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Morphological and mitochondrial variation of spur-thighed tortoises, *Testudo graeca*, in Turkey

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Testudo graeca has a wide distribution under different geographic, climatic and ecological conditions, and shows high morphological differences especially in the Asian (Middle Eastern and Caucasian) parts of the range. This study investigates morphometric and genetic differentiation in the *T. graeca* complex in Turkey using the densest sampling to date. We sequenced two mt-DNA loci (ND4 and cyt *b*) of 199 samples and combined them with previously published data. Bayesian analysis yielded six well-supported clades, four of which occur in Turkey (*ibera*, *terrestris*, *armeniaca* and *buxtoni*). The *armeniaca* mtDNA clade locally represents a morphometrically distinct burrowing ecomorph. However, previous studies have shown that individuals outside Turkey possessing *armeniaca* mtDNA lack the distinctive *armeniaca* morphotype we observed, precluding taxonomic conclusions.

Key words: mtDNA, morphometry, Testudinidae, *Testudo*, Turkey

INTRODUCTION

Spur-thighed tortoises (*Testudo graeca* Linnaeus 1758) occur on three continents (Europe, Africa, and Asia). Such a wide distribution under different geographic, climatic, and ecological conditions causes high morphological differences in shell shape, coloration and pattern, most notable in the Asian (Middle Eastern and Caucasian) parts of the range (Parham et al., 2006; Fritz et al., 2007; Türkozan et al., 2010). Based on these morphological differences, nine Asian taxa were recognised (Guyot, 2004), whereas molecular studies revealed discordance between morphological variation, with six mitochondrial clades (Parham et al., 2006; Fritz et al., 2007). Subsequently, Mikulíček et al. (2013, using nuclear AFLP), recovered four geographically well-defined units, which combine one or more of the mtDNA clades, and Mashkaryan et al. (2013, using nuclear microsatellites) revealed weak differences and extensive gene flow between the six mtDNA clades. These conflicting findings reflect the different levels of variation inherent to each marker, and a complex evolutionary history likely involving both vicariance and gene flow.

In addition to conflicting genetic evidence, contrasting patterns of morphological variation are also reported. Perälä (2002) divided *T. graeca* populations along the Mediterranean (southern) coast of Turkey into several species or subspecies based on morphometric data. Türkozan et al. (2010) showed that the populations along the Mediterranean coast of Turkey are morphometrically homogenous, but that some inland Turkish populations are morphometrically distinct, reflecting some of the genetic clade assignments of Parham et al. (2006) and Fritz et al. (2007).

The current study expands on Türkozan et al. (2010) and provides the most comprehensive and geographically dense comparison of genetic and morphological variation for the *T. graeca* complex in the Middle East and Caucasus to date. The higher genetic coverage in the region where the deepest mitochondrial clades and most divergent phenotypes come into contact allows us to compare geographically referenced morphological data with genetic information without the a priori assumption of subspecies based on mtDNA clades sensu Fritz et al. (2007).

MATERIALS AND METHODS

Sampling and DNA extraction

A total of 199 tissue samples (nail clippings) were collected across Turkey between 2002 and 2006, and used for the analyses of two mtDNA loci (194 and 199 samples were used for the amplification of cytochrome b (CytB) and NADH dehydrogenase (ND4), respectively). We combined our data with previously published ND4 sequences from Parham et al. (2006), and previously published CytB sequences from Fritz et al. (2007) and Mashkaryan et al. (2013). Genbank accession numbers

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Table 1. Primers used in this study

ND4				
Primer	**Primers**	**PCR**	**Sequence**	**Tm (˚C)**
L-ND4-TG	5´ GTA GAG GCC CCA ATT GCA G 3´ (Parham et al., 2006)	✓	✓	64.5
H-Leu-TG	5´ TGT ACT TTT ACT TGG AAT TGC ACC A 3´ (Parham et al., 2006)	✓	✓	65.1
CytB				
mt-A1	5´ CCC CCT ACC AAC ATC TCA GCA TGA TGA AAC TTC G 3´ (Fritz et al., 2007)	✓	✓	70.7
mt-a-neu2	5´ CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC 3´ (Fritz et al., 2007)	✓	✓	72.7
mt-Fr	5´ CTA AGA AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA 3´ (Fritz et al., 2007)	✓	✓	67.0
mt-c2	5´ TGA GGA CAA ATA TCA TTC TGA GG 3´ (Fritz et al., 2007)		✓	61.7
mt-E-Rev	5´ GCA AAT AGG AAG TAT CAT TCT GG 3´ (Fritz et al., 2007)		✓	60.4

Table 2. Uncorrected p distances (percentages) between clades of *T. graeca*

	ibera	***terrestris***	***armenica***	***buxtoni***	***zarudnyi***
ibera	---				
terrestris	0.022	---			
armenica	0.048	0.044	---		
buxtoni	0.04	0.039	0.042	---	
zarudnyi	0.035	0.036	0.037	0.03	---
African	0.039	0.04	0.042	0.041	0.038

and sample locations are shown in Appendix 1. Total genomic DNA was extracted from ethanol preserved nails with the standard phenol-chloroform protocol (Sambrook et al., 1989). The primers used in this study are shown in Table 1.

PCR amplification was carried out using Taq DNA polymerase (Fermentas) and performed in a 50ul volume (1X Taq Buffer, 0.2mM dNTPs, 0.4mM each primer, 1.5mM $MgCl_2$, and 1ul Taq polymerase). The thermal profile for ND4 consisted of an initial 4 min denaturation step at 94 °C, 35 cycles of denaturation at 94 °C for 45 sec, annealing at 57 °C for 30 sec, and extension at 72 °C for 80 sec, with a final 10 min extension step at 72 °C. The thermal profile for CytB consisted of an initial 4 min denaturation step at 94 °C, 35 cycle of denaturation at 94 °C for 45 sec, annealing at 57 °C for 1 min, extension at 72 °C for 2 min, and a final 10 min extension step at 72 °C. PCR products were purified using the SIGMA GenElute PCR Clean-Up Kit. Each DNA fragment was sequenced in both directions, using the BigDye Terminator (Applied Biosystem) kit. Sequencing products were analysed on an AB3730xl automated DNA sequencer (Macrogen, Seoul, South Korea).

Data Analysis

Partial sequences of ND4 (849bp) and partial CytB (986 bp) mitochondrial DNA sequences were aligned using Clustal X (Thompson et al., 1997) with default parameters and ambiguous bases resolved by eye using BioEdit (Hall, 1999).

A Maximum likelihood analysis (ML) was performed using RAxML Version 8.1.11 (Stamatakis, 2014). The dataset was partitioned by codon position for protein coding regions and by whether a region consisted of protein coding sequences or not. Due to limitations of model selection in RAxML, all partitions were set to GTRGAMMA. Automatic settings were used and all ML analyses were run for 1000 bootstraps. Bayesian inference were conducted in Mr. Bayes 3.2.3 (Ronquist et al., 2012). The alignment was partitioned as in the RAxML analysis, and the partitions were model tested in jModeltest2 (Darriba et al., 2012, Guindon & Gascuel, 2003), using the best-fitting model under the Akaine Information Criterion (AIC, CytB: HKY + I + G for codon position 1, GTR + G for codon position 2, GTR + G for codon position 3, K80 for the tRNA partition; ND4: GTR for the first and third codon positions, HKY + I + G for the second position, and HKY + G for the tRNA partition). The models used for the concatenated analysis were the same, except that tRNA from both fragments was dealt with as a single partition with the model HKY + G. All Bayesian inference analyses were run with one cold chain and three hot chains for 10 million generations and sampled every 1000 generations with 25% of samples discarded as burn-in. Tracer 1.6 (Rambaut et al., 2014) was used to determine that the analysis had converged, and analyses were rerun with different number seeds for a total of four times to determine that the analysis converged on the same answer. All Maximum-likelihood and Bayesian inference analyses were conducted on the CIPRES Science Gateway (Miller et al., 2010). Intergroup and intragroup uncorrected genetic distances were calculated under default settings in MEGA7 (Kumar et al., 2016).

Population genetic statistics were calculated in DnaSP Version 5.10.1 (Librado and Rozas, 2009). Default parameters were used after manually setting the datasets to mitochondrial and haploid data, as well as annotating coding and noncoding codon positions and assigning codon positions to protein coding regions. CytB and ND4 regions were examined separately due to missing sites for some samples. In order to identify new haplotypes, similar haplotypes were collapsed in DnaSP. A list was

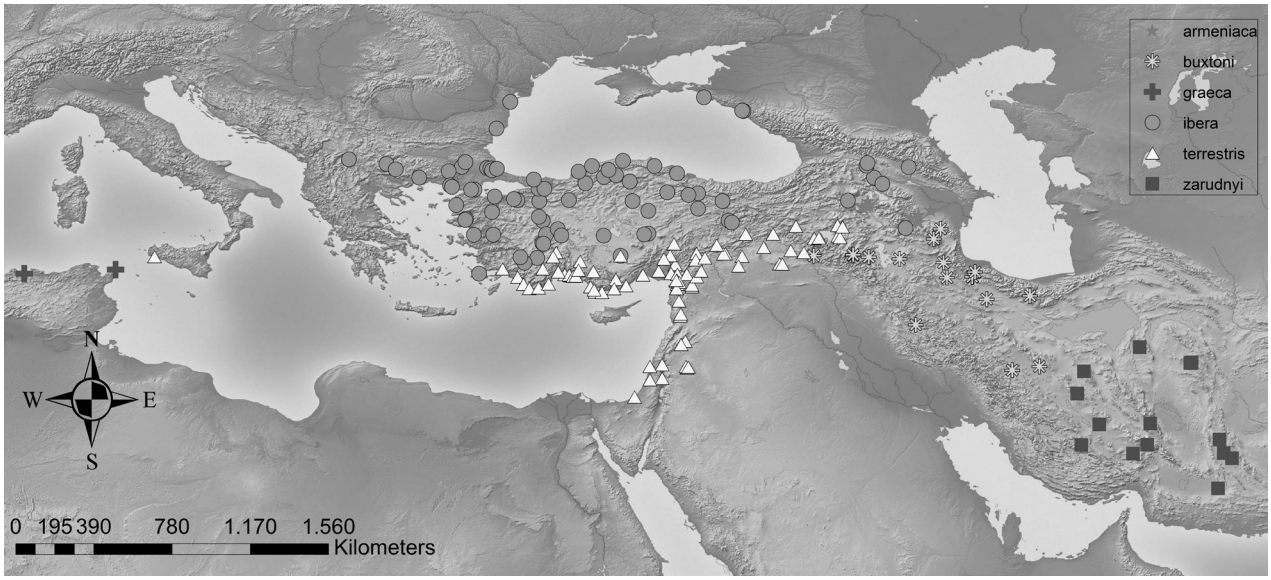

Figure 1. Map showing the localities of *T. graeca* samples used in this study with coloured symbols representing the different mitochondrial clades.

generated and manually checked for haplotypes that did not contain previous Genbank sequence. The CytB dataset was pruned of samples that did not meet a threshold of less than 5% missing data in the alignment. Intergroup and intragroup uncorrected genetic distances were calculated under default settings in MEGA7 version 7 (Kumar et al., 2016).

