhibits CTmax about 2 °C lower. The choice of warmer

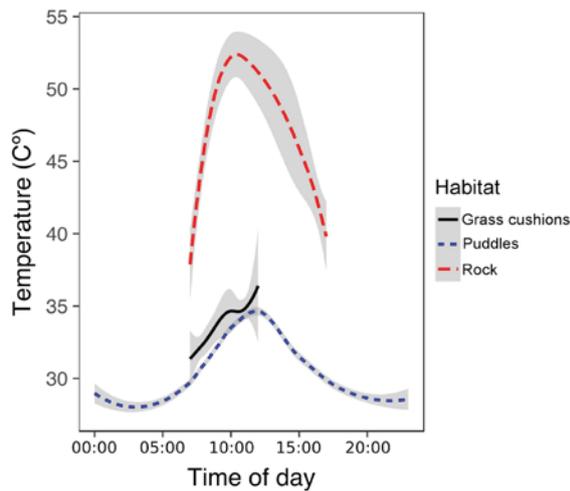


Figure 2. Daily rock (red dashed line; $n = 135$ measurements), grass cushions (black solid line; $n = 60$ measurements) and water (blue dashed line; $n = 12$ puddles) temperatures recorded at the site of study. Water temperatures were recorded in puddles where tadpoles of *L. lithonaetes* were found. Shaded region indicates 95 % confidence interval.

puddles for oviposition and larval development has been in turn interpreted as an adaptation to complete development in a shorter period of time (Berven et al., 2007). Indeed, the availability of appropriate breeding sites for *L. lithonaetes* at the study site is profoundly affected by rains. During both the dry and the rainy seasons, puddles may dry out after a few days or a few weeks without rain, due to high water evaporation on hot rocky substrates; the low probability of tadpole survival to puddle desiccation may favour the evolution of rapid developmental rates.

In the tropics, however, the presence of *L. lithonaetes* tadpoles in hot puddles could also reflect the advantages of lower predation and competition (Wassersug, 1975; Abe & Neto, 1991): shallow, ephemeral and thermally challenging puddles are known to be colonized by a lower number of species, compared to permanent ponds (Holt, 1977; Sih et al., 1985). In fact, just in a few rock puddles, *L. lithonaetes* tadpoles were found co-existing with one species of libellulid dragonfly and one of belostomatid bug, both alleged predators of anuran larvae (Heyer et al., 1975). Also, the tadpoles were almost exclusively found in the puddles formed on the exposed rock, despite cooler puddles and ponds were available in the forest and grassland habitats, less than 50 meters away from the hot rocky habitat.

In other anurans, tolerance limits of tadpoles are reduced during the metamorphosis stage (stages 43-44; Gosner, 1960; Cupp, 1980; Noland & Ultsh, 1981), which may reflect the adjustment for a new life form. However, the adaptation of tadpoles to high temperatures may also be reflected in the CTmax of the adult frogs (Hoppe, 1978). Contrary to our expectations, water temperatures were significantly lower to rock temperatures and comparable to grass cushions temperatures (Fig. 2). Our data suggest that the thermal profile experienced by adults was generally higher than that experienced by tadpoles. Nevertheless,

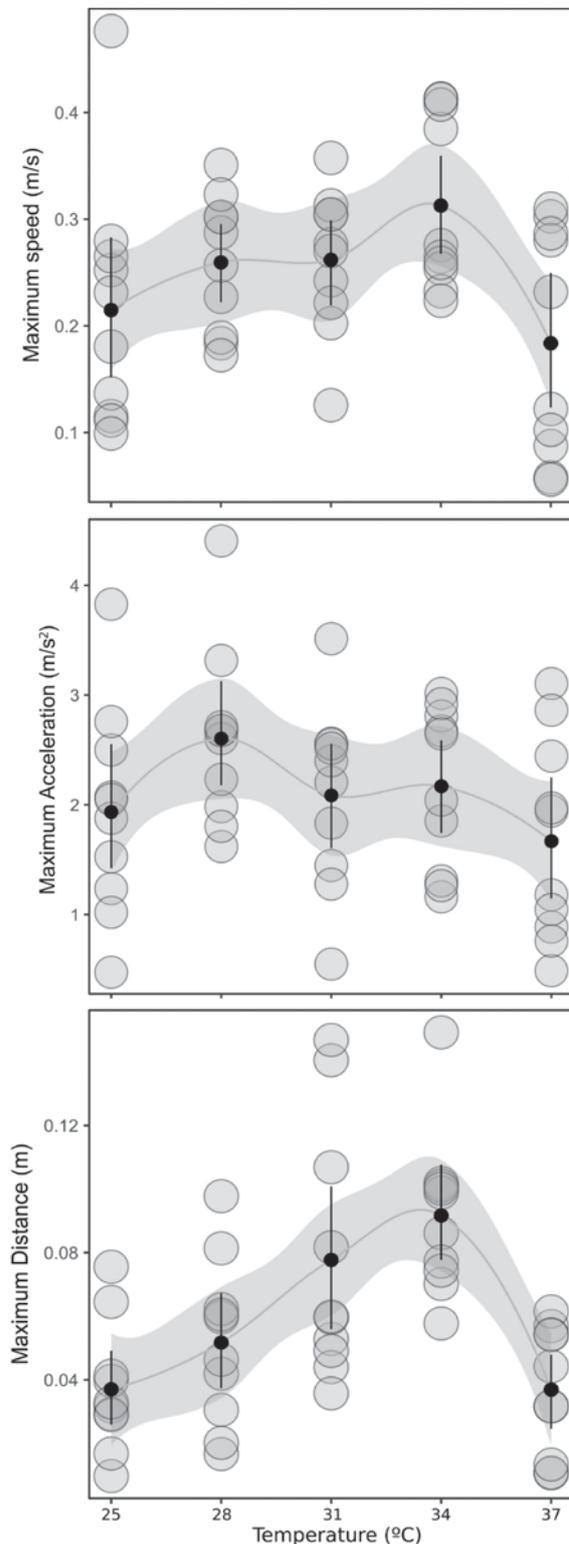


Figure 3. Effect of water temperature on locomotor performance of *L. lithonaetes* tadpoles. Grey lines denote loess smoothness of the model. Shaded regions and vertical black lines indicates 95 % confidence intervals ($n = 10$ individuals).

L. lithonaetes tadpoles were more tolerant of higher temperatures than adults. These results could reflect tadpole adaptations to extreme puddle temperatures during the dry season due to high water evaporation rates. However, our results should be treated carefully

because (1) we did not measure puddle temperatures during the dry season and (2) we are assuming that adults hide in rock and grass cushions during the day. Adults are rarely observed during the day thus, it is not completely certain that these are the only microhabitats chosen by them. Tadpoles are instead restricted to water bodies just a few centimetres in depth and therefore have arguably poorer opportunities to thermoregulate by microhabitat selection. If this argument holds, the microhabitat preferences of the adults would explain why tadpoles have higher thermal tolerances compared to adults. A telemetry study where information is collected on thermal conditions experienced by adults would provide crucial evidence to corroborate this hypothesis.

As we expected, maximum distance of locomotor performance in *L. lithonaetes* tadpoles peaked between 31–34 °C (Fig. 3), which coincided well with a range of ecologically relevant temperatures for this species (Fig. 2), like has been observed in *Rhinella (Bufo) granulosa* and *Hyperolius viridiflavus*, species that inhabit arid environments (Reviewed by Navas et al., 2008). Muscle force is known to increase with temperature in a non-linear fashion, possibly because temperature reduces the accumulation of inorganic phosphates (Rome & Swank, 1992; Ranatunga, 1998; Navas et al., 2006; Allen et al., 2008). Burst power and resistance of frog muscles also depend strongly on temperature (Phillips et al., 2006; James et al., 2012). The asymmetry of the locomotor performance curves can be explained by the “warmer is better” hypothesis (Bennett, 1987) for at least two of the three measured traits: the locomotor performance is improved by higher body temperatures, since aerobic speed is highly temperature dependent via stamina consumption. The locomotor responses we elicited here probably represent the ability of tadpoles to escape from predators effectively and therefore, should peak within the range of temperatures that tadpoles experience in the field. However, to evaluate the relationship between the optimal temperature and the maximal locomotor performance in *L. lithonaetes* tadpoles, an intra and interspecific comparison is needed.

Acclimation may play a role in an animals CTmax and locomotor performance (Hutchison, 1961; Brattstrom, 1968; Wilson & Franklin, 1999; Wilson et al., 2000), a plastic response that we did not study here. We acknowledge that most of the studies in this research area use longer acclimation periods under known regimes temperatures, which facilitate comparisons between populations and/or species. However, tropical amphibians are likely to have limited metabolic acclimation capacities (Feder & Lynch 1982; Navas et al., 2008; but see Brattstrom, 1968) and our main objective was to show differences in high-temperature tolerance between tadpoles and adults. Therefore, we consider that our results are ecologically relevant because i) the animals were acclimated to the thermal profile of their natural environment and ii) the range of temperatures, i.e. thermal profile, experienced by tadpoles and adults (puddle vs. grass cushion temperatures, respectively) was comparable. Moreover, it is unlikely that the difference between rock and puddle temperatures is responsible for the lower CTmax of adults since, to our knowledge, all the research in amphibians

has shown that acclimation temperature has either no effect or a positive effect on CTmax (Hutchinson & Ferrance, 1970; Simon et al., 2015). Thus, our results suggest that the observed differences in CTmax between tadpoles and adults are likely due to actual physiological differences between stages rather than a different short-term acclimation. A follow-up study using a standardised acclimation period would be useful to control the effect of exposure to previous thermal profiles on the CTmax. Further research topics include the biochemical and molecular adaptations that allow tadpoles to tolerate higher temperatures than adults (Feder & Hofmann, 1999; Allen et al., 2008; Boussau et al., 2008), and the extent and importance of behavioural thermoregulation in the temperature regimes experienced by adults (Brattstrom, 1979; Brown, 2013). Both sources of information would provide substantial and more conclusive evidence for a case of adaptation in the thermal tolerances of *L. lithonaetes* tadpoles.

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Phylogenetic relationships and origin of the rattlesnakes of the Gulf of California islands (Viperidae: Crotalinae: *Crotalus*)

Eduardo Ruiz-Sanchez¹, Gustavo Arnaud², Oscar Roberto Cruz-Andrés² & Francisco Javier Garcia-De León³

¹ Departamento de Botánica y Zoología, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Camino Ing. Ramón Padilla Sánchez 2100, Nextipac, Zapopán, Jalisco 45200, Mexico

² Centro de Investigaciones Biológicas del Noroeste, S.C. Instituto Politécnico Nacional 195, Col. Playa Palo de Santa Rita, La Paz, B.C.S. 23090, Mexico

³ Laboratorio de Genética para la Conservación, Centro de Investigaciones Biológicas del Noroeste, Instituto Politécnico Nacional 195, Playa Palo de Santa Rita, La Paz, B.C.S. 23096, Mexico

The islands of the Gulf of California are divided into three categories – oceanic, continental, and landbridge – and were formed from the Middle Miocene to the Holocene. The species of the *Crotalus* genus are an important ecological element of the endemic fauna of these islands. This study is the first to include several island-endemic species in a phylogenetic framework. We seek to understand the phylogenetic relationships among these species, and in particular, whether these species are more related to the Baja California peninsula or continental Mexican species, and whether the divergence times for these relationships are consistent with the formation of the islands. We performed a phylogenetic analysis and estimated divergence times using Bayesian inference and two mitochondrial 12S and 16S genes. The analyses show a new relationship between *Crotalus angelensis* and *C. pyrrhus*. *Crotalus lorenzoensis* was nested with *C. ruber* individuals, making this species a paraphyletic species. A novel relationship was also detected in that *C. estebanensis* was sister to *C. basiliscus*. The divergence time for all island-endemic *Crotalus* species is consistent with the formation of these islands. In addition, the insular species are related to their congeners in the Baja California peninsula or mainland Mexico.

Keywords: Baja California Peninsula; Bayesian inference; island-endemic; mtDNA; rattlesnakes

INTRODUCTION

The Baja California peninsula began to form during the Middle Miocene due to the expansion process that occurred between the Pacific and North American tectonic plates. As far as we know, the peninsula was formed progressively at the end of the Pliocene and the beginning of the Pleistocene, approximately 2.58 million years ago (Smith, 1991; Riddle et al., 2000a; Holt et al., 2000; Oskin & Stock, 2003; Wood et al., 2008; Murphy & Aguirre-León, 2002; Murphy & Méndez de la Cruz, 2010). This also led to the formation of the Gulf of California (GC).

The Gulf of California has a complex geological history with two stages of formation: the first during the Miocene and the second in the Pliocene (Carreño & Helenes, 2002). The islands constitute one of the Gulf of California's most interesting features and are classified into three types: oceanic, continental, and landbridge (Grismer, 2002). The oceanic islands of the GC have never been connected to Baja California (BC) or mainland Mexico. The continental islands were once connected to the Baja California peninsula and/or mainland Mexico but became separated as a result of tectonic displacements along coastal fault zones, and broke off the trailing edge

of the peninsula as it moved north-west. The landbridge islands are the commonest and youngest of all the islands in the Gulf of California and were connected to Baja California or mainland Mexico, but for the most part, were cut off by a rise in the sea level (Grismer, 2002). These three types of islands can be separated into three regions: the northern, central midriff, and southern gulf areas (Carreño & Helenes, 2002).