Morphology

For this study, specimens were classified into taxa (subspecies of Fritz et al., 2007) according to mitochondrial lineages determined in this study. The same data set from Türkozan et al. (2010) was used to test for morphological differences among molecular clades, only using samples collected at sites where mtDNA clades were identified. Of the 171 (81♂, 90♀) samples included in the analysis, 26 (15♂, 11♀) were *armeniaca*, 11 (3♂, 8♀) were *buxtoni* 80 (36♂, 44♀) were *ibera* and 54 (27♂, 27♀) were *terrestris*.

Body measurements were taken with either wooden (accuracy ± 1mm) or dial calipers (accuracy ± 0.02mm). All individuals were photographed, measured, and subsequently released. The morphometric measurements taken are the same as Türkozan et al. (2010): straight carapace length (SCL), from the outermost projection of the cervical scale to the outermost projection of the posteriors marginals; plastron length (PL), from the outermost projection of the gulars to the posterior end of the anals; median carapace width (CW), at the center of the carapace; maximum carapace width (MCW), at posterior marginals 7-9; carapace height (CH), the vertical measurement between the most dorsal point of carapace and the most ventral point of plastron; length of bridge (LB), between axillary and inguinal scales; maximum (not midline) gular scale length (MGSL); maximum (combined left plus right) gular scale width (MGSW); maximum humeral scale width (CHSW); maximum pectoral scale width (CPSW); maximum abdominal scale width (CAbSW); maximum femoral scale width (CFSW); maximum anal scale width (CSAW); gular suture length (GSL), Humeral suture length (HSL); pectoral suture length (PSL); abdominal suture length (AbSL); femoral suture length (FSL); anal suture length (ASL); nuchal length (NL), straight-line measurement from the anterior edge of cervical scale to posterior end; nuchal width (NW), the width of cervical at the posterior end; maximum width of first vertebral scale (VW1); maximum width of second vertebral scale (VW2); maximum width of third vertebral scale (VW3); maximum width of fourth vertebral scale (VW4); maximum width of fifth vertebral scale (VW5); maximum median length of first vertebral scale (VL1); maximum median length of second vertebral scale (VL2); maximum median length of third vertebral scale (VL3); maximum median length of fourth vertebral scale (VL4); maximum median length of fifth vertebral scale (VL5); maximum dorsal width of supracaudal scale (DSW); maximum ventral width of supracaudal (VSW); maximum median length of supracaudal length (SL); first pleural scale along length as the minimum straight line distance between the anteriormost and posteriormost contact points with adjacent (normally first and fifth) marginal scales (CL1); length of second pleural scale along the marginal (CL2); length of third pleural scale along the marginal (CL3); length of fourth pleural scale along the marginal (CL4); and maximum inner height of anterior shell opening parallel to median axis (IHASO).

We performed discriminant function analysis (DFA) with stepwise selection (*F* for entry: 3,84; for removal: 2,71) to obtain a subset of variables that provided best discrimination. Since the DFA is sensitive to number of samples only females were analyzed. Measurements used in DFA were standardised for CL. Furthermore, principal component analysis (PCA) were performed to raw measurements to further support the morphological differences among lineages. Significance levels for all tests were set at $p < 0.05$. All statistical analyses were performed using STATISTICA 7.0.

Table 3. Genetic diversity within *T. graeca*. n = number of sequences, S = number of polymorphic sites, k = average number of pairwise nucleotide differences, π = nucleotide diversity (Standard Deviation in parenthesis), h = number of haplotypes, Hd = haplotype diversity (Standard Deviation in parenthesis), - = test could not be run or statistic could not be calculated due to low sequence or haplotype number, NS = Not significant.

	Clade	N	s	k	π (SD)	h	Hd (SD)	Fu's F	Fu & Li's D	Fu & Li's F	Tajimas's D
CytB	CytB	299	192	21.683	0.0257 (0.0010)	74	0.935 (0.007)	-4.555	-4.6482 (P<0.02)	-3.3691 (P<0.02)	-1.1023 (P>0.10) NS
	African	1	-	-	-	1	-	-	-	-	-
	armeniaca	33	17	2.917	0.0031 (0.0004)	9	0.703(0.056)	-0.572	-2.4588 (0.10>P<0.05) NS	-2.34919 (0.10>P>0.05) NS	-1.0196 (P>0.10) NS
	buxtoni	22	70	14.416	0.0151 (0.0018)	13	0.931 (0.036)	0.899	-1.2321 (P>0.10) NS	-1.3874 (P>0.10) NS	-1.08001 (P>0.10) NS
	ibera	115	54	2.846	0.0030 (0.0005)	21	0.714 (0.035)	-6.849	-6.4613(P<0.02)	-5.6736 (P<0.02)	-2.3026 (P<0.01)
	terrestris	121	74	4.255	0.0049 (0.0004)	33	0.896(0.016)	-14.428	-4.1561 (P<0.02)	-3.9998 (P<0.02)	-2.2340 (P<0.01)
	zarudnyi	7	4	1.714	0.0016 (0.0004)	4	0.810 (0.130)	-0.428	-0.0686 (P>0.10) NS	0.00000 (P>0.10) NS	0.2390 (P>0.10) NS
ND4	ND4	229	109	12.011	0.0147(0.0008)	50	0.858 (0.019)	-5.155	-3.6783(P<0.02)	-2.9461(P<0.05)	-1.1299(P>0.10) NS
	African	2	10	10.000	0.0149 (0.0074)	2	1.000 (0.500)	-	-	-	-
	armeniaca	7	5	2.952	0.0035 (0.0007)	5	0.905 (0.103)	-0.737	0.5967 (P>0.10) NS	0.5479 (P>0.10) NS	0.1726 (P>0.10) NS
	buxtoni	18	12	5.124	0.0063 (0.0008)	4	0.725 (0.055)	5.316	0.7402 (P>0.10) NS	1.0485 (P>0.10) NS	1.32699 (P>0.10) NS
	ibera	94	23	0.793	0.0001 (0.0002)	12	0.311 (0.063)	-8.259	-5.3457 (P<0.02)	-5.0810 (P<0.02)	-2.4484 (P<0.01)
	terrestris	97	44	2.939	0.0035 (0.0004)	28	0.886 (0.018)	-16.284	-5.2281(P<0.02)	-4.7522 (P<0.02)	-2.0729 (P<0.05)
	zarudnyi	11	0	0.000	0.00000	1	0.000	-	-	-	-

Table 4. Classification matrix from discriminant function analysis for morphologic populations (rows: observed classifications, columns: predicted classifications) ARM: *armeniaca*, BUX: *buxtoni*, TER: *terrestris*, IBE: *ibera*

	Percent Correct	ARM	BUX	IBE	TER
ARM	100	11	0	0	0
BUX	0	0	0	5	3
IBE	76	0	1	29	8
TER	43	0	0	13	10
Total	62,5	11	1	47	21

RESULTS

All phylogenetic methods used in this study supported similar tree topologies with regard to the major clades, and therefore only the BI tree is shown. Bayesian analysis yielded six well supported clades (see Figure 1) corresponding to the mtDNA clades of Parham et al. (2006; Figs. 2-5). Of these clades, four occurred in Turkey (Fig. 1). The *ibera* and *terrestris* mtDNA clades are sister groups, and the *armeniaca* mtDNA clade is sister to the *graeca* mtDNA clade. The *buxtoni* mtDNA clade is sister to the *zarudyni* mtDNA clade. The *ibera* mtDNA clade includes tortoises from the Caucasus, Bulgaria, Greece, Republic of Macedonia, Romania, Russia, and Anatolia together with European Turkey. The *terrestris* mtDNA clade includes tortoises from southeastern Turkey, the Levant, Syria, Israel, and the Mediterranean coast of Turkey. The westernmost specimen of the *terrestris* mtDNA clade is from Kızılağaç village, Province Muğla, Turkey; the easternmost specimen is from Sağlarca village near Eruh. The northern distribution of the *terrestris* mtDNA clade is limited by the Taurus Mountain range. Seven samples from Karapınar (Meke Lake), central Anatolia, were assigned to two different mtDNA clades (*terrestris* and *ibera*). The *buxtoni* mtDNA clade includes tortoises from Şırnak, Turkey (westernmost point and 40 km away from the nearest *terrestris* record) and western Iran. The *zarudyni* mtDNA clade includes tortoises only from east Iran. The *armeniaca* mtDNA clade contained individuals from eastern Turkey, Armenia, Nagorno Karabag and Azerbaijan. The *greaca* mtDNA clade includes tortoises from Algeria and Tunisia. The uncorrected distances among clades ranged from 0.022 (*ibera-terrestris*) to 0.048 (*ibera-armeniaca*) (Table 2). The intra clade variation was between 0.0006 (*zarudyni*) and 0.015 (African).

Of the 50 ND4 haplotypes found, 34 were previously unsampled. Twenty-one of those haplotypes were assigned to the *terrestris* mtDNA clade, nine were assigned to the *ibera* mtDNA clade, and two were assigned to the *buxtoni*, and *armeniaca* mtDNA clades each. For CytB, after thirty-six sequences from Genbank were removed for missing more than 5% of the sequence used in the alignment (See Online appendices, Appendix 2), the alignment consisted of 299 samples with 1059 sites, 1042 which were protein coding and the rest consisted of non-coding flanking tRNA. Of the 74 CytB haplotypes found, 32 were previously unrecorded. Sixteen of those haplotypes were assigned to the *terrestris* mtDNA clade, eight were assigned to the *ibera* mtDNA clade, and eight were assigned to the *buxtoni* mtDNA clade. Low nucleotide diversity, high haplotype diversity, negative Tajima'D and Fu's F suggest a recent population expansion (Table 3).

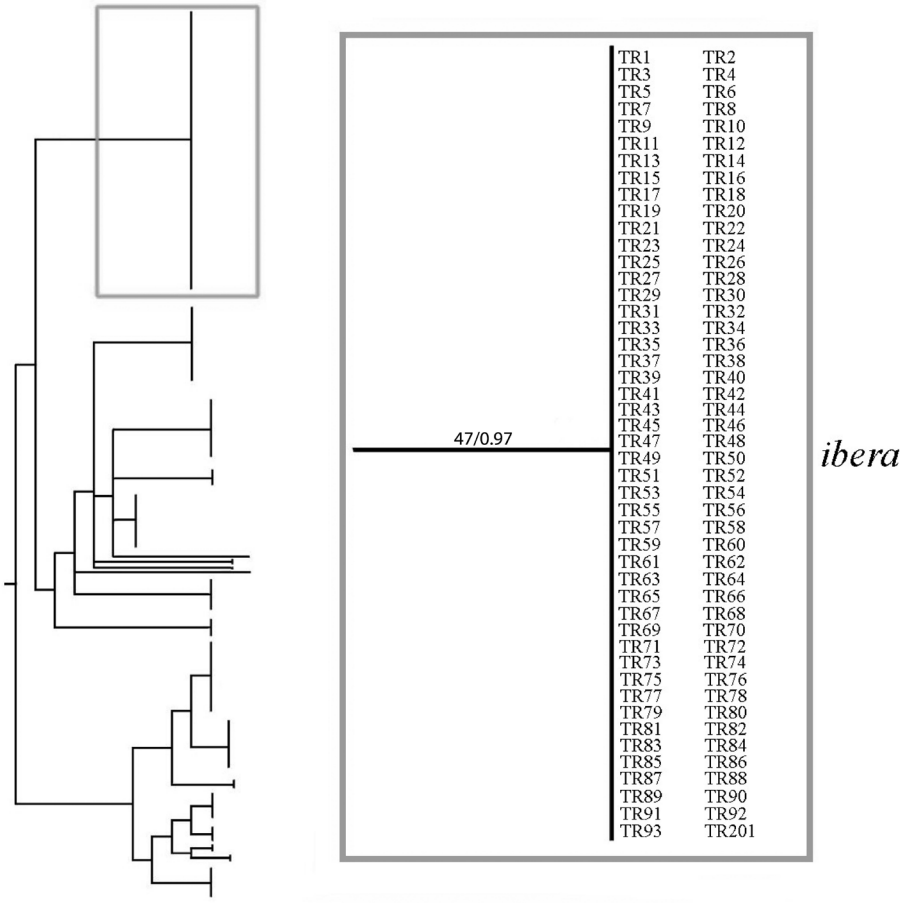

Figure 2. Extended Bayesian tree of samples of the *ibera* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

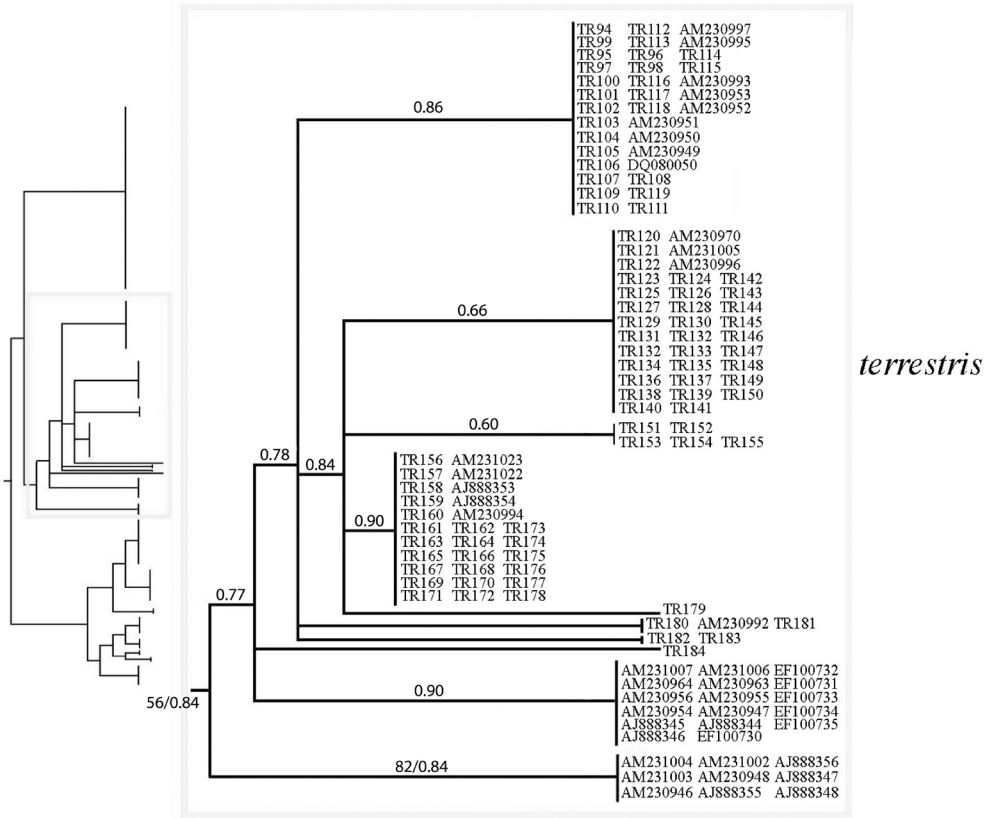

Figure 3. Extended Bayesian tree of the *terrestris* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

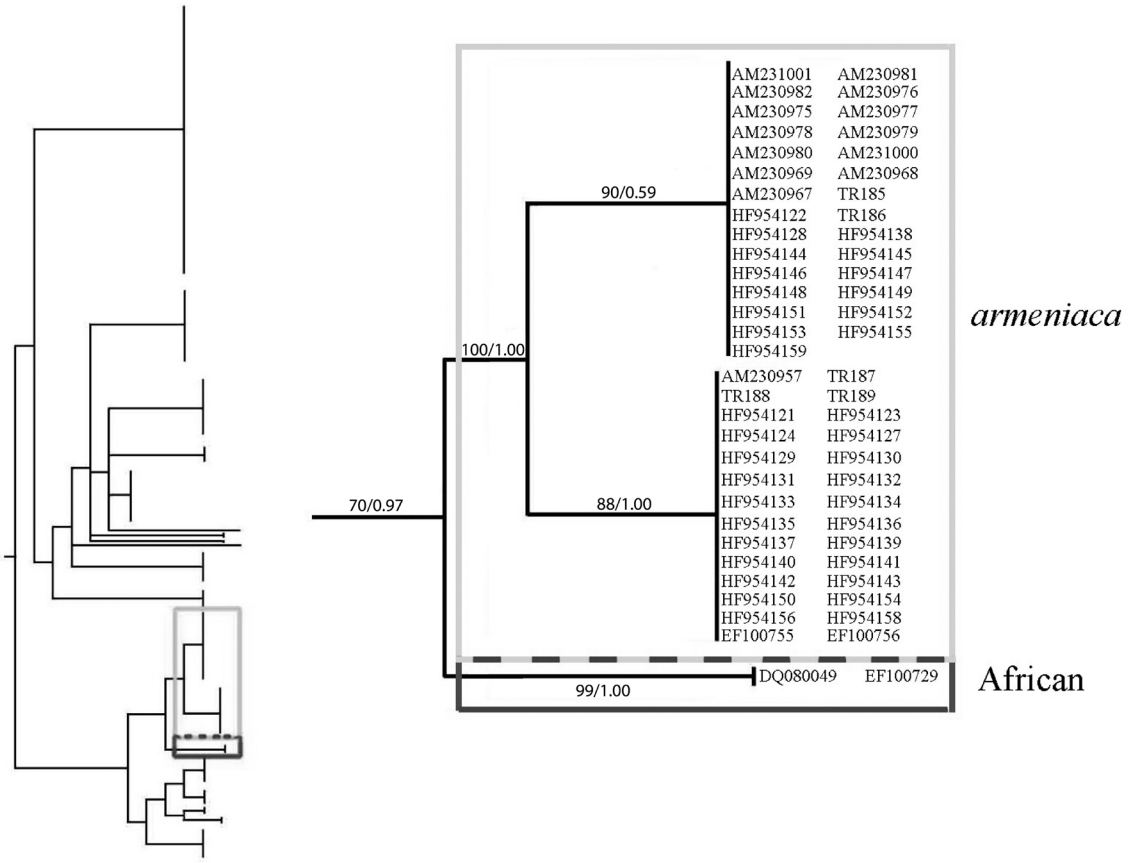

Figure 4. Extended Bayesian tree of the *armeniaca* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

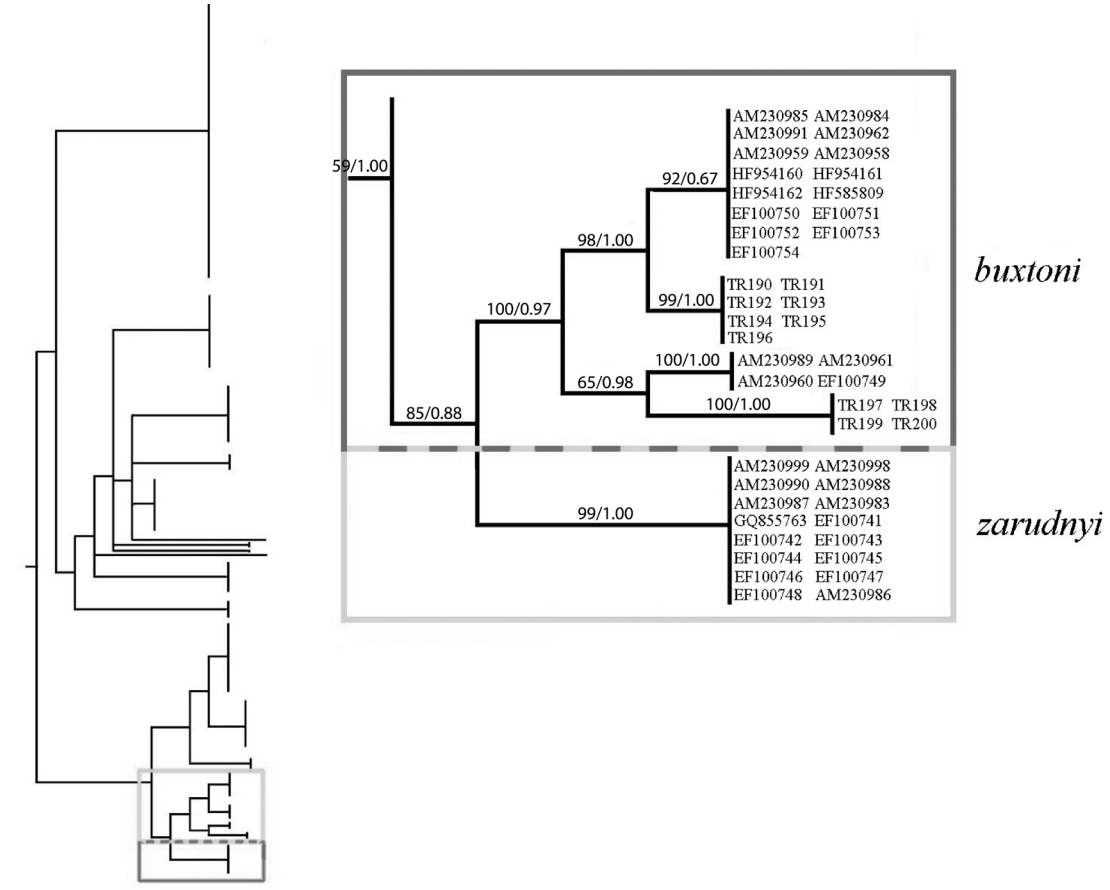

Figure 5. Extended Bayesian tree of the *buxtoni* mtDNA and the *zarudyni* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

Table 5. Principal component loadings of morphometric characters.