The northern islands were formed by sedimentation of sediment delivery from the Colorado River. Some of these islands were formed in the Holocene, while the rest were the result of volcanic activity during the early Pleistocene (Carreño & Helenes, 2002). The central islands emerged during the middle to late Miocene (15-10 and 10-5 Ma). Those islands were uplifted due to the many faults in the region from the Pleistocene to the Holocene. Finally, volcanic activity, granitic rock or oceanic sediments from the Pliocene formed the southern islands. Altogether, this led to the formation of about 100 islands and islets, most of which were colonised by species of flora and fauna from Baja California and mainland Mexico. According to Grismer (2002), there are 161 species of native and non-native species of herpetofauna in the Baja California peninsula and the Pacific and GC islands. Four of these species are salamanders, 13 frogs, 4 turtles, 84 lizards,

Correspondence: Francisco Javier Garcia-De León (fgarciadl@cibnor.mx) ; Gustavo Arnaud (garnaud04@cibnor.mx)

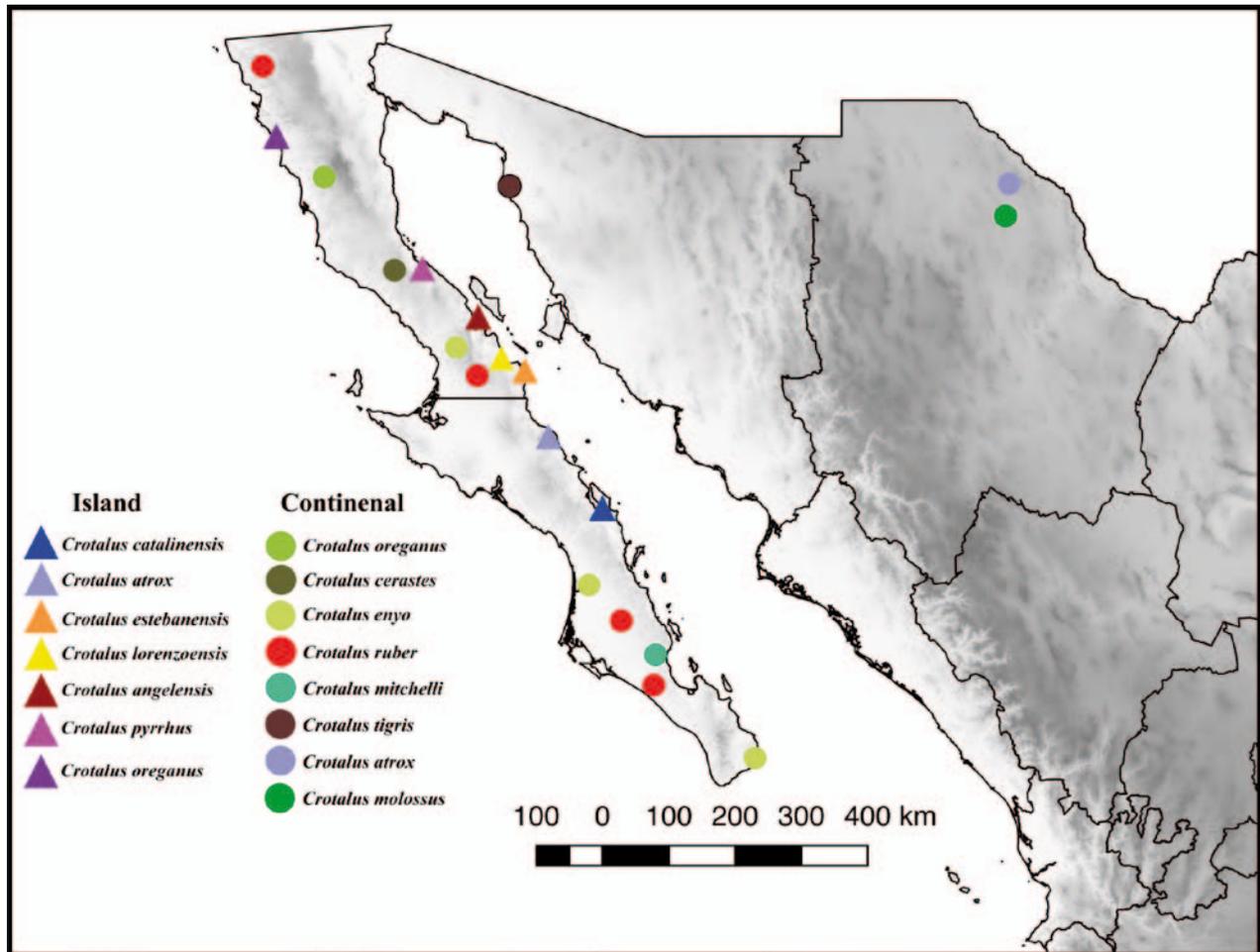


Figure 1. Distribution of species of rattlesnakes from the Gulf of California islands, the California peninsula, and mainland Mexico. Symbols represent sampling locations and indicate the corresponding species.

and 57 snakes. Among the snakes, the Viperidae family is represented by 17 *Crotalus* species (Grismer, 2002; Meik et al., 2018). Ten of these are island-endemic and one is endemic to the peninsula (Table 1; Grismer, 2002; Ernst & Ernst, 2012; Meik et al., 2018). Five species are shared between California and the Baja California peninsula (*C. atrox*, *C. cerastes*, *C. mitchellii*, *C. oreganus*, and *C. ruber*), and four island species are shared between the United States and mainland Mexico (*C. atrox*, *C. cerastes*, *C. mitchellii*, and *C. molossus*; Ernst & Ernst, 2012).

Mexico is considered the center of diversification of *Crotalus* snakes, since about 92 % of the species of this group are found in the country (Klauber, 1972; Armstrong & Murphy, 1979; Greene, 1997; Flores-Villela & Canseco-Márquez, 2004; Paredes-García et al., 2011). The *Crotalus* genus probably originated during the Middle Miocene (Bryson et al., 2010). The Sierra Madre Occidental and the Mexican Plateau have the highest diversity of *Crotalus* species (Campbell & Lamar, 2004), with the former considered the ancestral area for the genus (Place & Abramson, 2004). *Crotalus* species in mainland Mexico and Baja California may be the ancestors of the endemic island species. However, to date there has been no single phylogenetic hypothesis that includes all the *Crotalus* species endemic to the islands.

Previous molecular phylogenetic analyses using four mitochondrial genes (12S, 16S, ND4, and cytB) and 61

Table 1. *Crotalus* species present in the Baja California peninsula (BCP), Gulf de California Islands (GCI), Pacific Islands (PI), Mainland Mexico (MM), and the United States of America (USA), based on Grismer (2002), Ernst and Ernst (2012), and Meik et al. (2018).

Species	BCP	GCI	PI	MM	USA
<i>Crotalus angelensis</i>		X			
<i>Crotalus atrox</i>	X	X		X	X
<i>Crotalus catalinensis</i> ¹		X			
<i>Crotalus caliginis</i> ¹			X		
<i>Crotalus cerastes</i>	X	X		X	X
<i>Crotalus enyo</i> ²	X	X	X		
<i>Crotalus estebanensis</i> ¹		X			
<i>Crotalus lorenzoensis</i> ¹		X			
<i>Crotalus pyrrhus</i>	X	X		X	X
<i>Crotalus mitchellii</i>	X	X	X	X	X
<i>Crotalus molossus</i>		X		X	X
<i>Crotalus oreganus</i>	X				X
<i>Crotalus polisi</i> ¹		X			
<i>Crotalus ruber</i>	X	X	X		X
<i>Crotalus thalassoporus</i> ¹		X			
<i>Crotalus tortugensis</i> ¹		X			
<i>Crotalus tigris</i>		X		X	X
Total	7	15	4	6	8

¹ Island-endemic, ² BCP-endemic



Figure 2. *Crotalus* species sampled in this study. **1.** *C. oreganus*, **2.** *C. angelensis*, Ángel de la Guarda Island **3.** *C. catalinensis*, Santa Catalina Island, **4.** *C. enyo*, **5.** *C. ruber*, **6.** *C. estebanensis*, San Esteban Island, **7.** *C. lorenzoensis*, San Lorenzo Island, **8.** *C. mitchellii*, **9.** *C. enyo* (La Paz). **10.** *C. cerastes*, **11.** *C. pyrrhus*, El Muerto Island, **12.** *C. atrox*, Tortuga Island. Pictures by Gustavo Arnaud, Francisco Javier García-De León and Fernando Pozas.

Crotalinae species, of which four were *Crotalus* species, found that this genus was monophyletic and sister to the *Sistrurus* genus (Parkinson et al., 2002). Murphy et al. (2002) conducted a more extensive phylogenetic analysis using 27 *Crotalus* species. In their sampling, they included *Crotalus catalinensis* from Santa Catalina Island, *C. atrox* from Santa Cruz Island and (*C. tortugensis* = *C. atrox*) from Tortuga Island. They found sister relationships between *C. catalinensis* and *C. ruber* and (*C. tortugensis* = *C. atrox*) was nested in a clade with two *C. atrox* species, and was sister to *C. atrox* from Santa Cruz Island. Castoe et al. (2007) performed a population study of *C. atrox* and found that (*C. tortugensis* = *C. atrox*) and *C. atrox* from Santa Cruz Island are nested within mainland lineages of *C. atrox*. Finally, in a population study of *Crotalus viridis* that included *C. viridis caliginis* from Coronados Sur Island

and *C. viridis helleri* from Santa Catalina Island (USA), Ashton & de Queiroz (2001) found strong support for the separation of *C. viridis* from *C. oreganus*.

To date, not all *Crotalus* island species in the GC and the Pacific have been included in a phylogenetic study. Therefore, here we include all the insular *Crotalus* species represented by more than one individual, except the newly described *C. polisi* and *C. thalassoporus* species (Meik et al., 2018), with the aim of answering the following questions: 1) “What phylogenetic relationships exist between insular species and other *Crotalus* species?”, 2) “Are insular species more closely related to species from Baja California or species from mainland Mexico?”, and 3) “What are the divergence times of the insular species and are these correlated with the formation processes of the islands?”

METHODS

Taxon sampling and laboratory methods

Between 2008 and 2012, we collected 42 samples of 14 *Crotalus* species (Table 2) from the Baja California peninsula, its associated islands, and mainland Mexico (Figs. 1, 2). These were considered the ingroup taxa. Additionally, we used all the *Crotalus* specimens previously analysed by Murphy et al. (2002; Table 2) as outgroup taxa. Based on recent phylogenetic analyses (Murphy et al., 2002; Castoe & Parkinson, 2006; Wüster et al., 2005; Bryson et al., 2010), we used *Sistrurus catenatus*, *S. miliarius*, as a sister genus to *Crotalus* and *Agkistrodon contortrix*, *A. piscivorus* as a sister genus to the *Crotalus*+*Sistrurus* clade (Wüster et al., 2005), and *Gloydus brevicaudus* to root the tree (Wüster et al., 2005; Bryson et al., 2010).

Crotalus, *Sistrurus*, and *Agkistrodon* are genera belonging to the Crotalinae subfamily. *Crotalus* and *Sistrurus* share some morphological features such as the rattle (a corneal structure in the terminal region), which *Agkistrodon* lacks. *Sistrurus* is characterised by the presence of nine large scales, arranged symmetrically on the back of the head (Gloyd, 1940). *Sistrurus* shares this morphological feature with members of the subfamily Azemiopinae, to which *Gloydus brevicaudus* belongs (Brattstrom, 1964).

We sequenced two mitochondrial regions (12S and 16S). These gene regions have been shown to be informative at different levels of divergence within rattlesnakes (Murphy et al., 2002; Wüster et al., 2005; Bryson et al., 2010). Total genomic DNA was extracted from blood and liver, muscle or skin using the chloride salting-out method (Aljanabi & Martinez, 1997; Riera et al., 2010). All gene regions were amplified via polymerase chain reaction (PCR) in a 15 µL reaction volume containing 0.4 mM of deoxynucleoside triphosphates (dNTPs), 0.4 µM for each primer for the 12S rDNA and 0.3 µM for the 16S rDNA, buffer 1x, 0.4 mM of MgCl₂, 1.0 U Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 100 ng template DNA. Previously published primer sequences are given in Murphy et al. (2002; 12S, 16S). Initially, DNA was denatured at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50-52 °C for 1 min, and 72 °C for 1 min. A final extension phase of 72 °C for 5 min finalised the protocol. Sequences were amplified in both directions using the GENEWIZ facilities in New Jersey, USA. Samples were analysed with an ABI Prism 3730xl Genetic Analyser (Applied Biosystems). Forward and reverse sequences for each individual were edited and manually aligned using CodonCode Aligner 5.0.2. (Codon Code Corporation).

Phylogenetic analysis

We analysed our sequence data using Bayesian inference (BI). BI analyses were conducted using MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003) on the combined mtDNA dataset, implementing separate models for each gene region (12S and 16S). jModelTest v2.1.10 (Darriba et al., 2012) was used to select a best-fit model of evolution for each partition, based on the Akaike information criterion (AIC). Two independent runs were performed for the

partitioned data matrix. For each run, we employed one cold and three heated chains, which were set to run for 40,000,000 generations, sampling one tree every 2,000 generations. The convergence of runs was confirmed by effective sample size (ESS) >200 for all parameters, using Tracer v1.6 (Rambaut et al., 2013). Sample points collected prior to stationarity were eliminated (25 %). Posterior probabilities (PP) for supported clades were determined by a 50 % majority-rule consensus of the trees retained after burn-in. BI analyses were run in the CIPRES Science Gateway (Miller et al., 2010).

Divergence time estimates

Divergence times were estimated using a Bayesian approach as implemented in BEAST v. 1.8 (Drummond & Rambaut, 2012). The model selected was GTR + I + G, based on the results of the AIC run in jModeltest 2.1.10, under an uncorrelated lognormal relaxed clock model. We used three calibration points and treated them as minimum age constraints. Two fossil calibrations for the tree were obtained for North American pit vipers. The first was the earliest record of *A. contortrix* in the Late Miocene (Late Hemphillian; Holman, 2000). The second was the oldest fossil from the *Sistrurus* genus, from the Late Miocene (Parmley & Holman, 2007). For both nodes, we used a hard upper-bound constraint with lognormal distributions following recommendations by Ho & Phillips (2009).