Variable	Factor 1	Factor 2	Factor 3
CL	-0,98527	0,056033	0,005615
PL	-0,98558	0,071031	-0,009379
CW	-0,98154	0,067987	-0,061550
MCW	-0,94933	0,040551	-0,063126
CH	-0,97242	0,066687	0,100431
LB	-0,92397	-0,429031	0,059865
MGSL	-0,89300	0,026598	-0,300681
CGSW	-0,87002	0,082015	0,300361
CHSW	-0,91160	-0,424941	-0,093412
CPSW	-0,95597	0,134011	-0,161781
CabSW	-0,94377	0,107487	-0,228006
CFSW	-0,96614	0,035017	-0,036663
CASW	-0,94322	0,090684	-0,067086
GSL	-0,81353	-0,001996	-0,386924
HSL	-0,72475	0,064444	0,535875
PSL	-0,16531	0,980750	-0,096608
AbSL	-0,92265	-0,333960	-0,036133
FSL	-0,71413	0,253066	0,386709
ASL	-0,75506	-0,213520	-0,421773
NL	-0,84634	-0,044661	0,150913
NW	-0,42599	-0,228330	0,311541
VW1	-0,86649	-0,050379	0,190464
VW2	-0,92513	-0,020171	0,194272
VW3	-0,96004	-0,009998	0,147988
VW4	-0,97307	0,006054	0,044585
VW5	-0,88770	0,115711	-0,207919
VL1	-0,95869	0,034584	0,085344
VL2	-0,96272	-0,019458	0,117758
VL3	-0,95731	0,026555	0,196068
VL4	-0,92217	-0,003989	0,090103
VL5	-0,93449	0,067368	0,019106
DSW	-0,79902	-0,021963	-0,325218
VSW	-0,91959	0,075979	-0,202497
SL	-0,91183	0,121743	0,013802
CL1	-0,96673	0,028291	-0,065187
CL2	-0,95450	-0,022425	-0,078485
CL3	-0,95929	-0,015095	0,007065
CL4	-0,90012	-0,016643	0,197550
IHASO	-0,91842	-0,015981	-0,166726

Morphology

Three discriminant functions were produced, and only the first two made significant contributions to distinguishing morphologically based taxa ($p < 0.001$; CGSW contributes most, followed by HSL, CPSW and GSL). Plastral measurements are major variables that allow us to discriminate between tortoise clades. The first discriminant function (DF) accounted for 94% of between-group variability. The first DF is weighted most heavily by CGSW and CPSW. The second function is shaped by CGSW and to a lesser extent HSL and GSL. The first and second DF discriminate mostly between *armeniaca* and the other clades (Fig. 6). Overall, the DFA resulted in 62.5% of classification corresponding to their clade (Table 4). Out of eight *buxtoni* samples, five classified as *ibera* and three as *terrestris*, while of the 38 *ibera* eight were classified as *terrestris*, one as *buxtoni*, and the rest as *ibera* (see Table 4).

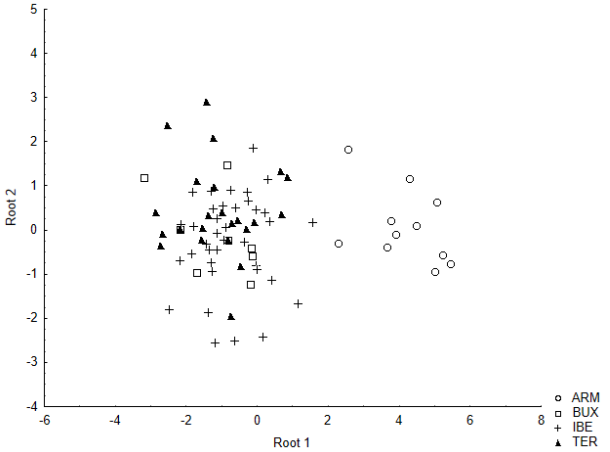

Figure 6. Specimens of females from the *armeniaca*, *buxtoni*, *ibera*, and *terrestris* mtDNA clades plotted in canonical variate space number of variables in the model: 4. Wilk's λ = 0.16175, F(12.148) = 12.257, P < 0.00001

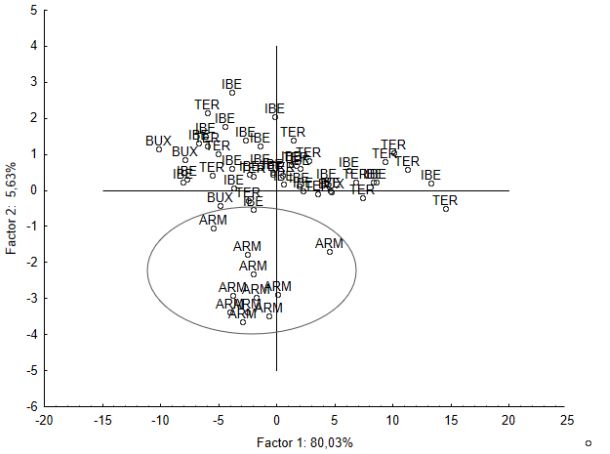

Figure 7. Scattered diagram of PCA along PC1 and PC2 based on 39 morphometric characters (names of clades abbreviated as in Tables 3 and 4)

The PCA results further support the morphological difference of *armeniaca* (80.3%) from other lineages (Fig. 7). The factor loadings of each variable are presented in Table 5.

DISCUSSION

The mitochondrial distance was highest between *ibera* and *armeniaca*, which are syntopic in Caucasus where they hybridise with each other (Mashkaryan et al., 2013). Nuclear AFLP data, on the other hand, found the highest genetic differentiation between the western Mediterranean (north Africa) and central-eastern Iranian clusters (*zarudnyi* mtDNA clade, Mikulíček et al., 2013), a difference likely linked to different evolutionary history of genetic markers and extensive gene flow among mtDNA clades.

Compared to the other mtDNA clades, *ibera* has a

range wide distribution from west to east, whereas the *terrestris* mtDNA follows the Taurus range across Turkey (except one locality out of this range where *terrestris* and *ibera* are syntopic). This clade comes into close contact with the *buxtoni* mtDNA clade at the Anatolian Diagonal in the east, one of the most effective physical and ecological barriers for many species (reviewed in Gür, 2016). The distribution of the *buxtoni* mt clade is limited by the Zagros Mountain forest steppe, one of the five ecoregions in the Irano-Anatolian hotspots. The *armeniaca* mtDNA clade in Turkey is limited to lowlands of the Araxes Valley characterised by Mediterranean climate. However, syntopic occurrences of *ibera* and *terrestris* (this study), *ibera* and *armeniaca* (Mashkaryan et al., 2013), and *armeniaca* and *buxtoni* (Javanbakht et al., 2017) combined extensive gene flow among mtDNA clades (Mashkaryan et al., 2013; Mikulíček et al., 2013), and suggests the existence of hybrid zones and parapatric distributions. Fritz et al. (2007) found the highest genetic diversity in Transcaucasia, which is considered to be a radiation center of *Testudo*, while the present study shows that overall genetic diversity is higher in Levantine and western Iran (Table 3).

Türkozan et al. (2010) previously reported the distinctiveness of three mt clades based on only morphology, namely *ibera*, *buxtoni* (i.e., perses), and *armeniaca* in Turkey. However, they expressed their results with caution due to a lack of genetic sampling within Turkey and morphometric studies outside of Turkey. In this current study, the only morphometrically distinct mtDNA clade is *armeniaca*, which is represented a highly specialised burrowing ecomorph in Turkey and parts of Armenia. The recovery of a distinct morphology for turtles assigned to the *armeniaca* mtDNA clade, one of the most divergent mtDNA lineages of the *T. gracea* complex in Asia, has raised the possibility of a distinct species based on Turkish samples alone (Türkozan et al., 2010). Other studies (Parham, et al., 2006; Mashkaryan et al., 2013) show that the *armeniaca* mtDNA haplotype extends beyond the geographic range of the morphologically specialised *armeniaca* morphotype, to the western Caspian Sea region where they lack the burrowing ecotype. For the remainder of the mtDNA clades, it appears that morphology cannot discriminate them from each other (Parham et al., 2006; Fritz et al., 2007, Türkozan et al., 2010). We therefore recommend that the subspecies status of *Testudo* clades in west Asia should not be used, given that they are based conflicting evidence derived from mtDNA differentiation alone (Mashkaryan et al, 2013; Mikulíček et al., 2013).

There is a clear evidence that the ranges of *T. graeca* in Asia have been contracting and expanding through time. Similarly, Anadon et al. (2015) found clear niche differences among five subspecies of *T. graeca* in Africa, with rainfall playing a primary role in shaping the distribution of clades, as in Iran and Transcaucasia (Javanbakht et al., 2017). The presence of glacial refugia (Atalay, 2002), refugia within refugia (Schmitt, 2007), wide elevational range (0-5137 m), and various macro and microclimates and vegetation types (Atalay, 2002) have impacted on the distribution of *T. graeca* complex in Turkey. The extent to which these factors resulted in the currently observed genetic clades, however, remains unresolved. Future studies should focus on geographically dense genomic-scale markers such as RADSeq in combination with phenotypic data.

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Intra-individual variation in exploration behaviour in a largely aquatic frog: effects of sex and personality traits

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Behavioural plasticity is important for survival and to adapt to a dynamic environment. However, it is known that many animals exhibit fixed behavioural responses termed behavioural syndromes. That said, even when exhibiting such fixed behavioural responses, animals still show variability in their behaviour. We here evaluate the variability in exploration behaviour in the frog *Silurana* (*Xenopus*) *tropicalis* by quantifying two different metrics of variability: the absolute difference between two sets of measurements, and the individual stability statistic. Our results show differences in the intra-individual variability between groups of frogs that can be assigned to different behavioural syndromes. Marked differences in variability also occur between males and females, with males being more stereotyped in their responses. Frogs identified as belonging to different behavioural groups (i.e. shy, intermediate, and bold) differed in the variability of the expression of these strategies, with bold individuals being more stereotypic in the exploration of an identical, novel environment. These observations may have implications for the evolution of behaviour in natural populations.

Key words: locomotion, amphibian, variability, behaviour, exploration

INTRODUCTION

Behaviour is a major component of life and is related to fitness and population survival (Smith & Blumstein, 2008; Wolf & Weissing, 2012). Behaviour impacts feeding and locomotion (Sustaita et al., 2013), hunting (Steele & Anderson, 2006), reproductive success (Moore et al., 2005), territory defence (Wells, 1977), the exploration of novel environments (Simmons & Thomas, 2004), as well as anti-predator responses (Millot et al., 2009). Although behaviour was initially considered to be highly variable, research over the past few decades has shown that fixed individual behavioural strategies exist, typically termed personality traits or behavioural syndromes (Briffa & Weiss, 2010). These behavioural syndromes describe the persistence of a behavioural strategy across time and behavioural contexts. Behavioural syndromes are of interest because they have been documented across the entire animal kingdom and suggest a genetic basis of behaviour (Sih et al., 2004). Despite the fact that individuals may have fixed behavioural strategies, behavioural responses are not invariant and variability exists even within a given behavioural strategy.