The *A. contortrix* node was given a zero offset of 6 million years ago (Ma), a lognormal mean of 0.01, and a lognormal standard deviation of 0.42. The *Sistrurus* stem was constrained with a zero offset (hard upper bound) of 8 Ma, a lognormal mean of 0.01, and a lognormal standard deviation of 0.76 (Bryson et al., 2010). For the root node, we used a mean stem age of 17 Ma and a standard deviation of 1.0, with a normal distribution (Ho & Phillips, 2009) based on previous molecular estimates by Bryson et al. (2010). One independent 40,000,000-generation run was performed with random starting trees, sampling every 2,000 generations. Tracer 1.5 (Rambaut et al., 2013) was used to assess convergence and estimate effective sample sizes (ESS) for all parameters. Results were summarised in a single tree using TreeAnnotator 1.8 (Rambaut & Drummond, 2015), and visualised with FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS

Phylogenetic analysis

The total number of aligned base pairs (bp) for each mtDNA marker was 511 bp for 12S and 1328 bp for 16S. The concatenated and aligned mtDNA matrix was 1839 bp.

The partitioned molecular mtDNA (12S + 16S) analysis using BI retrieved a 50 % majority-rule consensus tree in which *Crotalus* is a monophyletic genus (PP = 0.99) with five well-supported clades (hereafter named clades 1, 2, 3, 4, and 5), and sister to the *Sistrurus* genus (Fig. 3). Clade 1 is made up of *Crotalus angelensis*, *C. pyrrhus* from El Muerto Island, one *C. pyrrhus* individual from California, and *C. mitchellii* (PP = 0.96). *C. pyrrhus* was sister to *C.*

Table 2. *Crotalus* species used in this study.

Species	Individuals	Voucher	Locality	Acronym	Species	Individuals	Voucher	Locality	Acronym
<i>Crotalus adamanteus</i>		ROM 18130 *	Commercially purchased				ROM 18141-42 *	Veracruz, Mexico	
<i>Crotalus angelensis</i>	Ca1	Ca 01	Ángel de la Guarda Island, B. C.	IAG	<i>Crotalus oreganus</i>	Co1	Co UABC144	Ensenada, B. C.	ENS
<i>Crotalus aquilus</i>		ROM 18117 *	Mexico, San Luis Potosí			Co2	Co UABC124	Ensenada, B. C.	ENS
<i>Crotalus atrox</i>	Cat1	Cat D2jul2011	Ciudad Juárez, Chihuahua.	CJ		Co3	Co UABC076	Ensenada, B. C.	ENS
	Ct1	Ct1 2011	Tortuga Island, B. C. S.	IT		Cca1	Cca 060610	Coronados Sur Island, B. C.	ICS
	Ct2	Ct2 2011	Tortuga Island, B. C. S.	IT		Cca2	Cca 010610	Coronados Sur Island, B. C.	ICS
	Ct3	Ct3 2011	Tortuga Island, B. C. S.	IT		Cca3	Cca 030610	Coronados Sur Island, B. C.	ICS
	Ct4	Ct 040110	Tortuga Island, B. C. S.	IT		Cca4	Cca 040610	Coronados Sur Island, B. C.	ICS
		ROM 18192 *	Tortuga Island, B. C. S.		<i>Crotalus polystictus</i>		ROM-FC 263 *	Mexico City, Mexico	
		ROM 18224 *	Santa Cruz Island		<i>Crotalus pricei</i>		ROM-FC 2144 *	Nuevo León, Mexico	
		ROM 18144 *	Riverside Co., CA.		<i>Crotalus pusillus</i>		ROM-FC 271 *	Michoacán, Mexico	
<i>Crotalus basiliscus</i>		ROM 18188 *	Nayarit, Mexico		<i>Crotalus pyrrhus</i>	Cm1	Cm 03062010	El Muerto Island, B. C.	IM
<i>Crotalus catalinensis</i>	Cc1	Cc 11sep08	Santa Catalina Island, B.C.S.	ISC		Cm2	Cm 03032010	El Muerto Island, B. C.	IM
	Cc2	Cc 04012011	Santa Catalina Island, B.C.S.	ISC		Cm3	Cm 051109	El Muerto Island, B. C.	IM
	Cc3	Cc 04032011	Santa Catalina Island, B.C.S.	ISC		Cm4	Cm 05409	El Muerto Island, B. C.	IM
	Cc4	Cc 04092011	Santa Catalina Island, B.C.S.	ISC			ROM 18178 *	Imperial Co., CA.	
		ROM 18250 *	Santa Catalina Island, B. C. S.		<i>Crotalus ruber</i>	Cr1	Cr n=2	San Antonio, B. C. S.	SA
<i>Crotalus cerastes</i>	Cce1	Cce 09209	El Huerfanito, B. C.	EH		Cr2	Cr Ab22013	El Comitán, B. C. S.	COM
	Cce2	Cce 050109	El Huerfanito, B. C.	EH		Cr3	Cr UABC184	Ensenada, B. C.	ENS
	Cce3	Cce 09109	El Huerfanito, B. C.	EH		Cr4	Cr 09012012	San Francisquito, B. C. S.	SF
		ROM-FC 2099 *	(no data collected)				BYU 34753-54 *	Cedros Island	
<i>Crotalus enyo</i>	Ce1	Ce pocitas	Las Pocitas, B. C. S.	POC			ROM 18197-98 *	Riverside, Co., CA.	
	Ce2	Ce2 0211	Cañón de la Zorra, B. C. S.	CZ	<i>Crotalus tigris</i>	Cti1	Cti 1jul2011	Sonoyta, Sonora.	SONO
	Ce3	Ce 3	Cañón de San Dionisio, B. C. S.	CSD			ROM 18167-68 *	Sonora, Mexico	
	Ce5	Ce Ba01	Bahía de Los Angeles, B. C.	BLA	<i>Crotalus transversus</i>		KZ-shed skin*	Mexico, specific locality unknown	
		ROM 13648 *	Baja California Sur		<i>Crotalus triseriatus</i>	1	LG: ROM 18114 *	Llano Grande, Mexico City, Mexico.	
<i>Crotalus durissus</i>		ROM 18138 *	Venezuela			2	Xo: ROM 18120 *	Xochimilco ¹ , Mexico City, Mexico	
<i>Crotalus estebanensis</i>	Ces1	Ces 09022012	San Esteban Island, Sonora	ISE		3	To: ROM 18121 *	Toluca, Mexico	
<i>Crotalus horridus</i>	1	ROM 18132-33 *	New York		<i>Crotalus unicolor</i>		ROM 18150 *	Aruba Island (captive-born)	
	1	UTA R-14697 *	Arkansas		<i>Crotalus vegrandis</i>		ROM 18261 *	Venezuela (purchased from Brazil)	
<i>Crotalus intermedius</i>		ROM-FC 223 *	Veracruz, Mexico		<i>Crotalus viridis</i>		ROM 19656 *	Los Angeles Co., CA.	
<i>Crotalus lepidus klauberi</i>		ROM 18128 *	Chihuahua, Mexico		<i>Crotalus willardi</i>	1	ROM 18183 *	Sonora, Mexico	
<i>Crotalus lorenzoensis</i>	Cl1	Cl 09042012	San Lorenzo Island, B. C.	ISL		2	ROM-FC 363*	Santa Cruz Co., AZ.	
	Cl2	Cl 04012011	San Lorenzo Island, B. C.	ISL		3	HWG 2575 *	Cochise Co., AZ.	
	Cl3	Cl 09032012	San Lorenzo Island, B. C.	ISL	<i>Sistrurus catenatus</i>		ROM-FC 243, 245 *	Ontario, Canada	
	Cl4	Cl 09052012	San Lorenzo Island, B. C.	ISL	<i>Sistrurus miliarius</i>		ROM 18232 *	Florida (commercially purchased)	
<i>Crotalus mitchellii</i>	Cmi1	Cmi 9	Cañón de la Zorra, B. C. S.	CZ	<i>Agkistrodon contortrix</i>		ROM 18230 *	(commercially purchased)	
	Cmi5	Cmi 22jun10	Cañón de la Zorra, B. C. S.	CZ	<i>Agkistrodon piscivorus</i>		ROM-FC 5599 *	(commercially purchased)	
<i>Crotalus molossus</i>	Cmo1	Cmo SJ90411	Sierra Juárez, Chihuahua.	SJ	<i>Gloydus ussuriensis</i>		ROM 20459 *	Jilin Prov. China	

¹ Incorrectly referred to as "Xochomiko" in Murphy et al. (2002) and subsequent citations (e.g. Castoe et al., 2006). * Samples downloaded from GenBank and used by Murphy et al. (2002).



Figure 3. Bayesian 50 % majority consensus tree based on combined fragments of partitioned mitochondrial DNA (12S and 16S), showing the relationships among insular, California peninsula and continental US and Mexico species of *Crotalus*. The numbers below the branches are posterior probabilities.

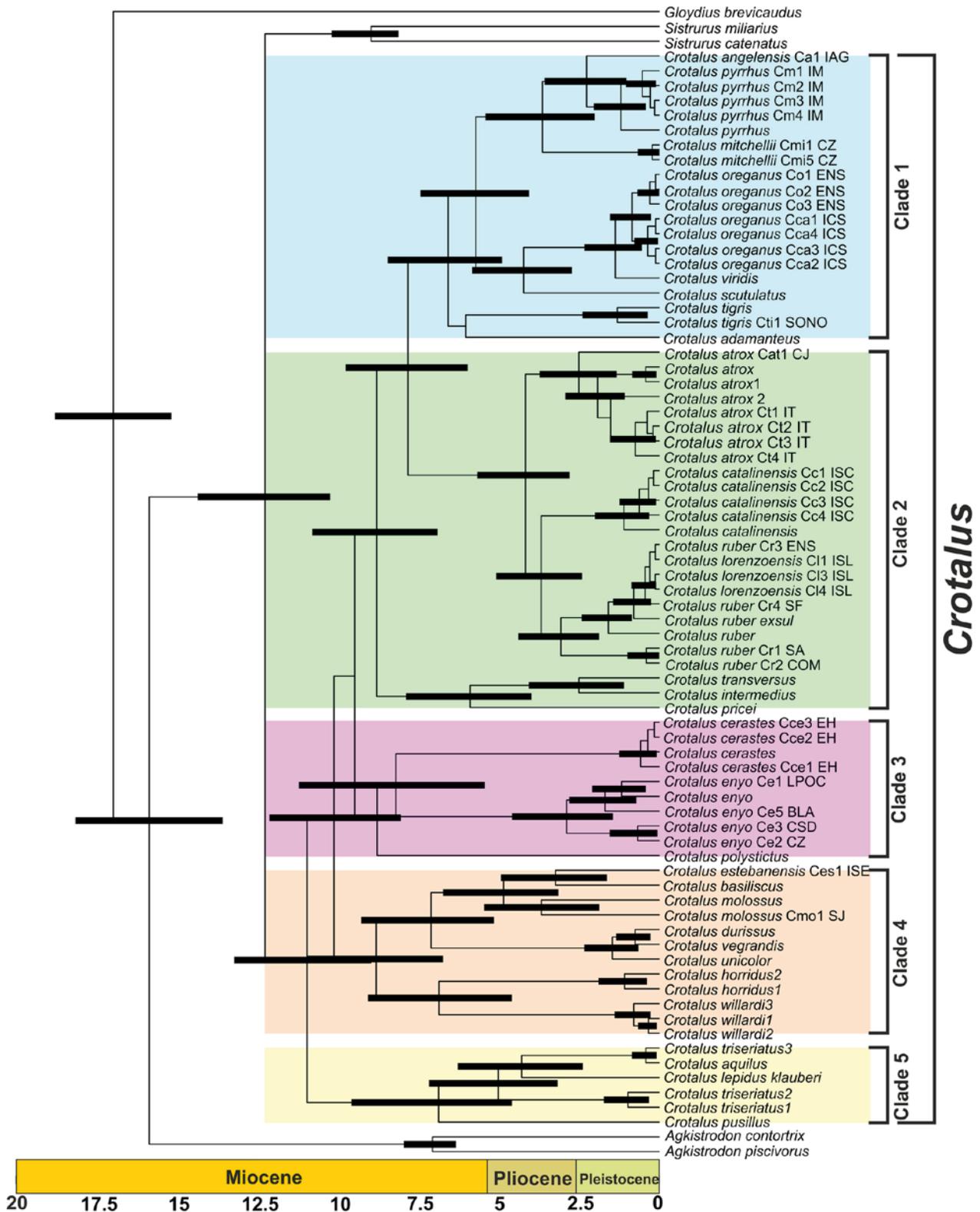


Figure 4. Chronogram based on a Bayesian approach to the *Crotalus* species of the Gulf of California, the California peninsula, and mainland USA and Mexico, from partitioned mitochondrial (12S and 16S) gene markers. Black bars indicate highest posterior density (HPD) intervals at 95 % for node age estimates.

angelensis from Ángel de la Guarda Island (PP = 0.87) and both were sister to two *C. mitchellii* individuals from Baja California Sur (PP = 0.99; Fig. 3). *Crotalus oreganus* from Ensenada formed a sister clade to *Crotalus oreganus* from Coronados Sur Island, and this species was sister to *C. viridis* (PP = 1.0; Fig. 3).

Clade 2 is made up of *C. atrox*, *C. catalinensis*, *C. lorenzoensis*, *C. ruber*, *C. polystictus*, *C. intermedius*, *C. transversus*, and *C. pricei*. All the *C. atrox* individuals from Tortuga Island were nested with the rest of the *C. atrox* individuals (PP = 1; Fig. 3). *C. catalinensis* was found to be a monophyletic species (PP = 1) sister to a clade comprising *C. lorenzoensis* from San Lorenzo Island and *C. ruber*, with low support (PP = 0.77; Fig. 3).