This variability in behavioural response is directly linked to the adaptive nature of behaviour in contexts such as predation or predator avoidance (Niemelä et al., 2012; Furtbauer et al., 2015). Phenotypic plasticity characterises the component of a behavioural response that is context dependent, and reaction norms describe the behavioural shift occurring due to behavioural plasticity. These are two key elements describing potential variability in behaviour. Depending on individuals, reaction norms can be different and illustrate dynamic responses to variable environmental contexts (Dingemanse & Wolf, 2013). Behavioural plasticity has been observed in several species (Dingemanse et al., 2009) and is intimately linked to the evolutionary responses of animals in variable environments (Foster & Sih, 2013; Snell-Rood, 2013). In this context, understanding behavioural plasticity and the complexity of behavioural responses to variable and changing environments is of interest given the major and global modifications of natural habitats due to anthropogenic factors, including global warming and habitat fragmentation and destruction.

Here, we analyze exploration behaviour in the frog *Silurana* (*Xenopus*) *tropicalis* (Gray, 1864). Exploration behaviour is a complex behaviour that is fitness relevant (Smith & Blumstein, 2008). Previously, we (Videlier et al., 2014) characterised three distinct and repeatable exploration syndromes in this species: shy, intermediate and bold. Moreover, we demonstrated significant differences between the sexes, with males being bolder than females (Videlier et al., 2015) and similar

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exploration syndrome clusters within the sexes. Yet, the variability in the behavioural responses within each sex or exploration syndrome in a fixed behavioural context has not been explored. Here, we use two different metrics to analyse the variability shown by individuals in the expression of behavioural syndromes: the absolute difference between two or more sets of measurements (Diff-values) and a more complex indicator of variability termed the individual stability statistic (ISS) (Dingemanse et al., 2009). We use these to quantify variability in behaviour between sexes and individuals that show stable exploration syndromes.

MATERIALS AND METHODS

Animals

Silurana tropicalis (*N* = 86) were caught in the wild in Cameroon in 2009 (permit number 000117/ MINRESI/B00/C00/C10/C13). Species identification was confirmed through genetic analysis (V. Gvoždík, personal communication). An additional ten individuals that were bred in captivity were added to the data set. As no qualitative differences were observed in exploration behaviour between the captive bred and wild-caught individuals we decided to pool them for further analyses. Animals were housed by sex in groups of 8 to 12 individuals at the Muséum National d'Histoire Naturelle (MNHN) in Paris and maintained in 21-L tanks mounted on three-shelf stand-alone frog racks (Aquaneering, Inc., San Diego, CA, USA) with the water temperature set at 24 °C. This temperature is close to the optimal performance temperature of *S. tropicalis* (Herrel & Bonneaud, 2012) and similar to temperatures measured under field conditions in ponds in the forest (Careau et al., 2014). Animals were fed with beef heart and mosquito larvae twice weekly. All individuals were pit-tagged (Nonatec, Rodange, Luxembourg) for unique identification. Fifty nine females and thirty seven males were used for the present study, which was performed in 2014. All experiments were performed in accordance with European ethical and legal regulations related to animal welfare and experimentation. Behavioural recordings were approved by the local institutional ethics committee at the MNHN (#68-25). All individuals were in good condition and alive at the end of the experiments with no signs of weight loss.

Behavioural analyses

All frogs were maintained in the laboratory under identical conditions for four years before testing, and had not been used for behavioural tests prior to these experiments. For each trial, animals were released in a rectangular container (height: 40 cm, length: 100 cm, width: 20 cm) with a water level of 20 cm maintained at a temperature of 24 ± 2 °C (Videlier et al., 2014). Water was changed with water from the home cages between recordings. Animals were introduced in a clean tank and left quietly for 5 min before the onset of the recordings. Shelters (opaque ceramic 'turtle huts' of 12 x 12.5 x 5.5 cm; ZooMed, San Luis Obispo, CA, U.S.A.) were placed at the two extremities. Frogs were filmed for 60 minutes with a Quickcam Pro 500 (Logitech, Inc. at Romanel-sur-Morges, Switzerland) set at 15 frames per second. Each individual was tested three times at different times of the day (morning: 09:00 am to 12:00 pm; early afternoon: 12:00–04:00 pm; late afternoon: 04:00–08:00 pm) in a randomised way. This allowed us to test the repeatability of behaviour across different activity periods and also to test the variability in behaviour. Videos were analysed using the ProAnalyst software (Xcitex, Inc., Cambridge, MA, USA) by tracking all movements of frogs during their exploration of the environment. Coordinates of the snout-tip as a marker of individuals were extracted and used to quantify the movements of each individual from which a number of variables were extracted: the total distance moved in 60 minutes (cm); the number of all movements and complete roundtrips; the maximal, minimal, and average speed of movement (cms-1); the latency of the first, the second and the last movement (s); the average, minimal, and maximal duration of a roundtrip with pauses (s); the entire duration of exploration with and without pauses(s); the total and average duration spent hidden between two roundtrips(s); the average number of pauses and the number of roundtrips away from the wall of the aquarium. See Videlier et al. (2014, 2015) for a definition of the variables.

Measures of variability

Two methods were used to quantify the variability in the behavioural parameters listed higher.

The first is the measure of the absolute difference in the $\log_{10}$-transformed values between the three trials for each individual (i.e. difference between trials 3 and 1; between trials 3 and 2; and between trials 2 and 1), hereafter named Diff-values; where X is the Log_{10}-transformed value of one of the eighteen behavioural traits and t1 and t2 are two of the three recorded trials. The repeatability of these Diff-values was tested across the three possible combinations (the difference between trials 3 and 1; between trials 3 and 2; and between trials 2 and 1) using Pearson correlations (Pearson, 1909). Low Diff-values indicate a stable behaviour and high Diff-values indicate more labile traits with high variability.

$$\text{Diff-values } X_{t1\text{-}t2} = X\,(_{t1}) - X\,(_{t2})$$

The second method used was the ISS (individual stability statistic) proposed by Asendorp (1990) and reviewed by Dingemanse and collaborators (2009); where X is the standardized Log_{10}-transformed value of one of the eighteen behavioural traits and t1 and t2 are two of the three trials. If the ISS value is close to one, the behavioural parameter is stable and shows little variability. To test the repeatability of the ISS we also used Pearson correlations between all possible combinations.

$$\text{ISS } X_{t1\text{-}t2} = 1 - ([X\,(_{t1}) - X\,(_{t2})]^{2} / 2)$$

Diff and ISS values were tested for normality and homoscedascity before further statistical testing. ANOVAs and Tukey HSD post-hoc tests were performed using Diff-values and ISS values to test for differences

between the sexes and between individuals categorised into different behavioural syndromes within each sex (Tables 2 and 3). All analyses were performed in R (R Development Core Team, 2010). Bonferroni corrected results are indicated in Tables 1-3 by an asterisk.

RESULTS

Absolute differences in trait values

The Diff-values (trial 3 vs. trial 2, trial 3 vs. trial 1, trial 2 vs. trial 1, further referred to as 3-2, 3-1, 2-1) for each behavioural parameter were significantly correlated (all $P < 0.05$; Table 1) with correlations between these behavioural parameters ranging from 0.38 to 0.68 (Table 1). Absolute Diff-values showed a difference between sexes (Table 2). For the Diff-set composed of the first two observations (2-1), speed (maximal, minimal and average), the duration of a roundtrip (maximal, minimal and average), the latency of the last movement, the total time of exploration, and the total distance traveled during the exploration showed significant differences between the sexes, with females scoring significantly higher than males (i.e. being more variable). When considering the other difference sets (3-1 and 3-2), the number of behavioural parameters showing a sex difference decreased, with only the variability of parameters linked to speed remaining significantly different between the sexes.

Each sex can be divided into three behavioural groups or syndromes as demonstrated previously (Videlier et al., 2015). For females, behavioural groups show differences in all Diff-values except for the minimal speed (Table 3). For males, syndromes are different with the exception of Diff set 2-1. Male differences were observed for most variables except for the number of complete roundtrips, the duration of exploration without pauses, the average number of pauses, and the number of movements away from the walls. For both males and females, the Diff-values were greater for shy individuals relative to those for other behavioural groups (Table 3) indicating that individual behavioural variability was greater for shy frogs.

Individual Stability Statistics

All ISS parameters show a correlation between at least two of the three sets calculated ($P < 0.05$; see Table 1). The correlation coefficients varied between 0.16 and 0.55. As observed with the Diff-values, the ISS between the first two measures (2 vs. 1) for speed (average, maximal and minimal), the duration of a roundtrip (maximal, average and minimal), the latency of the first movement and the last movement, the duration of the total exploration, and the distance explored are different between sexes (Table 2). We also observe a decrease over time in the number of variable behavioural parameters that differs significantly between sexes, with only the variability

Table 1. Results of the Pearson correlations testing for the repeatability of the Diff and ISS values

	Diff-values	**ISS**											
	Diff3-1vs-Diff2-1	**Diff3-2vs-Diff2-1**		**Diff3-1vsDiff3-2**			**Diff3-1vsDiff2-1**		**Diff3-2vsDiff2-1**		**Diff3-1vsDiff3-1**	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
number of complete roundtrips	0.47	**< .001***	-0.53	**< 0.001***	0.47	**< 0.001***	0.34	**<.001***	0.24	**0.02**	0.18	**0.08**
number of movements	0.44	**<.0001***	-0.52	**<.0001***	0.53	**<.0001***	0.24	**0.02**	0.22	**0.03**	0.29	**<.01**
total distance moved	0.41	**<.0001***	-0.48	**<.0001***	0.60	**<.0001***	0.25	**0.01**	0.27	**0.01**	0.58	**<.0001***
average speed	0.41	**<.0001***	-0.48	**<.0001***	0.60	**<.0001***	0.32	**<.01**	0.55	**<.0001***	0.29	**<.01**
minimal speed	0.44	**<.0001***	-0.45	**<.0001***	0.60	**<.0001***	0.21	**0.04**	0.11	**0.29**	0.68	**<.0001***
maximal speed	0.44	**<.0001***	-0.45	**<.0001***	0.60	**<.0001***	0.31	**<.01**	0.52	**<.0001***	0.30	**<.01**
number of movements away from the wall	0.53	**<.0001***	-0.44	**<.0001***	0.52	**<.0001***	0.26	**0.01**	0.16	**0.01**	0.08	**0.11**
average number of pauses	0.43	**<.0001***	-0.68	**<.0001***	0.37	**0.0002***	0.23	**0.02**	0.6	**<.0001***	0.13	**0.19**
average duration of a roundtrip	0.44	**<.0001***	-0.49	**<.0001***	0.56	**<.0001***	0.35	**<.001***	0.26	**0.01**	0.46	**<.0001***
minimal duration of a roundtrip	0.46	**<.0001***	-0.55	**<.0001***	0.48	**<.0001***	0.45	**<.0001***	0.29	**<.01**	0.42	**<.0001***
maximal duration of a roundtrip	0.45	**<.0001***	-0.44	**<.0001***	0.60	**<.0001***	0.21	**0.04**	0.29	**<.01**	0.49	**<.0001***
latency of the first movement	0.44	**<.0001***	-0.50	**<.0001***	0.56	**<.0001***	0.42	**<.0001***	0.28	**<.01**	0.29	**<.01**
latency of the second movement	0.45	**<.0001***	-0.51	**<.0001***	0.53	**<.0001***	0.26	**0.01**	0.36	**<.001***	0.39	**<.001***
latency of the last movement	0.44	**<.0001***	-0.42	**<.0001***	0.63	**<.0001***	0.31	**<.01**	0.27	**<.01**	0.62	**<.0001***
duration of all movements with pauses	0.41	**<.0001***	-0.48	**<.0001***	0.60	**<.0001***	0.18	**0.09**	0.27	**<.01**	0.57	**<.0001***
duration of exploration without pauses	0.40	**<.0001***	-0.53	**<.0001***	0.56	**<.0001***	0.08	**0.41**	0.35	**<.001***	0.38	**<.001***
total time spent hidden	0.42	**<.0001***	-0.53	**<.0001***	0.55	**<.0001***	0.25	**0.01**	0.24	**0.02**	0.46	**<.0001***
average time spent hidden	0.43	**<.0001***	-0.53	**<.0001***	0.54	**<.0001***	0.27	**<.01**	0.29	**<.01**	0.38	**<.001***

bold values are significant. * significant after Bonferroni correction.

Table 2. Results of the ANOVAs testing for differences between the sexes in behavioural plasticity (Diff and ISS values). Bold values indicate significant *P*-values and highlighted cells indicate variables with significant differences. d.f. = 1,95 for all tests.

	Set 2 vs. set 1						Set 3 vs. set 1						Set 3 vs. set 2					
	Diff-Values			ISS			Diff-Values			ISS			Diff-Values			ISS		
	F	P		F	P		F	P		F	P		F	P		F	P	
number of complete roundtrips	1.13	0.29	m=f	1.32	0.25	m=f	0.57	0.45	m=f	0.27	0.60	m=f	1.64	0.20	m=f	2.37	0.13	m=f
total number of movements	3.17	0.08	m=f	3.04	0.08	m=f	0.49	0.49	m=f	0.52	0.47	m=f	0.95	0.33	m=f	1.96	0.17	m=f
average speed	23.49	**<0.001***	**f>m**	13.36	**<0.001***	**m>f**	6.85	**0.01**	**f>m**	4.07	**0.05**	**m>f**	11.76	**<0.001***	**f>m**	**6.78**	**0.01**	**m>f**
maximal speed	22.18	**<0.001***	**f>m**	14.38	**<0.001***	**m>f**	6.18	**0.01**	**f>m**	4.08	**0.05**	**m>f**	14.51	**<0.001***	**f>m**	**10.00**	**<0.01**	**m>f**
minimal speed	22.96	**<0.001***	**f>m**	13.39	**<0.001***	**m>f**	14.97	**<0.001***	**f>m**	8.30	**<0.01**	**m>f**	10.84	**<0.01**	**f>m**	**6.86**	**0.01**	**m>f**
average duration of a roundtrip	11.74	**<0.001***	**f>m**	9.86	**<0.01**	**m>f**	2.47	0.12	m=f	3.23	0.08	m=f	1.94	0.17	m=f	2.49	0.12	m=f
maximal duration of a roundtrip	8.72	**<0.001***	**f>m**	8.70	**<0.01**	**m>f**	1.56	0.22	m=f	2.50	0.12	m=f	1.90	0.17	m=f	2.78	0.10	m=f
minimal duration of a roundtrip	6.06	**0.02**	**f>m**	5.89	**0.02**	**m>f**	1.37	0.25	m=f	3.27	0.07	m>f	2.57	0.11	m=f	2.33	0.13	m=f
latency of the first movement	4.38	**0.04**	**f>m**	5.96	**0.02**	**m>f**	3.27	0.07	m=f	4.33	**0.04**	**m>f**	1.44	0.23	m=f	2.35	0.13	m=f
latency of the second movement	2.31	0.13	m=f	3.15	0.08	m=f	0.21	0.65	m=f	0.70	0.41	m=f	0.27	0.61	m=f	0.47	0.49	m=f
latency of the last movement	9.85	**<0.01**	**f>m**	8.56	**<0.01**	**m>f**	2.69	0.10	m=f	2.31	0.13	m=f	2.91	0.09	m=f	2.57	0.11	m=f
duration of all movements with pauses	9.83	**<0.01**	**f>m**	8.29	**<0.01**	**m>f**	2.44	0.12	m=f	1.41	0.24	m=f	1.92	0.17	m=f	1.20	0.28	m=f
duration of exploration without pauses	1.50	0.22	m=f	1.50	0.22	m=f	0.14	0.71	m=f	0.01	0.93	m=f	0.01	0.93	m=f	0.21	0.65	m=f
total time spent hidden	2.95	0.09	m=f	2.50	0.12	m=f	0.54	0.47	m=f	0.35	0.56	m=f	0.55	0.46	m=f	0.38	0.54	m=f
average time spent hidden	3.39	0.07	m=f	3.61	0.06	m=f	0.05	0.82	m=f	0.00	0.95	m=f	0.23	0.64	m=f	0.06	0.81	m=f
average number of pauses	2.09	0.15	m=f	1.92	0.17	m=f	0.00	0.99	m=f	0.09	0.77	m=f	1.46	0.23	m=f	1.70	0.20	m=f
number of movements away from the wall	0.35	0.55	m=f	0.07	0.79	m=f	0.08	0.77	m=f	0.27	0.60	m=f	0.48	0.49	m=f	0.24	0.63	m=f
total distance moved	6.51	**0.01**	**f>m**	6.54	**0.01**	**m>f**	2.57	0.11	m=f	2.09	0.15	m=f	3.08	0.08	m=f	3.32	0.07	m=f

* significant after Bonferroni correction for multiple testing. m = male; f = female

in speed remaining different in all cases (Table 2). In addition, the ISS also differs between behavioural groups. As observed with Diff-values, there was no significant difference in minimal speed, the number of complete roundtrips, the total number of movements, and the number of movements away from the wall for female behavioural groups. For males ISS, all syndromes show differences except for the set 2-1 as observed for the Diff-values. However, for both females and males, the shy group has lower ISS values compared to the bold or intermediate group (Table 3) suggesting again a greater variability for shy frogs.

DISCUSSION

Sex differences in individual variation in behaviour

Sexual dimorphism is observed across the entire animal kingdom and is a direct response to the optimisation of reproduction in the two sexes (Lande, 1980; Hedrick & Temeles, 1989). Although sexual dimorphism in morphological traits is well documented, other phenotypic traits including behaviour, metabolism, and performance can also be sexually dimorphic (Shine, 1979; Payne, 1984; Post et al., 1999; Shillington, 2005; Labus et al., 2013; Tomlinson & Phillips, 2015). In frogs, sexual dimorphism in body size, morphology, and performance has been documented (Le Galliard & Ferrière, 2008; Herrel et al., 2014; Gordon et al., 2015). Moreover, distinct differences in the behaviour of the two sexes has been demonstrated in frogs (Kelley, 1988). *Silurana tropicalis* is no exception with females being larger than males, but males having relatively longer limbs and a relatively greater endurance capacity (Herrel et al., 2012). Moreover, females of this species are shyer than males (Videlier et al., 2015).

Our analyses (Table 2) also show that females display larger differences between the first two sets of measurements and generally lower ISS values than males. Surprisingly this suggests that females show a greater variability in the expression of their behaviour than males. Shy individuals are averse to risk taking and explore their environment less. Yet, despite restricting their movements, females show more intrinsic variability in the way they explore their environment. Interestingly, over time the difference between the sexes is reduced to variables relating to differences in the variability in the speed of their exploration. Locomotor speed often differs between the sexes, including in snakes (Shine et al., 2003), lizards (Lailvaux et al., 2003), and frogs (Herrel & Bonneaud, 2012; Herrel et al., 2012). However, these

Table 3. ANOVAs testing for differences in behavioral plasticity between behavioural groups. Both sexes were tested separately. Bold values indicate significant *P*-values and highlighted cells indicate variables with significant differences. d.f. = 2,95 for all tests.

MALE // Diff values	Set 2 vs. set 1			Set 3 vs. set 1			Set 3 vs. set 2		
	F	P		F	P		F	P	
number of complete roundtrips	2.15	0.13	Bold=Interm=Shy	0.12	0.89	Bold=Interm=Shy	0.80	0.46	Bold=Interm=Shy
total number of movements	1 .22	0.31	Bold=Interm=Shy	**5.20**	**0.01**	**(Bold=Interm)<Shy**	2.95	0.07	Bold=Interm=Shy
average speed	1.86	0.16	Bold=Interm=Shy	**13.65**	**<0.001***	**(Bold=Interm)<Shy**	**21.51**	**<0.001***	**(Bold=Interm)<Shy**
maximal speed	0.87	0.43	Bold=Interm=Shy	**10.64**	**<0.001***	**(Bold=Interm)<Shy**	**15.39**	**<0.001***	**(Bold=Interm)<Shy**
minimal speed	2.15	0.13	Bold=Interm=Shy	**6.11**	**<0.01**	**(Interm<Shy)=Bold**	**8.46**	**<0.01**	**(Bold=Interm)<Shy**
average duration of a roundtrip	0.57	0.57	Bold=Interm=Shy	**9.87**	**<0.001***	**(Bold=Interm)<Shy**	**24.77**	**<0.001***	**(Bold=Interm)<Shy**
maximal duration of a roundtrip	0.28	0.75	Bold=Interm=Shy	**8.76**	**<0.001***	**(Bold=Interm)<Shy**	**27.55**	**<0.001***	**(Bold=Interm)<Shy**
minimal duration of a roundtrip	1.42	0.25	Bold=Interm=Shy	**4.36**	**0.02**	**(Interm<Shy)=Bold**	**10.29**	**<0.001***	**(Bold=Interm)<Shy**
latency of the first movement	2.00	0.15	Bold=Interm=Shy	2.07	0.14	Bold=Interm=Shy	**4.49**	**0.02**	**(Bold<Shy)=Interm**
latency of the second movement	1.52	0.23	Bold=Interm=Shy	**7.71**	**<0.01**	**(Bold=Interm)<Shy**	2.22	0.12	Bold=Interm=Shy
latency of the last movement	0.29	0.75	Bold=Interm=Shy	**19.29**	**<0.001***	**(Bold=Interm)<Shy**	**17.05**	**<0.001***	**(Bold=Interm)<Shy**
duration of all movements with pauses	2.79	0.08	Bold=Interm=Shy	**27.56**	**<0.001***	**(Bold=Interm)<Shy**	**35.75**	**<0.001***	**(Bold=Interm)<Shy**
duration of exploration without pauses	**4.11**	**0.02**	**(Interm<Shy)=Bold**	2.10	0.14	Bold=Interm=Shy	**4.08**	**0.02**	**(Bold<Shy)=Interm**
total time spent hidden	2.21	0.13	Bold=Interm=Shy	**15.71**	**<0.001***	**(Bold=Interm)<Shy**	**7.03**	**<0.01**	**(Bold=Interm)<Shy**
average time spent hidden	22.45	0.10	Bold=Interm=Shy	**11.89**	**<0.001***	**(Bold=Interm)<Shy**	**7.67**	**<0.01**	**(Bold=Interm)<Shy**
average number of pauses	0.27	0.77	Bold=Interm=Shy	0.26	0.77	Bold=Interm=Shy	0.25	0.78	Bold=Interm=Shy
number of movements away from the wall	0.90	0.42	Bold=Interm=Shy	0.17	0.85	Bold=Interm=Shy	1.07	0.35	Bold=Interm=Shy
total distance moved	2.02	0.15	Bold=Interm=Shy	**17.60**	**<0.001***	**(Bold=Interm)<Shy**	**26.90**	**<0.001***	**(Bold=Interm)<Shy**