Clade 3 is represented by only two monophyletic sister species: *C. enyo* and *C. cerastes* (PP = 0.95). Clade 4 comprises eight species, with *C. estebanensis* from San Esteban Island sister to *C. basiliscus* (PP = 0.93) and nested in a clade with *C. molossus* (PP = 0.99; Fig. 3). Finally, Clade 5 contains the rest of the species, none of which were collected in this study (Fig. 3).

Divergence time estimates

The BEAST analyses suggest that the split between *Crotalus* and *Sistrurus* occurred 12.26 Ma (95 % High Probability Density (HPD) 12.03–8.1 Ma; Fig. 4). The stem age of *Crotalus* was 10.95 Ma (95 % HPD 13.2–8.95 Ma; Fig. 3). The speciation events occurred from the late Miocene to the Pleistocene (Fig. 4). In clade 1, the divergence between *C. pyrrhus* from El Muerto Island and the *C. pyrrhus* individual from California occurred 1.19 Ma (95 % HPD 2.02–0.41 Ma). *C. angelensis* diverged from *C. pyrrhus* 2.26 Ma (95 % HPD 3.54–1.02 Ma). The two *C. mitchellii* individuals from BCS diverged from the rest of the species 3.63 Ma (95 % HPD 5.39–2.02 Ma; Fig. 4). *C. oreganus* from Ensenada diverged from its congeners on Coronados Sur Island 0.84 Ma (95 % HPD 1.51–0.26 Ma). Finally, the divergence between *C. oreganus* and *C. viridis* occurred 1.36 Ma (95 % HPD 2.31–0.55 Ma; Fig. 4).

In clade 2, the divergence between *C. atrox* (including individuals from Tortuga Island) and *C. catalinensis*, *C. lorenzoensis*, and *C. ruber* occurred 4.16 Ma (95 % HPD 5.64–2.79 Ma). *C. atrox* first arrived on Tortuga Island 1.91 Ma (95 % HPD 2.90–1.07 Ma; Fig. 4). *Crotalus catalinensis* separated from the clade comprising *C. ruber* and *C. lorenzoensis* 3.67 Ma (95 % HPD 5.06–2.40 Ma). One *C. ruber* individual from Ensenada, BC, is nested with *C. lorenzoensis* individuals. The colonisation of San Lorenzo Island by *C. lorenzoensis* took place 0.43 Ma (95 % HPD 0.84–0.10 Ma; Fig. 3). In clade 3, the divergence between *C. cerastes* and *C. enyo* occurred 8.19 Ma (95 % HPD 11.2–5.43 Ma; Fig. 4). Finally, in clade 4, *C. estebanensis* diverged from *C. basiliscus* 3.22 Ma (95 % HPD 4.91–1.63 Ma; Fig. 4).

DISCUSSION

The islands of the Gulf of California are divided into three groups based on their origin: the northern, central midriff, and southern gulf areas (Carreño & Helenes, 2002). However, Case (2002) classified the islands into two groups: the Sonora islands (Tiburón, San Pedro Mártir,

San Esteban, and San Pedro Nolasco), which are closer to mainland Mexico; and the Baja California islands (all other islands), which are closer to the Baja California peninsula. Case (2002) also divided reptiles, including rattlesnakes, into three groups: species distributed in mainland Mexico and Baja California, species restricted to Sonora and adjacent regions, and species restricted to Baja California and the west coast of the USA. In our phylogenetic and molecular dating analyses, we included rattlesnakes with those three geographical affinities (Grismer, 2002).

We recovered the monophyly of the *Crotalus* genus with strong support (PP = 0.99), and this was sister to the *Sistrurus* genus (PP = 1). Previous molecular analyses recovered the same relationship (Parkinson et al., 2002; Murphy et al., 2002; Wüster et al., 2005). The inclusion of three rattlesnake species not previously included in any phylogenetic analysis (*C. angelensis*, *C. estebanensis*, and *C. lorenzoensis*) plus several *C. atrox* individuals from Tortuga Island, *C. mitchellii* from Baja California, *C. pyrrhus* from El Muerto Island, *C. oreganus* from BC and Coronados Sur Island, *C. ruber* from BC, *C. catalinensis* from Santa Catalina Island, and *C. enyo* from BC, yielded new phylogenetic relationships that differed from previous studies (Murphy, 2002).

The novel relationships found in clade 1 involve the phylogenetic position of *Crotalus angelensis*, which was sister to the clade comprising *C. pyrrhus* from El Muerto Island and *C. pyrrhus* from California, USA, and sister to this clade are the *C. mitchellii* individuals from Cañon de la Zorra, BCS. Meik et al. (2018) conducted a phylogenetic analysis focusing on the *C. mitchellii* complex and found sister relationships between the newly described species *C. polisi* and *C. angelensis*, with this clade being sister to *C. pyrrhus*.

In clade 2, we found *C. lorenzoensis* nested, with low support (0.85), in the same clade as *C. ruber* individuals from both BC and BCS (Fig. 3). This novel relationship makes *C. ruber* a paraphyletic species. However, *C. lorenzoensis* could be an isolated population of *C. ruber*. Murphy et al. (2002) included a sample from Cedros Island named *Crotalus "exsul"*, that sample was sister to *C. ruber*. In our phylogenetic results, the *C. "exsul"* resulted sister to the clade formed by *C. lorenzoensis* and *C. ruber* individuals. Cedros Island diamond rattlesnake (*C. exsul*) now is a subspecies of *C. ruber* (*C. ruber exsul*). These results highlight the need to use SNP (single nucleotide polymorphisms) markers and an integrative taxonomy similar to that used by Meik et al. (2018) to resolve the boundaries of the species in this complex group. The four *C. atrox* individuals from Tortuga Island included in this study were nested together but have different haplotypes to the individual previously included in the phylogeny reported by Murphy et al. (2002).

We recovered *C. catalinensis* as sister to the *C. ruber*-*C. lorenzoensis* clade, while Murphy et al. (2002) recovered *C. catalinensis* as sister to *C. ruber*. Grismer (2002) proposed taxonomic relationships between *C. angelensis* and *C. mitchellii* that were also found in our study. Additionally, Grismer (2002) proposed a sister relationship between *C. mitchellii* and *C. cerastes*, but in our phylogenetic reconstruction, the latter is sister to *C. enyo* and this relationship is well supported (Fig. 3).

These findings are consistent with Murphy et al. (2002). The close relationships between *C. atrox*, *C. catalinensis*, *C. lorenzoensis*, and *C. ruber* found here are consistent with the taxonomic relationships proposed by Grismer (2002). *C. tortugensis* was recognised as a different species from *C. atrox* (Grismer, 2002; Murphy et al., 2002). However, Castoe et al. (2007) found that *C. tortugensis* was nested with *C. atrox* individuals from the continent. Our results, which include more *C. atrox* individuals from Tortuga Island, support the findings published by Castoe et al. (2007).

Grismer (2002) proposed relationships between *C. molossus* and *C. estebanensis*. However, we found a novel relationship in that *C. estebanensis* was sister to *C. basiliscus* from Nayarit, Mexico, and nested within the same clade as *C. molossus* (Fig. 3). *Crotalus basiliscus* and *C. molossus* were found to be sister species in the phylogeny reported by Murphy et al. (2002). *Crotalus oreganus* individuals from Ensenada, BC, and individuals from Coronados Sur Island were sister species to *C. viridis* (Fig. 3), in keeping with previous results by Asthon & de Queiroz (2001).

Our phylogenetic results support the hypothesis put forward by Case (2002) with respect to invasion from areas near the islands, in which *C. estebanensis* from San Esteban Island (a Sonora island) is related to species from mainland Mexico (*C. basiliscus* and *C. molossus*). Meanwhile, the remaining island species (*C. catalinensis*, *C. angelensis*, and *C. lorenzoensis*) are related to species distributed in the Baja California peninsula and California, USA. *Crotalus atrox* from Tortuga Island differs from the rest of the island species, because individuals from mainland Mexico (Murphy & Aguirre-Léon, 2002) colonized the island.

Our phylogenetic hypothesis presented here based on two mitochondrial fragments shows a partial evolutionary history of the island endemic (some of them never studied before) and peninsular rattlesnake's species. Recent studies using UCEs (ultraconserved elements) or SNPs in continental, peninsular and island endemic *Crotalus* species (Blair et al., 2018; Meik et al., 2018) have been shown to be useful to resolve species limits in *Crotalus* lineages. Thus, the relationships found here could change with the use of nuclear markers (e.g. UCEs and SNPs).

Divergence time estimates

Vicariance has been the most recurrent hypothesis to explain the phylogeographic patterns of the flora and fauna of the Baja California peninsula. For example, the separation of the Baja California peninsula was an important factor that promoted genetic differentiation in peninsular vertebrates (Riddle et al., 2000a, 2000b; Grismer, 2002; González-Rubio et al., 2016). Another vicariance event evoked is the formation of the seaway at the Isthmus of La Paz (3 Ma; Riddle et al., 2000b). However, little is known about the invasion of the Gulf of California islands and the western part of the Baja California peninsula. Here we report results obtained from the variation of the mitochondrial DNA of rattlesnakes as a proxy to determine the temporality and origin of the invasions of the islands. Our molecular divergence time estimates indicate that the divergence

of *C. angelensis* from *C. pyrrhus* occurred at 2.26 Ma, which is consistent with the separation of the continental Ángel de la Guarda Island from Baja California during the Pleistocene (Carreño & Helenes, 2002). The colonisation of Coronados Sur Island by *C. oreganus* from BC occurred during the Pleistocene. Carreño & Helenes (2002) remarked that this island first separated from BC during the Pliocene-Pleistocene, so if our divergence times are correct, this island may have formed before 0.84 Ma.

The oceanic Tortuga Island was formed during the Pleistocene (Carreño & Helenes, 2002), which is consistent with the colonisation of this island by *C. atrox* (Murphy & Aguirre-Léon, 2002). San Lorenzo Island first separated from BC during the Pliocene (Carreño & Helenes, 2002). However, the divergence of *C. lorenzoensis* from *C. ruber* occurred during the Pleistocene. Santa Catalina Island first separated during the Pliocene (Carreño & Helenes, 2002), which is consistent with the divergence of *C. catalinensis* from *C. ruber*-*C. lorenzoensis*, 3.66 Ma. Finally, *C. estebanensis* diverged from *C. basiliscus* 3.22 Ma. This date is consistent with the origin of San Esteban Island during the Miocene-Pliocene, but not with the first separation during the Pleistocene (Carreño & Helenes, 2002). Although estimates of divergence time may vary depending on the size of the effective population and the fastest coalescence times for the case of mitochondrial genes (Arbogast et al., 2002); Blair et al. (2018) using UCE, found estimations of divergence times similar to mitochondrial genes. Therefore, the addition of nuclear genes in the study of the endemic rattlesnakes of the islands could lead to similar estimates, but with reduced variance (Edwards & Beerli, 2000).

We also found a south-north geographic pattern of separation between *C. mitchellii* individuals from BCS and CA. That separation occurred at 3.66 Ma, during the Pliocene. *C. ruber* individuals from BC also exhibited a geographical separation from individuals from BCS, which occurred 3.05 Ma, during the late Pliocene (Fig. 3). A south-north geographic pattern of separation has been found in other reptile species (Murphy & Aguirre-Léon, 2002). Additionally, an interesting pattern of genetic variation and substructure geographic was found between *C. enyo* individuals from the Cape Region and the rest of the individuals from central BCS. The separation occurred at 2.87 Ma, also during the late Pliocene. This pattern was also found with *Urosaurus nigricaudus* (Murphy & Aguirre-Léon, 2002). These findings require a more precise phylogeographic examination of these last species to determine the causative agents and temporality of the pattern of genetic variation.

CONCLUSIONS

This is the first study that includes all the insular species of rattlesnake in the Gulf of California. Our molecular tree recovered novel phylogenetic relationships, highlighting sister relationships between *Crotalus lorenzoensis* and *C. ruber*, and between *C. estebanensis* and *C. basiliscus*. With the exception of *C. atrox* from Tortuga Island and *C. estebanensis* from San Esteban Island, which are related to mainland species, the rest of the insular species are

related to species from the California peninsula. Our phylogenetic findings support most of the taxonomical relationships proposed by Grismer (2002). The Gulf of California islands were formed between the Pliocene and the Pleistocene. Our molecular divergence time estimates for the different clades in which the insular rattlesnake species are nested indicate that those endemic insular species colonized the islands after their formation, assuming realistic estimates. Finally, further phylogeographic analyses of some rattlesnake species distributed in the California peninsula are needed in order to understand whether those species were affected by climate oscillations in the Pleistocene.