* significant after Bonferroni correction for multiple testing.

MALE // ISS values	Set 2 vs. set 1			Set 3 vs. set 1			Set 3 vs. set 2		
	F	P		F	P		F	P	
number of complete roundtrips	0.87	0.43	Bold=Interm=Shy	0.18	0.84	Bold=Interm=Shy	0.43	0.66	Bold=Interm=Shy
total number of movements	1.19	0.32	Bold=Interm=Shy	4.21	0.02	Bold=Interm=Shy	1.71	0.20	Bold=Interm=Shy
average speed	2.35	0.11	Bold=Interm=Shy	**18.59**	**<0.0001***	**Shy<(Bold=Interm)**	**16.95**	**<0.0001***	**Shy<(Bold=Interm)**
maximal speed	1.89	0.17	Bold=Interm=Shy	**16.73**	**<0.0001***	**Shy<(Bold=Interm)**	**14.17**	**<0.0001***	**Shy<(Bold=Interm)**
minimal speed	1.15	0.33	Bold=Interm=Shy	**6.93**	**<0.01**	**Shy<(Bold=Interm)**	**10.26**	**<0.001***	**Shy<(Bold=Interm)**
average duration of a roundtrip	0.91	0.41	Bold=Interm=Shy	**10.07**	**<0.001***	**Shy<(Bold=Interm)**	**24.70**	**<0.0001***	**Shy<(Bold=Interm)**
maximal duration of a roundtrip	0.47	0.63	Bold=Interm=Shy	**11.00**	**<0.001***	**Shy<(Bold=Interm)**	**30.50**	**<0.0001***	**Shy<(Bold=Interm)**
minimal duration of a roundtrip	1.05	0.36	Bold=Interm=Shy	3.20	0.05	Bold=Interm=Shy	**6.20**	**0.01**	**Shy<(Bold=Interm)**
latency of the first movement	2.68	0.08	Bold=Interm=Shy	2.07	0.14	Bold=Interm=Shy	**6.36**	**<0.01**	**Shy<(Bold=Interm)**
latency of the second movement	2.11	0.14	Bold=Interm=Shy	**7.05**	**<0.01**	**Shy<(Bold=Interm)**	2.52	0.0953	Bold=Interm=Shy
latency of the last movement	0.47	0.63	Bold=Interm=Shy	**21.56**	**<0.0001***	**Shy<(Bold=Interm)**	**20.60**	**<0.0001***	**Shy<(Bold=Interm)**
duration of all movements with pauses	2.46	0.10	Bold=Interm=Shy	**28.80**	**<0.0001***	**Shy<(Bold=Interm)**	**46.52**	**<0.0001***	**Shy<(Bold=Interm)**
duration of exploration without pauses	3.13	0.06	Bold=Interm=Shy	1.07	0.35	Bold=Interm=Shy	3.10	0.06	Bold=Interm=Shy
total time spent hidden	2.66	0.08	Bold=Interm=Shy	**18.71**	**<0.0001***	**Shy<(Bold=Interm)**	**8.31**	**<0.01**	**Shy<(Bold=Interm)**
average time spent hidden	3.03	0.06	Bold=Interm=Shy	**17.24**	**<0.0001***	**Shy<(Bold=Interm)**	**11.69**	**<0.001***	**Shy<(Bold=Interm)**
average number of pauses	0.29	0.75	Bold=Interm=Shy	0.14	0.87	Bold=Interm=Shy	0.31	0.732	Bold=Interm=Shy
number of movements away from the wall	0.69	0.69	Bold=Interm=Shy	0.03	0.97	Bold=Interm=Shy	0.61	0.55	Bold=Interm=Shy
total distance moved	1.21	0.31	Bold=Interm=Shy	**20.56**	**<0.0001***	**Shy<(Bold=Interm)**	**25.59**	**<0.0001***	**Shy<(Bold=Interm)**

* significant after Bonferroni correction for multiple testing.

FEMALE // Diff values	Set 2 vs. set 1			Set 3 vs. set 1			Set 3 vs. set 2		
	F	P		F	P		F	P	
number of complete roundtrips	**3.67**	**0.03**	**(Shy<Interm)=Bold**	**4.50**	**0.01**	**Shy<(Bold=Interm)**	**4.12**	**0.02**	**(Shy<Bold)=Interm**
total number of movements	**3.61**	**0.03**	**(Bold<Interm)=Shy**	1.54	0.22	Bold=Interm=Shy	0.56	0.58	Bold=Interm=Shy
average speed	**7.31**	**<0.001***	**Bold< (Interm=Shy)**	**4.44**	**0.02**	**(Bold<Interm)=Shy**	**4.88**	**0.01**	**(Bold<Interm)=Shy**
maximal speed	**5.81**	**<0.001***	**(Bold<Interm)=Shy**	**4.56**	**0.01**	**(Bold<Interm)=Shy**	**7.89**	**<0.001***	**(Bold<Interm)=Shy**
minimal speed	1.53	0.022	Bold=Interm=Shy	0.22	0.8	Bold=Interm=Shy	0.25	0.78	Bold=Interm=Shy
average duration of a roundtrip	**15.00**	**<0.001***	**Bold<Interm<Shy**	**9.93**	**<0.001***	**Bold<Interm<Shy**	**5.19**	**<0.01**	**(Bold<Interm)=Shy**
maximal duration of a roundtrip	**12.56**	**<0.001***	**Bold< (Interm=Shy)**	**5.71**	**<0.01**	**(Bold<Shy)=Interm**	**3.44**	**0.04**	**(Bold<Interm)=Shy**
minimal duration of a roundtrip	**16.55**	**<0.001***	**Bold<Interm<Shy**	**10.72**	**<0.001***	**(Bold=Interm)<Shy**	3.15	0.05	Bold=Interm=Shy
latency of the first movement	**10.01**	**<0.001***	**(Bold=Interm)<Shy**	**10.41**	**<0.001***	**Bold<Interm<Shy**	3.07	0.05	Bold=Interm=Shy
latency of the second movement	**6.89**	**<0.01**	**(Bold=Shy)<Interm**	**7.89**	**<0.001***	**Shy<Bold<Interm**	**6.26**	**<0.01**	**Shy<Bold<Interm**
latency of the last movement	**9.64**	**<0.001***	**Bold<Interm<Shy**	**8.70**	**<0.001***	**Bold<(Interm=Shy)**	**5.79**	**<0.01**	**(Bold<Interm)=Shy**
duration of all movements with pauses	**10.45**	**<0.001***	**Bold<(Interm=Shy)**	**6.13**	**<0.01**	**Bold<(Interm=Shy)**	**4.01**	**0.02**	**(Bold<Interm)=Shy**
duration of exploration without pauses	**5.81**	**<0.01**	**Bold<(Interm=Shy)**	2.27	0.11	Bold=Interm=Shy	1.99	0.15	Bold=Interm=Shy
total time spent hidden	**9.64**	**<0.001***	**(Bold=Shy)<Interm**	**9.18**	**<0.001***	**(Bold=Shy)<Interm**	**5.20**	**<0.01**	**(Bold=Shy)<Interm**
average time spent hidden	**9.08**	**<0.001***	**(Bold=Shy)<Interm**	**8.38**	**<0.001***	**(Bold=Shy)<Interm**	**5.58**	**<0.01**	**(Bold=Shy)<Interm**
average number of pauses	**7.75**	**<0.01**	**Bold<(Interm=Shy)**	**5.37**	**<0.01**	**Shy <(Bold=Interm)**	**5.01**	**<0.01**	**(Bold<Shy)=Interm**
number of movements away from the wall	**4.55**	**0.01**	**(Shy<Interm)=Bold**	**3.74**	**0.03**	**(Shy<Bold)=Interm**	**3.58**	**0.03**	**(Shy<Bold)=Interm**
total distance moved	**4.84**	**0.01**	**(Bold<Interm)=Shy**	3.56	0.03	Bold=Interm=Shy	2.43	0.10	Bold=Interm=Shy

* significant after Bonferroni correction for multiple testing.