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Observations of threatened Asian box turtles (*Cuora* spp.) on trade in Vietnam

Thong Pham Van¹, Benjamin Leprince¹, Hong Luong Xuan², Quyen Nguyen Thu³, Olivier Le Duc¹, Cedric Bordes¹, Manh Vuong Tien⁴ & Luca Luiselli^{5,6,7}

¹Turtle Sanctuary and Conservation Center, 19 rue Béranger 75003 Paris, France

²Hanoi Wildlife Rescue Center, Tien Duoc, Soc Son district, Hanoi, Vietnam

³Thai Nguyen University of Agriculture and Forestry, Quyet Thang commune, Thai Nguyen city, Thai Nguyen province, Vietnam

⁴CITES Vietnam, B9 building, 2 Ngoc Ha street, Ba Dinh district, Hanoi, Vietnam

⁵Institute for Development, Ecology, Conservation and Cooperation, via G. Tomasi di Lampedusa 33 - 00144 Rome, Italy

⁶Department of Applied and Environmental Biology, Rivers State University of Science and Technology, P.M.B. 5080, Port Harcourt, Nigeria

⁷Département de Zoologie and Animal Biology, Faculty of Sciences, University of Lomé, B.P. 1515, Lomé, Togo

Asian box turtles (genus *Cuora*; Geoemydidae) are among the most threatened Asian turtles. Here, we present (i) a field investigation from 2014-2018 on the *Cuora* individuals traded by high-level traders in six provinces of Vietnam and (ii) the estimated numbers of traded individuals determined through interviews with traders. In addition, we (iii) provide an analysis of the confiscated individuals that were carried out at the main wildlife rescue centre of the Socialist Republic of Vietnam. Overall, the number of traded individuals observed was relatively small ($n = 481$), with *C. bourreti* being by far the most frequently observed in trade. During the same period, the interviewed traders estimated that about 3,400 individuals were traded through the period 2014-2018, most of them being *C. bourreti*. During 2010-2018, a total of 281 individuals were carried to the wildlife rescue centre, with over 60 % of the total rescued individuals being *C. galbinifrons*. Overall, the numbers of traded *Cuora* individuals recorded in the present study were very small compared to the huge estimates from other Asian countries. However, this lower traded number does not imply that the status of the target species are not affected, as their population sizes are already catastrophically reduced in Vietnam.

Keywords: turtle trade, Vietnam, interviews, field surveys

INTRODUCTION

Asian box turtles (genus *Cuora*; Geoemydidae) number 13 known species worldwide (Turtle Taxonomy Working Group, 2017). Some *Cuora* species are overexploited (Fig. 1) (e.g. Stuart, 2004; Schoppe, 2009; Nijman, 2010) and presumably in heavy decline (Stanford et al., 2018), are therefore considered as Critically Endangered by the IUCN (2018). Six *Cuora* species are native to Vietnam, with another species (*C. zhoui*) still in doubt (Turtle Taxonomy Working Group, 2017). Of these, *Cuora galbinifrons* was originally described by Bourret (1941) from a specimen collected from Vietnam. Based on its distinctive morphological features, this species was then separated into three subspecies (Obst & Reimann, 1994), that were subsequently even considered as distinct species by most of the authors: *Cuora galbinifrons*, *C. bourreti*, and *C. picturata* (Stuart & Parham, 2004).

Of these three species, *C. galbinifrons* is known from southern China, and the northern and central parts (southernmost are Quang Binh province) of Vietnam and Laos (Fritz et al., 2002; McCormack et al., 2016), while *C. bourreti* is found in Quang Binh Province to Kon Tum

province and southern Laos (McCormack & Stuart, 2016; Stuart et al., 2011). *C. picturata* is endemic to Binh Dinh, Khanh Hoa and Phu Yen provinces, in central Vietnam (Ly et al., 2011; Duong et al., 2014). Thus, apparently the three species exhibit a continuously allopatric or contiguously parapatric distribution in Vietnam: *C. galbinifrons* in the northern provinces, *C. bourreti* in the central provinces, and *C. picturata* in the southern provinces of Vietnam (IUCN, 2018). There is still doubt concerning the coexistence between *C. bourreti* and *C. picturata* which, apparently, could coexist in Gia Lai province (McCormack & Stuart, 2016), however species delimitations are often difficult in this turtle genus due to a great propensity for natural and human-induced hybridisation (Spinks et al., 2012). Interestingly, the sympatric occurrence of *C. galbinifrons* and *C. bourreti* was instead confirmed in Phong Nha Ke Bang National Park, Quang Binh province (Fritz et al., 2002). Overall, Vietnam is one of the most important countries in east Asia as for the conservation prospects of these declining species.

The preferred habitat of the three above-mentioned *Cuora* species is montane moist forest (Fig. 1 and see, e.g.

Correspondence: Luca Luiselli (l.luiselli@ideccngo.org)



Figure 1. Individuals of *C. bourreti* being traded at Quang Nam province (photo a), *C. bourreti* and a few *C. galbinifrons* at Da Nang city (photo b), *C. galbinifrons* (with also a few individuals of *C. mouhotii*) for sale in Quang Binh province in north Vietnam (photo c), and *C. galbinifrons* transferred to Hanoi Wildlife Rescue Center, Tien Duoc, Soc Son district in 2018 after confiscation from illegal wildlife trade (photo d). This plate also shows the general view of the habitat of *C. bourreti* in Bach Ma National Park (photo e) and *C. galbinifrons* at Phong Nha-Ke Bang National Park (photo f). Photo credit: (a), (b), (d), (e) Thong Pham Van; (c), (f) Benjamin Leprince.

Ly et al., 2013) at an altitudinal range of 500-1000 m a.s.l for *C. galbinifrons* (Blanck, 2013), slightly lower (300-700 m a.s.l.) for *C. bourreti* (Stuart et al., 2011; McCormack & Stuart, 2016) and from 346 to 561 m a.s.l. for *C. picturata* (Ly et al., 2011; McCormack et al., 2016). Thus, the protection of the rapidly disappearing mountain forest patches may represent the most important conservation action for securing the future of these threatened species in Vietnam. Other than habitat loss, the decline of Vietnamese *Cuora* species has also been driven by intensive exploitation since the 1990s to supply the Chinese food markets (Hendrie, 2000). In addition, all *Cuora* species were also traded for pet markets (Pham et al., 2019) and the Chinese traditional medicine markets (McCormack & Stuart, 2016).

Overall, there was an indirectly estimated population collapse of over 90 % during the past 60 years due to over exploitation and habitat loss (IUCN, 2018), with *C. galbinifrons* being now listed among the 25 most threatened turtle species of the world and *C. bourreti* and *C. picturata* among the top 50 (Stanford et al., 2018). Nonetheless, no direct field demographic data from any

population studies are available to confirm this presumed population collapse.

Here, we present quantitative data from (i) field investigations on individuals of three of *Cuora* species traded by high-level traders in six provinces of Vietnam, and (ii) on the estimated numbers of individuals on the basis of interviews with the same high-level traders. In addition, we (iii) provide data on the numbers of confiscated individuals that were carried out at the main wildlife rescue centre of the Socialist Republic of Vietnam (Hanoi Wildlife Rescue Center, Tien Duoc, Soc Son district) in 2010-2018.

METHODS

The field study was carried out in six big cities and provinces of Vietnam, including Da Nang, Nha Trang, Hanoi, Ho Chi Minh, Buon Ma Thuat and Quang Nam (Fig. 2). The survey was conducted in August to September 2014, April, July and December 2016, April 2017, January, March and September 2018. Each survey trip lasted for 10 to 20 days.

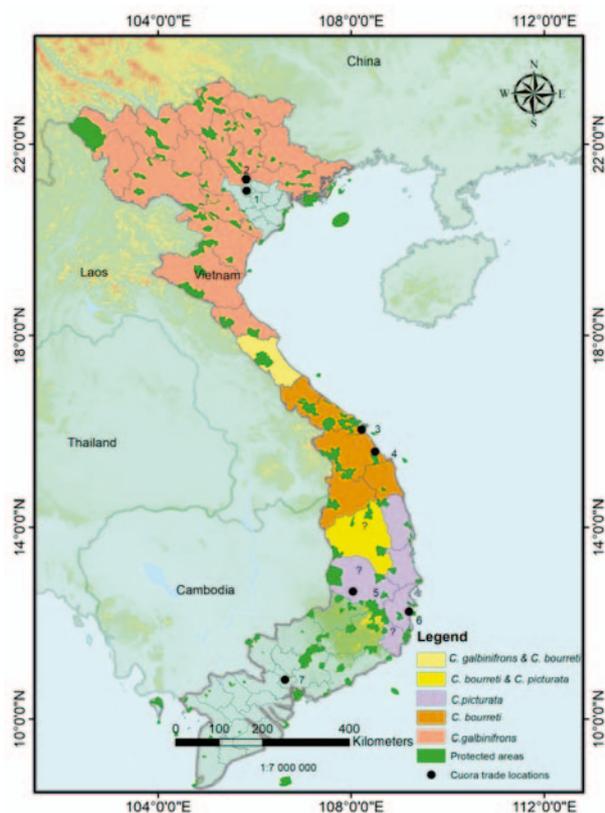


Figure 2. Map of Vietnam showing the study areas used for the field surveys with 1= Hanoi city, 2= Hanoi Wildlife Rescue Center, 3 = Danang city, 4 = Tam Ky city, Quang Nam province, 5 = Buon Ma Thuat city, Dak Lak province, 6 = Nha Trang city, Khanh Hoa province, 7 = Ho Chi Minh city. The map also shows the *Cuora* spp. distribution in Vietnam. Question marks (?) indicate areas where the species' presence is still unconfirmed

The field investigation, explicitly authorised by the CITES Vietnam and Soc Son Wildlife Rescue Center (Hanoi), was based on surveys of turtles in the hands of illegal traders actively involved in the pet market in Vietnam. We met these traders after contacting them through Facebook (Pham et al., 2019). Two of the co-authors (Leprince & Pham) conducted the interviews with each of the illegal traders in Hanoi from 2014. When possible, we examined the animals that they had for sale and also escorted them in the field. Each time we accompanied the trader in the field, we also examined the turtle individuals collected by other traders in their network and temporarily stored for sale in their private houses. Overall, we surveyed the turtle individuals in the hands of six traders of high level (level 4) out of about 20 operating at the country scale. According to Pham (2018), in each commune of Vietnam there are at least 1 to 2 small wildlife traders (level 1), each district has at least also 1-2 bigger traders (level 2), each province has at least 1-2 traders (level 3), and each region has usually one big trader (level 4), who collects all turtle individuals from lower-level traders. Thus, our survey was well representative of the volume of turtles on trade in the explored regions of Vietnam throughout the study period. When inside the traders' houses, we asked which species they keep and how many individuals. We took photos

of the various turtle individuals if the trader allowed us to do so. We also identified each individual to species level, and counted the number of traded individuals, also recording their body size (carapace length). If the trader did not allow us to take photos, we recorded all the other data as mentioned above. We also asked to the interviewees the following information: (i) where did each turtle individual come from? (ii) what was the price for each turtle individual for sale? (iii) where will they sell the turtle? (iv) what type of means do they use to transport turtles to customers? (v) in which months do they obtain the largest quantity of that given turtle species? (vi) how many individuals of that given species do they sell per year in the region? For the number of turtles traded per year, they were also asked to include the numbers traded by other traders of their province working under them.

We also accessed the database of the Soc Son Wildlife Rescue Center (Hanoi) to see how many *Cuora* ssp. individuals were transferred to the rescue centre when confiscated by the police from illegal trading. This rescue centre is the main governmental rescue centre of Vietnam, with all protected wildlife species being potentially housed in the centre when confiscated from the trade. In the period of survey, the centre housed such species as tigers, Malayan bears, king cobras, and turtles. The quantitative data presented here were obtained by the head of the rescue centre during the period 2010 to 2018. Differences between frequencies of observed individuals by species, as well as of estimated individuals by species, were evaluated by χ^2 test. Past software 3.0 version was used for performing any statistical tests.

RESULTS

Overall, we observed 481 individuals of *Cuora* ssp. on trade in Vietnam during the study period (Table 1). The numbers of observed individuals differed significantly among species ($\chi^2= 270.2$, $df = 2$, $P < 0.0001$), with *C. bourreti* being far more frequently observed in trade than the other two species. *Cuora bourreti* also dominated the estimated numbers of traded specimens according to the performed interviews ($\chi^2= 2219$, $df = 2$, $P < 0.0001$) (Table 1).

Interview data presented in this paper were obtained from six high-level traders (level 4) who operated in six provinces and cities. We found that turtle individuals came essentially from hunters living nearby the nature reserves (see also Le, 2007), and were then sold to local traders from districts and communes going to collect them directly in the hunters' villages. For example, the level 4 trader in Da Nang claimed that he normally orders *C. bourreti* individuals from the local traders (level 1 and level 2) from different sources such as protected areas: Song Thanh Nature Reserve (Quang Nam province), Sao La Nature Reserve, and Bach Ma National Park (Thua Thien Hue Province). Additionally, he even claimed that he ordered turtle individuals from Laos, and then illegally imported them to Vietnam through border gates in Nam Giang district. The same level 4 trader claimed that he usually orders *C. galbinifrons* individuals from traders of levels 3 and 4 in Quang Tri and Ha Tinh areas. The same

Table 1. Synopsis of the observations concerning the number of *Cuora* ssp. individuals on trade in Vietnam during the present study. In this table, both the number of individuals directly observed by us on trade and those estimated (per year) by the hunters/sellers during semi-structured interviews are presented, as well as their origin (province) according to the interviews with traders.

Date	Observation locations	Species	No. observed	No. adults + subad.	No. hatchlings + juv.	Estimated No. Turtles per yr	Source of turtle from provinces of Vietnam and Laos
11-Aug-14	Hanoi	<i>Cuora bourreti</i>	8	8	0	300	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
2-Sep-14	Da Nang city	<i>C. bourreti</i>	41	41	0	500	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
22-Jul-16	Buon Ma Thuot	<i>C. bourreti</i>	23	19	4	60	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
20-Dec-16	Da Nang city	<i>C. bourreti</i>	37	29	8	0	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
17-Dec-16	Da Nang city	<i>C. bourreti</i>	100	85	15	1000	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
17-Dec-16	Da Nang city	<i>C. bourreti</i>	5	5	0	0	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
19-Dec-16	Buon Ma Thuot	<i>C. bourreti</i>	53	53	0	30	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
15-Apr-16	Da Nang city	<i>C. bourreti</i>	34	34	0	500	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
15-Apr-17	Quang Nam Province	<i>C. bourreti</i>	6	5	1	50	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
20-Apr-17	Da Nang city	<i>C. bourreti</i>	22	22	0	0	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
20-Apr-17	Da Nang city	<i>C. galbinifrons</i>	24	24	0	400	Quang Binh, Ha Tinh, Nghe An, Thanh Hoa
9-Jan-18	buon Ma Thuot	<i>C. galbinifrons</i>	13	13	0	0	Quang Binh, Ha Tinh
9-Jan-18	Da Nang city	<i>C. galbinifrons</i>	16	16	0	0	Quang Binh, Ha Tinh, Nghe An, Thanh Hoa
9-Jan-18	Da Nang city	<i>C. galbinifrons</i>	41	41	0	200	Quang Binh, Ha Tinh, Laos, Nghe An, Thanh Hoa
25-Mar-18	Hanoi	<i>C. picturata</i>	2	2	0	100	Phu yen & Khanh Hoa
25-Mar-18	Ho Chi Minh	<i>C. picturata</i>	2	2	0	200	Phu yen & Khanh Hoa
25-Mar-18	Buon Ma Thuot	<i>C. picturata</i>	26	24	2	26	Kon Tum, Gia Lai, Quang Nam, Quang Ngai, Hue, Quang Binh, Quang Tri
24-Sep-18	Nha Trang	<i>C. picturata</i>	11	8	3	50	Phu yen & Khanh Hoa
24-Sep-18	buon Ma Thuot	<i>C. picturata</i>	17	16	1	17	Phu yen & Khanh Hoa
		TOTAL	481	447	34	3433	

information was also independently provided by the interviewed level 4 trader in Nha Trang city (Khanh Hoa province), thus confirming its reliability. Most individuals were sold in cities such as Da Nang, Quang Tri, and Hanoi (Table 1) before heading to China; and many individuals are also sold via Facebook (Pham et al., 2019).

All six level 4 traders agreed that turtles were collected by hunters from February to August in each year, thus indicating that this is the period of highest above-ground activity of these reptiles. These data were in good agreement with interview data from Espenshade III & Thien Duc (2000), which reported a main hunting

season between April and September. According to the traders, turtles were usually hunted by dogs. The price offered by traders for each turtle individual varied among years, sites and species. However, the average price of *C. galbinifrons*, *C. bourreti*, *C. picturata* were, respectively, US\$159.09, US\$134.09 and US\$209.09. According to the traders, the transportation of turtles from villages to cities is mainly made by bus. Turtles are also routinely exported to China by bus, car, and train, and generally illegally pass the political border with China via the provinces of Lang Son, Quang Ninh, and Lao Cai. In the Soc Son Rescue Center, a total of 281 individuals

were observed during 2010–2018 (Table 2). Well over 60 % of the total rescued individuals were *C. galbinifrons*, in contrast with the fact that *C. bourreti* dominated the traded sample in our field surveys.

Table 2. Numbers of *Cuora* individuals confiscated and transferred to the Soc Son Rescue Center, Hanoi, Vietnam, during the period 2010–2018.

Date	Species	No. of individuals	Source
31-Jul-14	<i>C. bourreti</i>	1	FPD Hanoi
24-Oct-14	<i>C. bourreti</i>	1	FPD Hanoi
25-Jul-15	<i>C. bourreti</i> & <i>C. galbinifrons</i>	8	FPD Hanoi
31-Jul-15	<i>C. bourreti</i> & <i>C. galbinifrons</i>	110	FPD Hanoi
13-Jan-10	<i>C. galbinifrons</i>	24	FPD Quang Ninh province
19-Oct-12	<i>C. galbinifrons</i>	18	FPD Lang Son province
30-Jul-14	<i>C. galbinifrons</i>	55	FPD Quang Ninh province
03-Aug-14	<i>C. galbinifrons</i>	1	FPD Quang Ninh province
07-Jan-16	<i>C. galbinifrons</i>	3	Hanoi police
22-Jun-16	<i>C. galbinifrons</i>	2	Hanoi police
15-Sep-16	<i>C. galbinifrons</i>	2	Hanoi police
16-Sep-16	<i>C. galbinifrons</i>	1	Hanoi police
15-Oct-16	<i>C. galbinifrons</i>	5	Hanoi police
27-Nov-17	<i>C. galbinifrons</i>	2	Hanoi police
09-Aug-18	<i>C. galbinifrons</i>	48	Vinh Phuc police
	TOTAL	281	

DISCUSSION

Our surveys revealed a clear difference between field surveys (with *C. bourreti* being by far the most intensely traded species) and the Soc Son Rescue Center surveys (with *C. galbinifrons* dominating the samples). We think that the field surveys do reflect the real pattern, i.e. that *C. bourreti* is by far the most intensely traded *Cuora* species in Vietnam, as it is probably also the commonest in the wild (Pham et al., unpublished observations). The preponderance of *C. galbinifrons* in the rescue centre likely reflects the current law enforcement in Vietnam, with only *C. galbinifrons* being fully protected (by national law Decree 160/2013/NĐ-CP). Thus, most of *Cuora* official rescue data were relative to this species because *C. galbinifrons* is the only *Cuora* species that can routinely be confiscated from traders/owners by police authorities. Similar patterns have also been observed in China, where local families very frequently raise captive *Cuora trifasciata* in Nanning City, but where an increased level of enforcement has led (i) to a decrease in the wild-caught individuals trade and (ii) to many captive-bred turtles also being confiscated (Shi et al., 2004).

Overall, the numbers of traded individuals recorded in this study were very small compared to the huge estimates on *Cuora* species traded in other Asian countries. Schoppe (2009), for instance, estimated that

about 2 million individuals were exported annually with an official quota amounted to only 18,000 individuals, thus showing that the majority of turtles were exported undeclared. However, Schoppe's (2009) data relate to *Cuora amboinensis*, and not to the Vietnamese species of *Cuora* studied herein. Concerning Vietnam, the number of *Cuora* officially traded according to the UNEP-WCMC CITES Trade Database was 2,377 live animals, including 17 *C. galbinifrons* individuals from 1984–2014. In 2000–2003, the number of *C. galbinifrons* recorded in trade in Hong Kong was 15,000 individuals (Cheung & Dudgeon, 2006), while during the same period, CITES only recorded 73 individuals traded around the world. A market survey by the Wildlife Conservation Society recorded 1,826 animals observed in food markets, and another 1,944 animals recorded in the local pet trade (years 2008–2011) in Guangzhou, China (S. Robertson, in litt. to Vietnam CITES Management Authority), with these individuals supplying the pet trade market globally and the Chinese traditional medicine market (McCormack & Stuart, 2016). Data for Vietnam are still uncertain, but in 1999, an estimated 150 *C. galbinifrons* were among specimens seized from a truck travelling from Central Vietnam to Hanoi (Hendrie, 2000). In general, our results would suggest that the current level of illegal trade for *Cuora* species is relatively modest in Vietnam (less than 500 individuals observed in four years, with an estimated 3,400+ individuals at the same sites according to the traders' interviews), at least in comparison with data provided from elsewhere (Schoppe, 2009). However, the relatively low number of traded turtles in this study does not imply that this reduced trade is not affecting the status of the target species, as their population size is already catastrophically reduced in Vietnam (IUCN, 2018; Stanford et al., 2018).

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Urinary corticosterone metabolite responses to capture and visual elastomer tagging in the Asian toad (*Duttaphrynus melanostictus*)

Edward J. Narayan¹ & Narahari P. Gramapurohit²

¹ School of Science and Health, Western Sydney University, Hawkesbury Campus, Penrith, NSW 2750, Australia

² Department of Zoology, Savitribai Phule Pune University, Ganeshkhind, Pune 411 007, India

Both authors contributed equally to this work

Herpetological research involving amphibians is increasingly using mark and recapture methods, employing various techniques such as toe-clipping and visible implant alphanumeric tags. Visual Implant Elastomer (VIE) is a new method available for herpetological surveys, involving a coloured dye inserted into the epidermal skin surface of frogs. Previously, there has been only one study which demonstrated that the VIE method does not generate a significant physiological stress response (using a faecal glucocorticoid method) in a captive amphibian species. Physiological stress can also be quantified non-invasively using urinary corticosterone metabolite (UCM) enzyme-immunoassay in amphibians. In this study, we tested the physiological stress response of a common amphibian species, wild caught Asian toads (*Duttaphrynus melanostictus*), by comparing UCM responses to capture handling, sham control or VIE marking method. Adult males (n = 38) were captured and sampled for baseline UCM (t = 0 h) then marked either using the VIE or sham (saline control), or only handled during capture. Subsequently, urine samples were collected at t = 2, 12 and 24 h for toads within each group. UCM levels were quantified using an enzyme-immunoassay (EIA) to determine differences among treatment groups and over time following capture. Toads generated acute stress responses to all three groups, showing a change in UCMs between baseline samples, 12 h, and 24 h samples. The mean UCM levels were not significantly different between the VIE method and the control groups (capture handling or sham operated). These results indicate that VIE method of tagging is no more stressful than routine handling of amphibians, hence in this context, the method does not have any additional welfare implications. Future research should explore the limitations of VIE tagging for long-term mark recapture studies, however, our current findings support its application as a minimally-invasive method for marking amphibians.

Keywords: amphibians, mark-recapture, welfare, stress, Visual Implant Elastomer (VIE)

INTRODUCTION

The physiological stress response involves activation of the hypothalamo-pituitary-adrenal-axis (HPA; Cockrem, 2007) (HPI-axis in amphibians; Narayan et al., 2013) and glucocorticoid secretion, which enables animals to cope with stressful situations through metabolic, physiological, and behavioural responses (Warne et al., 2011). Over-production of glucocorticoids can cause negative consequences on the ecological fitness of animals (Coddington & Cree, 1995). Recently, non-invasive hormone monitoring techniques, such as faecal and urinary based methods, are readily available and allow an alternative method of determining physiological stress in amphibians (Narayan et al., 2011). Urinary corticosterone metabolite (UCM) assessment allows non-invasive monitoring of the stress response from a physiological perspective (Narayan et al., 2011).

Mark-recapture is routinely used in herpetological

research for assessing population numbers, trends, and dynamics (Moore et al., 2010). Traditionally, toe-clipping (the removal of one or more toes; Schmidt & Schwarzkopf, 2010) has been used for amphibian mark-recapture studies (Halliday & Verrell, 1988). However, toe-clipping physically alters an animal's body, and elicits an unwarranted physiological stress response lasting several hours (Narayan et al., 2011). Whilst the toe-clipping method has historically been widely used in amphibian studies, the suitability and potentially detrimental effects of the method are a subject of debate. For example, toe-clipping is believed to affect foraging and locomotion and could also reduce body-weight (Schmidt & Schwarzkopf, 2010). Various alternative techniques are now available that are cheaper and easily accessible, such as visual implant elastomer (VIE) tags (Woods & James, 2003), passive integrated transponder (PIT) tags (Jørgensen et al., 2017), and visible implant alphanumeric (VIA) tags (Chan et al., 2006).

Correspondence: Edward Narayan (enarayan@csu.edu.au); Narahari Gramapurohit (naraharipg@unipune.ac.in)

VIE tags are small, flexible and bio-compatible (Northwest Marine Technology, Inc., 2018). The VIE tag is 'injected' as a liquid that cures into a pliable solid. The VIE tags are injected sub-cutaneous and remain externally visible. Unlike the method of toe-clipping to identify focal animals in the field, this technology is likely to be more humane. VIE tagging has been successfully used for individual marking in many vertebrate species such as salamanders, reptiles and fish as well as crustaceans (Davis & Ovaska, 2001; Marold, 2001; Woods & James, 2003; Curtis, 2006; Grant, 2008; Waudby & Petit, 2011). Furthermore, VIE is being used more commonly in the field for the marking of amphibians (Anholt et al., 1998; Nauwelaerts et al., 2000). Recently, Antwis et al. (2014) investigated the potential effects of VIE marking on physiological stress responses in the red-eyed tree frog (*Agalychnis callidryas*). The authors measured adrenal responses using faecal glucocorticoid metabolite concentrations. The results showed no effects of VIE method on adrenal response in the frog.

The aim of the current study was to determine whether VIE is a physical stressor that can elicit a stress response in a tropical amphibian. We examined the physiological stress by determining the changes in UCM levels of adult male *Duttaphrynus melanostictus* subjected to a standard short-term handling protocol, sham control or VIE tagging. We hypothesised that the VIE tagging would elicit a significant stress response in toads compared with the other groups. Thus, the results would verify the suitability of VIE tagging method for marking of common Asian toads.

METHODS

Ethics statement

Sampling was conducted on the S.P. Pune University Campus Maharashtra, India (18°55' N and 73°82' E). Verbal permission was obtained through the security office within the office of the Registrar, which provided unrestricted access to the study site on the campus. As NPG is a faculty of the S. P. Pune University, specific written permission and access to the study site was not required. The study did not involve any endangered or protected species. Moreover, no animals were sacrificed in this study and underwent minimal handling. All the toads were released at the site of capture after urine sampling. The study was carried out following the guidelines of the departmental committee for animal ethics.

Field methods

We sampled a natural sub-population of *D. melanostictus* on the S. P. Pune University campus during the breeding season (late July and early August, 2013). The weather conditions were moist with some light rain on the sampling nights. Toads were generally found in open grounds, gardens and crevices near buildings and construction sites on the campus. Sampling was conducted between 1900–2100 h.

The toads were located randomly and captured ($n = 38$ male toads, identified by the presence of nuptial pads and reddish throats) using torches to identify eye shine. Females were not used because of limited

sample size. Urine was collected immediately (within 2 min) from each toad. Each urine sample was collected via normal capillary action by gently inserting a sterile 200 μ L pipette tip (2 mm length) into the toads' cloaca. Generally, the toads urinated immediately upon capture, although occasionally gentle massaging of the underbelly abdomen was required to stimulate urination. Only uncontaminated toad urine samples were used, with possible contamination risks including faeces and rain water. Urine samples (sample volume ranging from 0.5 mL to 3 mL) were aliquoted into labelled, sterile 1.5 mL polypropylene Eppendorf tubes and kept on cold ice prior to transfer into the laboratory freezer. We refer to this first urine sample (time = 2 min) as the baseline sample.

Subsequently, toads were allocated into three experimental groups; (1) capture handling only; (2) sham control or (3) VIE tag. The toads in group (1) were placed in a clean, labelled Zip-lock® bag with tiny holes made for ventilation. Toads in group (2) were given a single intra-peritoneal injection of 100 μ L saline solution using a 1 mL syringe and 29-gauge needle and immediately returned to their labelled bags. Toads in group (3) were tagged by injection of VIE (Northwest Marine Technologies, Inc.) under the skin with an insulin syringe. The method was previously described by Brannelly et al. (2013). Briefly, tags were applied using 2–3 mm injections halfway between the knee and the pelvis on one hind limb. Combinations of three fluorescent silicon bead colours (pink, yellow and green) were used to uniquely tag individual toads. The locations of the tags, as identified using a handheld UV light, were recorded upon injection and again 24 h post-injection.

All toads were transported to the laboratory within 20 min of field procedures. In the laboratory, all toads were re-sampled (as described in the 'Field methods' section) for urine at 2, 12 and 24 h after field capture. All the toads were released at the site of capture after the final urine sampling.

Urinary corticosterone metabolite (UCM) enzyme-immunoassays

The enzyme-immunoassay (EIA) used was originally validated in our earlier research work (Narayan & Gramapurohit, 2016). Briefly, the antibodies used in this study were polyclonal, and the corticosterone antiserum (CJM006) and the conjugated horseradish peroxidase (HRP) label were standardised for a standard direct competitive EIA system (Munro & Stabenfeldt, 1984; Munro, 1985). Recovery of corticosterone standard was 89 %. The coefficients of variation for intra-run and inter-run assays were 3.2 % and 7.2 % respectively. Assay sensitivity was 0.55 pg/well.

Concentrations of UCM were determined using a polyclonal anticorticosterone antiserum (CJM006) diluted 1: 45 000, horseradish peroxidase conjugated CORT label diluted 1: 120 000 and CORT standards (1.56–400 pg well⁻¹). Cross reactivity of the antiserum was 100 % with CORT, 14.25 % with desoxycorticosterone and 0.9 % with tetrahydrocorticosterone (Munro, 1985). Samples were assayed on NuncMaxiSorp™ 96 well plates. For each assay, the plates were coated with 50 μ L of the antibody

diluted to an appropriate concentration in a coating buffer (50 mmol L⁻¹ bicarbonate buffer, pH 9.6) and incubated at 4 °C for 12 h. Plates were then washed thoroughly using phosphate-buffered saline containing 0.5 ml L⁻¹ Tween 20 to rinse away any unbound antibody. Stocks of standards, high- and low-binding internal controls, urine samples, and horseradish peroxidase labels were diluted to an appropriate concentration in assay buffer (39mM NaH₂PO₄·H₂O, 61mM NaHPO₄, 15mM NaCl and 0.1 % bovine serum albumin, pH 7.0). For each EIA, 50 µL of standard, internal control, and urine sample were added to each well, followed by the addition of 50 µL horseradish peroxidase label. The plates were incubated at ambient temperature for 2 h. Plates were then washed and 50 µL of a substrate buffer (0.01 % tetramethylbenzidine and 0.004 % H₂O₂ in 0.1 M acetate citrate acid buffer, pH 6.0) was added to each well. The reaction was stopped using 0.2 M H₂SO₄ and the optical density (OD) was read at 450 nm using an ELISA plate reader (Thermo Scientific Multiskan SK, Ascent software-version 2.6). The UCM concentrations were normalised to creatinine levels to control for water content using the methods described previously (Narayan et al., 2010). UCM levels were expressed as pg/µg Creatinine (pg/µg Cr).

Statistical analysis

All statistical analyses were carried out using Prism GraphPad (version 5.0). All the data was tested for normality using D'Agostino and Pearson omnibus normality tests before subjecting to statistical analyses. All the tests were two tailed and the significance level was set at 0.05 Differences in the mean UCM levels among groups and time-periods (0, 2, 12 h and 24 hrs) were analysed using repeated measures ANOVA, with time as a fixed factor, followed by Dunn's multiple pairwise comparison test.

RESULTS

The mean baseline UCM levels of the toads tagged with VIE was 32.07 ± 4.64 pg/µg Cr (range 28.42 – 41.50 pg/µg Cr). The sham operated group displayed a UCM range of 36.78 - 65.72 UCM pg/ug Cr and a mean baseline UCM level of 36.78 + 12.63 pg/µg Cr. The capture and handling control groups displayed a UCM range of 42.17 - 64.83 UCM pg/ug Cr, and a mean baseline UCM of 42.97 + 5.09 pg/ug Cr (Fig. 1).

Individual male toads showed variability in their UCM responses to the treatments (Fig. 1). Coefficient of variation (CV) in UCM levels for each of the treatment groups ranged as follows; VIE group (28-41 %), sham operated group (82-103 %) and capture-handling control group (45-90 %).

Overall, UCM concentrations varied significantly by time-period ($F_{3,96} = 5.69$, $P = 0.0012$) and by individual within each group ($F_{32,96} = 5.924$, $P < 0.0001$). However, they did not differ among treatment groups ($F_{2,96} = 1.22$, $P = 0.3000$).

Within the VIE tagged group, UCMs were significantly elevated at 12 h post capture ($P = 0.0065$) for comparisons between mean UCMs at time-periods 0 c.f. 12 hrs; 12 c.f.

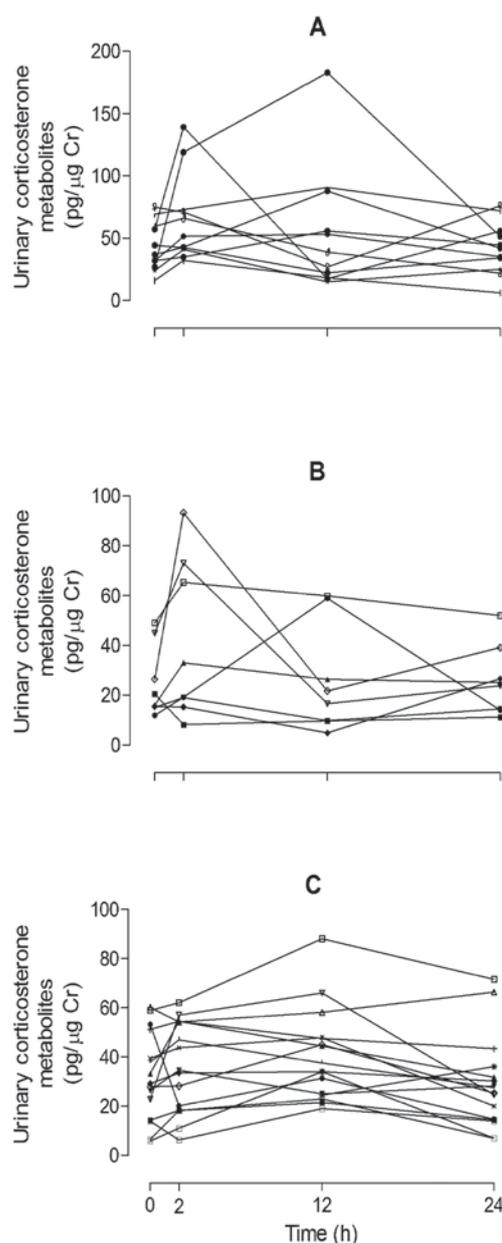


Figure 1. Individual urinary corticosterone metabolites in male Asian toads subjected to **(A)** capture handling control (n= 11), **(B)** sham (saline) control (n= 9), or **(C)** VIE method (n=16) at various time periods (0 h, 2 h, 12 h, 24 h) post-capture

24 h), and had returned to baseline concentrations by 24 h post-capture ($p > 0.05$ for comparisons between mean UCMs at time periods 0 c.f. 2 h; 0 c.f. 24 h; 2 c.f. 12 h; 2 c.f. 24 h; Fig. 2).

For the sham operated and capture handling only groups, the mean UCMs levels were comparable (neither increased or decreased) between any of the time periods (0, 2, 12 or 24 h; $p > 0.05$; Fig. 2).

DISCUSSION

Capture handling of wildlife in herpetological research is unavoidable as it enables the collection of crucial field data relating to the life-history and ecology of study species. As such, it is important that we understand that

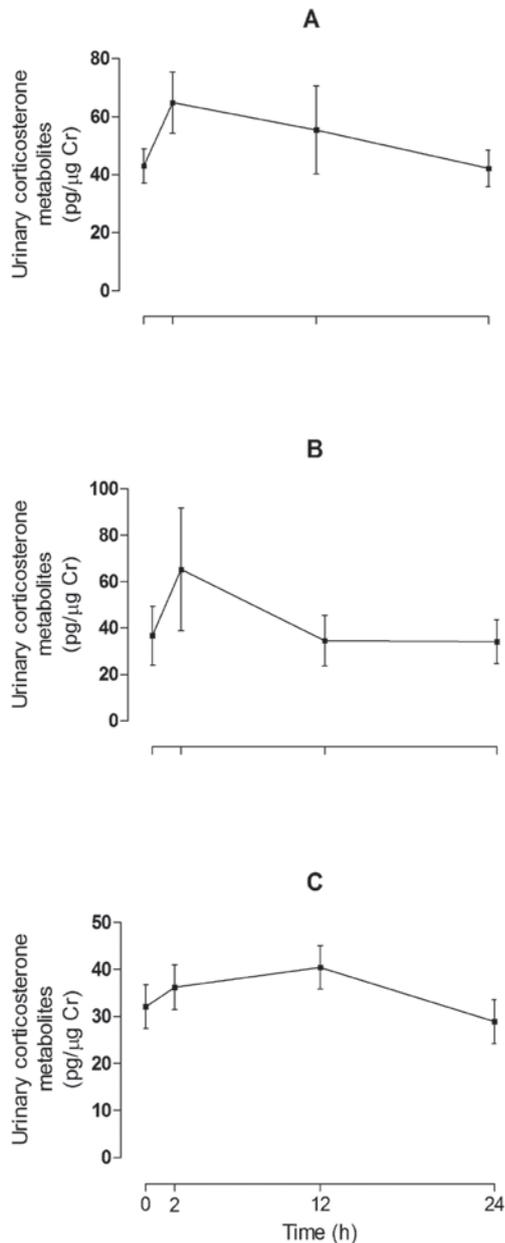


Figure 2. Mean (\pm S.E) urinary corticosterone metabolites in male Asian toads subjected to **(A)** capture handling control (n= 11), **(B)** sham (saline) control (n= 9), or **(C)** VIE tagging (n=16) at various time periods (0 h, 2 h, 12 h, 24 h) post-capture

even though ecological mark-recapture studies may not generate chronic stress in wildlife such as amphibians, wildlife can still perceive physical handling/experimental manipulation as a potential threat, and thus potentially elicit physiological stress. In this study, we tested whether the visual implant elastomer (VIE) technique generated physiological stress in male *D. melanostictus*. Our results show that although VIE tagging increased UCM levels of male toads, the mean levels of stress response were not different among groups that were subjected to either handling only or a sham (saline) injection. This result corroborates with previous work conducted on the impacts of VIE tagging on red-eyed tree frogs (Antwis et al., 2014).

Previously, adrenocorticotrophic hormone (ACTH)

stimulation tests have been used on other amphibian species to determine the lag-time for the activation of HPI-axis and metabolism of UCMs, and their subsequent appearance in urine samples. It has been demonstrated that amphibians start to generate significant changes in UCMs in response to mild capture handling protocols at 2 h, and serial sampling of 2 h intervals post-treatment have been shown to result in significant changes in UCMs (Narayan et al., 2010). In our study, the sampling regime of 0 h (pre-treatment) and 2, 12 and 24 h (post-treatment) should have detected changes in UCMs reflecting the metabolism-specific lag-time of UCMs (Narayan et al., 2010). A more robust sampling design (i.e. sequential urine sampling at every 1 or 2 h interval post-treatment for over 24 h) would have in fact been more detrimental, potentially influencing and masking any notable variation in UCMs.

Short-term or acute stress response in amphibians involves the activation of the HPI-axis and release of corticosterone from the inter-renal tissue. Corticosterone release helps in glucose mobilisation so that muscle cells can expend energy. This helps amphibians in various processes, such as vocalisation, foraging behaviour, and escaping predators. However, prolonged activation of the HPI-axis can be maladaptive for amphibians and result in chronic stress, leading to severe effects on the immune response, reproductive endocrine response, and negatively affecting behaviour and survival. Our results suggest that the VIE tagging method does not lead to chronic stress in male Asian toads as their UCM levels had returned to baseline by 24 h post treatment, despite being elevated at 12 h post treatment. Additionally, our findings demonstrate that the VIE tagging method induces a stress response to the same magnitude as simply capturing and handling a toad – an activity which is required at a minimum for the successful implementation of a mark and recapture method.

A corticosterone stress response will be apparent in any interference with animals in the wild, no matter which technique is used (Schmidt & Schwarzkopf, 2010). We have demonstrated that the VIE method keeps this stress response to a minimum, highlighting its applicability as a humane marking technique for amphibians. This is especially apparent when comparing the VIE method to the traditional toe-clipping method, which has been demonstrated to elicit a higher stress response (Narayan et al., 2011). Furthermore, previous studies have demonstrated that toe-clipping has a plethora of detrimental impacts other than prompting a stress response (i.e. reduced jumping ability, locomotion, and ability to adapt to foraging conditions), not only for frogs but a range of taxa including lizards, geckos, and salamanders (Davis & Ovaska, 2001; Schmidt & Schwarzkopf, 2010; Guimarães et al., 2014). Conversely, the VIE method has been concluded to be a safe, effective, and convenient tagging technique for most applicable species with little to no detrimental impacts (Woods & James, 2003; Sapsford et al., 2015). Thus, considering the previous literature surrounding the ‘VIE vs toe-clipping’ techniques and our findings for the VIE method, there is little doubt that VIE is currently the most appropriate technique for the mark and recapture of frogs.

To improve animal welfare, we must first improve our technology. We need to direct our attention away from invasive mark-recapture methods, as well as hormone measuring methods, which cause unnecessary stress and detrimental impacts on animals. The advancements that VIE provides can greatly improve the success of research involving mark and recapture techniques whilst also minimising stress responses. This study has provided key findings to make the use of VIE tagging, as well as UCM testing a common practice.

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Arm-wave display in a *Liolaemus* lizard

Natalin S. Vicente

Instituto de Herpetología, Unidad Ejecutora Lillo (UEL), Fundación Miguel Lillo, CONICET. San Miguel de Tucumán, Tucumán, Argentina.

Lizards show a great variety of visual displays. Among them, arm-waving is a behaviour that has received little attention and its role is still controversial. Here, I analyse the arm-waves of *Liolaemus pacha* lizards in their natural habitat with the aim of determining their function. Arm-waving was performed by both sexes, usually with the ventral body surface in contact with the ground. Furthermore, it was more frequent when alone, and during female-male interactions. The results of this study are suggestive but not conclusive regarding the possible function of this behaviour.

Keywords: communication; predator-prey interaction; reptiles; social signal; submissive; visual display

Identifying the role and the information-content of a signal can be challenging. In lizards, while some signals like dewlap displays (Nicholson et al., 2007; Driessens et al., 2015; Ingram et al., 2016), headbob displays (Macedonia et al., 2013; Ossip-Klein et al., 2013; Ossip-Drahos et al., 2018; Vicente, 2018) and colour patterns (Bastiaans et al., 2013; Klomp et al., 2016; 2017; Pérez i de Lanuza et al., 2014; Pérez i de Lanuza & Font, 2016) have been the focus of many studies, other behaviours have received less attention. This is the case of arm-waving, also known as foot-shakes or circumduction (sensu Carpenter & Ferguson 1977).

Arm-waves consist of the movement of both front arms (singly or alternately), which are raised and rotated in a circular or undulating motion (Carpenter & Ferguson, 1977). Arm-waving is shown in a variety of contexts and could, therefore, have different functions. For instance, some arm-waves are social signals, functioning as aggressive or submissive signals (Van Dyk & Evans, 2008; Woo & Rieucou, 2012). During male-male interactions, agamid lizards perform fast-waves combined with headbobs that are interpreted as signals of dominance status or aggression (Brattstrom, 1971; Van Dyk & Evans, 2008). However, when arm-waves are displayed at slow speed they function as appeasement or submissive signals (e.g. Woo & Rieucou, 2013). Arm-waves can also have a pursuit-deterrent function (Cooper et al., 2004; Cooper, 2010; Font et al., 2012). Pursuit-deterrent signals can inform the predator that it has been detected, or that the

signaller would be difficult or costly to capture (Hasson, 1991; Caro, 2005; Cooper, 2010). Finally, alternative functions have been proposed including removing a foot from contact with a hot substrate, maintaining individual distance, or inducing predators to move, revealing their location (Schall, 1974, Magnusson, 1996; Cooper et al., 2004).

Arm-waving has been described in lizards of the families Agamidae (e.g. Brattstrom, 1971; Ord et al., 2002; Van Dyk & Evans, 2008), Lacertidae (e.g. Font et al., 2012), Teiidae (e.g. Baird et al., 2003; Cooper et al., 2004), Dactyloidae (Jenssen, 1979), Iguanidae (Distel & Veazey, 1982), Phrynosomatidae (Carpenter, 1967) and Liolaemidae (Halloy & Castillo, 2006). Specifically, for the genus *Liolaemus*, two types of arm-waves, depending on the type of movement, have been described: one-arm forelimb displays and two-arm forelimb displays (Halloy & Castillo, 2006). *Liolaemus* two-arm wave displays are performed with the body in contact with the substrate, and consist of the lizard lifting both arms simultaneously and/or alternatively, similar to the butterfly and crawl stroke in swimmers (Fig. 1). However, their function is still unknown (Halloy & Castillo, 2006). In one-arm forelimb displays, the lizard lifts one arm up and down; these displays have been interpreted to have a role in territorial signalling. In the present study, I analyse 52 two-arm-wave displays of *Liolaemus pacha* with the aim of determining their function.

I made eight field trips, three during 2012 (October to December), three in 2013 (October to December), and two in November 2014 and 2015 to Los Cardones, located 20 km East from Amaicha del Valle, Department of Tafi del Valle, Province of Tucuman, Argentina (26°40'1.5''S, 65°49'5.1''W). The site is located at 2725 m above sea level on the western slope of Sierras Calchaquíes. The field trips lasted two to five days, and I made observations from 10:00 h until 16:00 h, a period adequate for observing social interactions in this species (Vicente, unpublished data). I walked along the study area (approximately 1 ha) in a fixed direction, approaching lizards at a slow speed (i.e. slow walking). I videotaped adult lizards at an average distance of 4 m, to minimise interference, using a digital camcorder (Sony Handycam HDR-Cx290). I used a focal animal sampling rule (Martin & Bateson 2007) such that each focal lizard was recorded during 15 min or until it went out of sight. As lizards were not captured and marked, and to avoid

Correspondence: Natalin Vicente (natalinvic@gmail.com)



Figure 1. Photograms extracted from a video recording of a displaying *L. pacha* lizard performing an arm-waving display. Numbers shown correspond to the respective frame.

recording the same individual twice, I started sampling each day from different points through the area and walking in different directions. Individuals with natural marks were recorded only once.

I analysed videotapes from 350 individuals (total of 13 h and 41m) and identified sequences in which the lizards performed arm-waves. The sequences of interest were cut from the original video, using .avi or .m2ts format videos (full-HD) with a resolution of 25 frames/second. I used the software TRACKER (Brown, 2009) for frame by frame analysis. In total, videotapes were obtained of 10 one-arm and 42 two-arm-wave displays. I recorded the sex and context (alone, male-male, and male-female interactions; for a detailed description of social contexts see Vicente, 2018). In this study, the context alone corresponds to those arm-waves displayed by lizards apparently alone, which might include arm-waves directed to an undetected receiver. Additionally, I classified the posture of the displaying lizard according to a scale ranging from zero to three (Fig. 2).



Figure 2. Examples of the four postures (0-3) described for *L. pacha*. The posture 0 is characterised by the complete ventral surface in contact with the substrate including throat and head (a). Posture 1 involved the head and throat contactless with the substrate (b). Posture 2 is characterised by the belly partially in contact with the substrate and the arms partially extended (c). Posture 3 is determined when the belly is fully exposed and the arms fully extended (d).

Table 1. GLM Analysis of arm-waves duration. Parameter estimates (PE ± SE), and *p*-value of explanatory variables (sex and context) describes variation in arm-wave duration. Estimate values represent the difference between sexes and contexts, with respect to the reference values (females and alone), when corresponding.

Response Variable	Explanatory variables	PE ± SE	<i>p</i>
Arm-wave duration	Intercept	3.62 ± 0.09	< 0.001
	Sex (males)	0.02 ± 0.11	> 0.05
	Context (female-male)	0.09 ± 0.11	> 0.05
	Context (male-male)	-0.01 ± 0.17	> 0.05

Table 2. Counts of arm-waves among contexts according to the following variables: laterality, orientation to the observer, quantity of arms used, posture, association with movement, and presence of headbobs.

		Alone	Male-Female interaction	Male-Male interaction	N total
Laterality	Left arm	5	9	3	17
	Right arm	21	12	2	35
Orientation to the observer	Orientated	15	9	3	27
	Non-orientated	11	12	2	25
Quantity of arms	One arm	6	4	0	10
	Two arms	20	17	5	42
	Zero	16	11	2	29
Posture	One	6	7	1	14
	Two	4	3	2	9
	Three	0	0	0	0
Movement	Stationary	13	11	3	27
	Movement	13	10	2	25
Headbobs displayed	With	7	11	4	22
	Without	19	10	1	30

I estimated the percentage of occurrence of arm-waving and the coefficient of variation (CV) for the arm-wave duration, considering that a CV of less than 30 % is stereotyped (Lehner, 1998). Then, for each arm-wave display I measured: a) arm-wave duration (seconds), b) lateralisation, determined as the type of arm that initiated the behavior (left or right hand), c) orientation to the observer, whether the arm that initiated the display was the one closer to the observer (oriented or non-oriented), d) movement, if lizards moved from one position to another before or after the arm-wave (movement or stationary), e) quantity of arms used, if lizards displayed with one or both arms (one or two), f) posture (0-3) and, g) presence of headbobs (presence or absence).

I evaluated the effect of sex and social context on the duration of arm-waving with a Generalized Linear Model (GLM), with duration as a response variable, and sex and social context as explanatory variables. The model was fitted using a negative binomial distribution because duration was transformed as a discrete variable and it also showed overdispersion (Logan, 2010). The differences in the frequency of lateralisation, orientation, movement, quantity of arms used, posture, and concurrence of headbobs between sexes and among contexts were estimated using Chi-square tests. I used $p \leq 0.05$ as the cut-off for statistical differences. All values are shown as mean ± SE.

Arm-waves in *L. pacha* are not a common behaviour; only 14.8 % of lizards showed this display (lizards videotaped = 350; arm-waves = 52). Duration of arm-waves was not stereotyped (37.09 ± 1.79 ds, CV = 34.7 %), and it was not influenced by sex (GLM, $p > 0.05$, Table 1; $n = 24$, 36.13 ± 2.23 ds; males $n = 28$, 37.93 ± 2.75 ds) or context (GLM, $p > 0.05$; alone context: $n = 26$, 35.23 ± 2.08 ds; female-male interaction: $n = 21$, 39.24 ± 3.58 ds; male-male interaction: $n = 5$, 36.13 ± 2.50 ds). Arm-waving was statistically more frequent in the alone context ($n = 26$) and female-male interaction ($n = 21$) than in male-male interactions ($n = 5$; Chi-square test, 13.79, $df = 2$, $p = 0.001$). Arm-waving frequency was not statistically different between males ($n = 28$) and females ($n = 24$; Chi-square test, 0.31, $df = 1$, $p > 0.05$; Fig. 3).

Arm-waves were performed and initiated more often with the right hand ($n = 35$, Chi-square test, 6.23, $df = 1$, $p = 0.01$) than with the left hand ($n = 17$; see Table 2 for differences among contexts). Lizard arm-waves oriented to an observer ($n = 25$) were performed at the same frequency as non-oriented displays ($n = 27$, Chi-square test, 0.08, $df = 1$, $p > 0.05$). Arm-waves associated with movement ($n = 25$) were performed at the same frequency as stationary ones ($n = 27$, Chi-square test, 0.08, $df = 1$, $p > 0.05$). Most arm-waves were displayed with both arms ($n = 42$, Chi-square Test, 19.69, $df = 1$, $p < 0.001$), and in a few occasions with one arm ($n = 10$). Posture 0 is the one adopted more often for performing arm-waves ($n = 29$, Chi-square Test, 12.81, $df = 2$, $p = 0.002$), while posture 1 ($n = 14$) and 2 ($n = 9$) were used less. No lizard used posture 3. Arm-waving was more often displayed without ($n = 32$) than with headbobs ($n = 20$, Chi-square test, 2.77, $df = 1$, $p > 0.05$).

The results of this study are suggestive but not

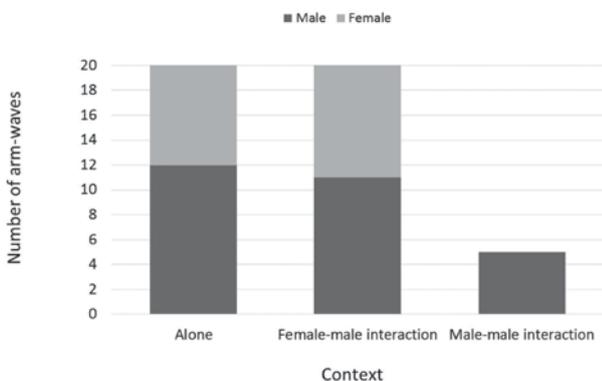


Figure 3. Bar plot showing the number of arm-waves performed by male and female *L. pacha* among the three contexts considered: alone, female-male interaction, male-male interaction.

conclusive regarding the potential functions of arm-waving. Arm-waving was displayed equally by both sexes and it was most frequently displayed in the contexts of alone and female-male interaction. Arm-waves could function as submissive signals. Most of the arm-waves were displayed with the ventral surface completely in contact with the substrate, head down, and in two cases with eyes closed, which can be considered as a submissive posture (Carpenter & Ferguson, 1977; Labra et al., 2007; Hamilton et al., 2013). Finally, arm-wave display orientation to the observer, which can be related to a pursuit-deterrent function (e.g. Hasson, 1991; Font et al., 2012), was not significant. Moreover, when *L. pacha* lizards were approached by an observer simulating a predator, arm-waves were not elicited (Salido & Vicente, 2019).

In conclusion, arm-waves in *L. pacha* are displayed by both sexes and their function is still unknown. Arm-waving could function as a social signal, as a submissive signal, or as a pursuit-deterrent signal. In either case, further studies are strongly needed including juveniles, and also, assessing possible differences in size and reproductive status between signaller and receiver, in order to investigate when arm-waving is elicited.

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