FEMALE // ISS values	Set 2 vs. set 1			Set 3 vs. set 1			Set 3 vs. set 2		
	F	P		F	P		F	P	
number of complete roundtrips	1.06	0.35	Bold=Interm=Shy	1.87	0.16	Bold=Interm=Shy	2.54	0.09	Bold=Interm=Shy
total number of movements	2.54	0.09	Bold=Interm=Shy	2.44	2.44	Bold=Interm=Shy	0.56	0.58	Bold=Interm=Shy
average speed	**3.58**	**0.03**	**Bold=Interm=Shy**	**3.30**	**0.04**	**(Interm<Bold)=Shy**	2.57	0.09	Bold=Interm=Shy
maximal speed	2.59	0.08	Bold=Interm=Shy	**4.0**	**0.02**	**(Interm<Bold)=Shy**	**4.66**	**0.01**	**(Interm<Bold)=Shy**
minimal speed	0.94	0.39	Bold=Interm=Shy	0.12	0.89	Bold=Interm=Shy	0.32	0.73	Bold=Interm=Shy
average duration of a roundtrip	**15.88**	**<0.0001***	**Shy<Interm<Bold**	**13.55**	**<0.0001***	**Shy<Interm<Bold**	**5.49**	**0.01**	**(Shy<Bold)=Interm**
maximal duration of a roundtrip	**10.43**	**<0.001***	**Shy<Interm<Bold**	**5.85**	**<0.01**	**Shy<Interm<Bold**	**3.70**	**0.03**	**Bold=Interm=Shy**
minimal duration of a roundtrip	**24.76**	**<0.0001***	**Shy<(Bold=Interm)**	**17.33**	**<0.0001***	**Shy<(Bold=Interm)**	**5.12**	**0.01**	**Shy<(Bold=Interm)**
latency of the first movement	**17.3**	**<0.0001***	**Shy<(Bold=Interm)**	**16.72**	**<0.0001***	**Shy<(Bold=Interm)**	**4.96**	**0.01**	**(Shy<Bold)=Interm**
latency of the second movement	**4.96**	**0.01**	**(Interm<Bold)=Shy**	**7**	**<0.01**	**Interm<Bold<Shy**	**5.98**	**<0.01**	**(Interm<Bold)=Shy**
latency of the last movement	**8.56**	**<0.001***	**Shy<Interm<Bold**	**8.56**	**<0.001***	**Shy<Interm<Bold**	**5.31**	**0.01**	**(Interm<Bold)=Shy**
duration of all movements with pauses	**8.2**	**<0.001***	**(Interm=Shy)<Bold**	**4.88**	**0.01**	**(Bold<Shy)=Interm**	**3.53**	**0.04**	**(Interm<Bold)=Shy**
duration of exploration without pauses	**4.5**	**0.02**	**(Interm<Bold)=Shy**	1.83	0.17	Bold=Interm=Shy	2.06	0.14	Bold=Interm=Shy
total time spent hidden	**7.53**	**<0.01**	**(Interm<Bold)=Shy**	**5.98**	**<0.01**	**(Interm<Bold)=Shy**	**3.33**	**0.04**	**Bold=Interm=Shy**
average time spent hidden	**6.57**	**<0.01**	**(Interm<Bold)=Shy**	**5.88**	**<0.01**	**(Interm<Bold)=Shy**	**4.24**	**0.02**	**(Interm<Bold)=Shy**
average number of pauses	**4.89**	**0.01**	**(Interm=Shy)<Bold**	2.35	0.11	Bold=Interm=Shy	**8.05**	**<0.001***	**Shy<(Bold=Interm)**
number of movements away from the wall	2.6	0.08	Bold=Interm=Shy	1.82	0.17	Bold=Interm=Shy	1.95	0.15	Bold=Interm=Shy
total distance moved	**3.42**	**0.04**	**(Interm<Bold)=Shy**	**4.00**	**0.02**	**(Interm<Bold)=Shy**	0.15	0.05	Bold=Interm=Shy

* significant after Bonferroni correction for multiple testing.

are typically measured as maximal locomotor capacity. Here, variability in the voluntary speeds selected during the exploration of a novel environment differed, with males selecting higher speeds that differ less from one trial to the next (Videlier et al., 2015). Voluntary speed can be different from maximal speed and be dependent on condition, on season, and on sex as observed in salamanders (Finkler et al., 2003). Male *S. tropicalis* showed a more stereotyped movement speed across trials. Females, in contrast, adapt their movement speed more based on their experience with the experimental condition. This suggests that both sexes show a different level of flexibility in their behavioural response, even under stable environmental conditions. Whereas behaviour in males thus appears more 'hard-wired', females show evidence of a more dynamic response in the expression of their behaviour.

Variability within behavioural syndromes

In *S. tropicalis* three different behavioural syndromes have been observed: bold, shy, and intermediate (Videlier et al., 2014). These three syndromes were moreover identified for both sexes (Videlier et al., 2015). Our two measures of behavioural variability (Diff and ISS) showed similar results when comparing the three syndromes within each sex. In both sexes, shy frogs show greater differences between two sets of measurements and lower ISS values compared to bold and intermediate frogs. Part of this greater variability may have been caused by the fact that shy frogs move very little. Consequently, any additional movement from one trial to another may impact the Diff or ISS scores to a greater degree than for animals that already move much more, such as intermediate or bold individuals. Bold individuals have been described as more proactive (Sih et al., 2004; Bell, 2007; Frost et al., 2007) with a curiosity to explore (Von Merten & Siemers, 2012) and are generally more aggressive (Kralj-Fišer & Schneider, 2012) compared to shy individuals. Despite the consistency of individuals in their behavioural response, intra-individual variation can been observed (Dingemanse et al., 2007; Highcock & Carter, 2014). Bold individuals appear more rigid in their behavioural pattern and show less variability from one trial to the next. This observation is pertinent and this has also been documented in mice and fish (Benus et al., 1990; Sih et al., 2004; Kareklas et al., 2016). To conclude, in general, proactive (bold) individuals appear more stereotyped than reactive (shy) ones. Interestingly, for males, differences between syndromes appear only when comparing the third set of measurements to sets one and two, contrary to females which show consistent differences across all sets of measurements. This further highlights the conservative nature of males showing more stereotyped movements and exploration behaviours.

Habitat fragmentation is known to strongly affect the tropical West-African rainforest belt which is the native home of these frogs (Achard et al., 2002; Wright, 2005). Habitat fragmentation is likely to act on exploration behaviour. Indeed, bold individuals may be selected for to counter the effect of an increase in the distance between isolated fragments (Berg et al., 2010; Buckley et al., 2013). Consequently natural selection may erode the variability in exploration behaviours observed if exploration behaviour is indeed heritable (Falconer, 1961). Several previous studies have highlighted the heritable nature of exploration behaviour (Dingemanse et al., 2002; Van Oers et al., 2004) suggesting that this may also be the case in *S. tropicalis*. The link between boldness and aggression (Quinn & Cresswell, 2005; Wilson & Godin, 2009; Thomson et al., 2011) could constitute another selective advantage of bold individuals, especially in the context of male-male competition and anti-predator behaviour. This may over time induce more stereotyped exploration behaviours in populations, resulting in an overall behavioural specialisation. However, in the context of global change resulting in more variable climatic contexts, generalist individuals may have higher fitness than specialists (Berg et al., 2010; Sih, 2013). Moreover, it has been suggested that generalists maintain gene flow more and can adapt faster to change (Simmons & Thomas, 2004). Thus, our laboratory study of the intra-individual variability of exploration behavioural in *S. tropicalis* highlights how habitat fragmentation could potentially impact this species in the wild. More stereotyped and specialised responses, in addition to the multiple problems already induced by habitat fragmentation, may impact the resilience of a population to environmental change leading possibly to reduced gene flow (Dixo et al., 2009), an increase in local adaptation (Huey et al., 2009), and possibly even species extinction (Hilliers et al., 2008).

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Autecology of neotropical lizard species *Anotosaura vanzolinia* (Squamata, Gymnophthalmidae) in a Caatinga region, north-eastern Brazil

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Here we investigate the autecology of the poorly known lizard *Anotosaura vanzolinia* (Squamata: Gymnophthalmidae) and describe diet, reproductive biology and morphological aspects, testing hypotheses of seasonality and ontogenetic differences. We collected 154 specimens (44 males, 41 females and 69 juveniles) from April 2011 to June 2014, where 101 were found buried in soil. Their diet consists mainly of arthropods found within its microhabitat, including ants and termites, but differences were found between adults and juveniles, and between seasons. Reproduction occurs during the wet season, even though reproductive males could be found in almost all months of the year. Females have fixed clutch size of two eggs, producing more than one clutch during the reproductive season; the incubation period is about 43 to 49 days.

Key words: microhabitat use, diet, reproduction, sexual dimorphism, hatchling size, semiarid.

INTRODUCTION

The family Gymnophthalmidae contains approximately 235 species distributed in 48 genera (Uetz, 2017). These species comprise small lizards (from 40 to 150 mm of snout-vent length) that occur from southern Mexico to Argentina, in Central and South America, and in the West Indies and a number of continental islands (Presch, 1980). They display a wide variety of ecological patterns, inhabiting varied Neotropical habitats, and consisting of terrestrial (e.g. *Vanzosaura* spp.) and fossorial (e.g. *Bachia* spp.) species, as well as semi-aquatic (e.g. *Potamites* spp.) and semi-arboreal (e.g. *Placosoma* spp.) species (Pianka & Vitt, 2003).

In Brazil, there are approximately 93 gymnophthalmid species distributed in 33 genera (Costa & Bérnils, 2015). *Anotosaura*, one of those genera, comprises two species: *A. vanzolinia* Dixon, 1974 (Fig. 1) and *A. collaris* Amaral, 1933, both inhabiting mesic environments in the Caatinga region. However, *A. vanzolinia* seems to be more widely distributed within the biome than *A. collaris*, which currently has only been recorded in mountain regions from northern Bahia State (Rodrigues et al., 2013). To date, *A. vanzolinia* has been recorded in the Brazilian states of Alagoas (Gonçalves et al., 2012), Bahia (Freitas & Silva, 2007; Garda et al., 2013), Pernambuco (Dixon, 1974; Pedrosa et al., 2014), Rio Grande do Norte (Gogliath et al., 2010) and Paraíba (Rodrigues, 1986; Delfim & Freire, 2007; Oliveira & Pessanha, 2013).

Currently, we know that *A. vanzolinia* is a semifossorial lizard that lives in leaf litter and feeds mainly on soil arthropods (Delfim & Freire, 2007; Freire et al., 2009; Oliveira & Pessanha, 2013). However, detailed ecological studies of this species are rare. Oliveira & Pessanha (2013) described both the microhabitat use and the diet of *A. vanzolinia*, and Oliveira et al. (2017) examed its endoparasitic fauna. Despite these, some important information, like reproductive ecology, morphological aspects and sexual dimorphism are still completely unknown for this species.

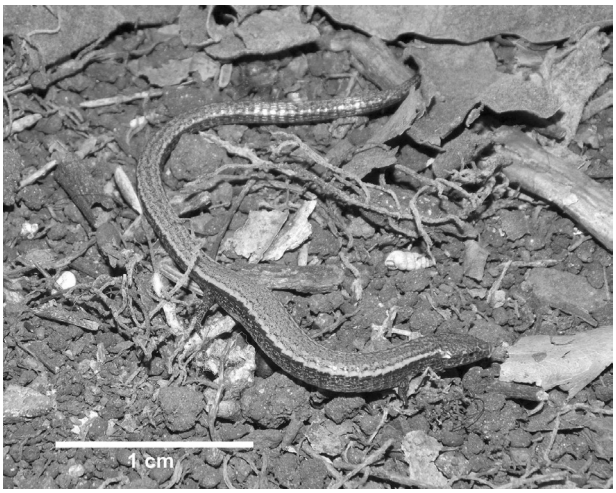

Figure 1. Adult *A. vanzolinia* from Caatinga in Campina Grande, Paraíba, Brazil.

Here we perform a detailed study examining the

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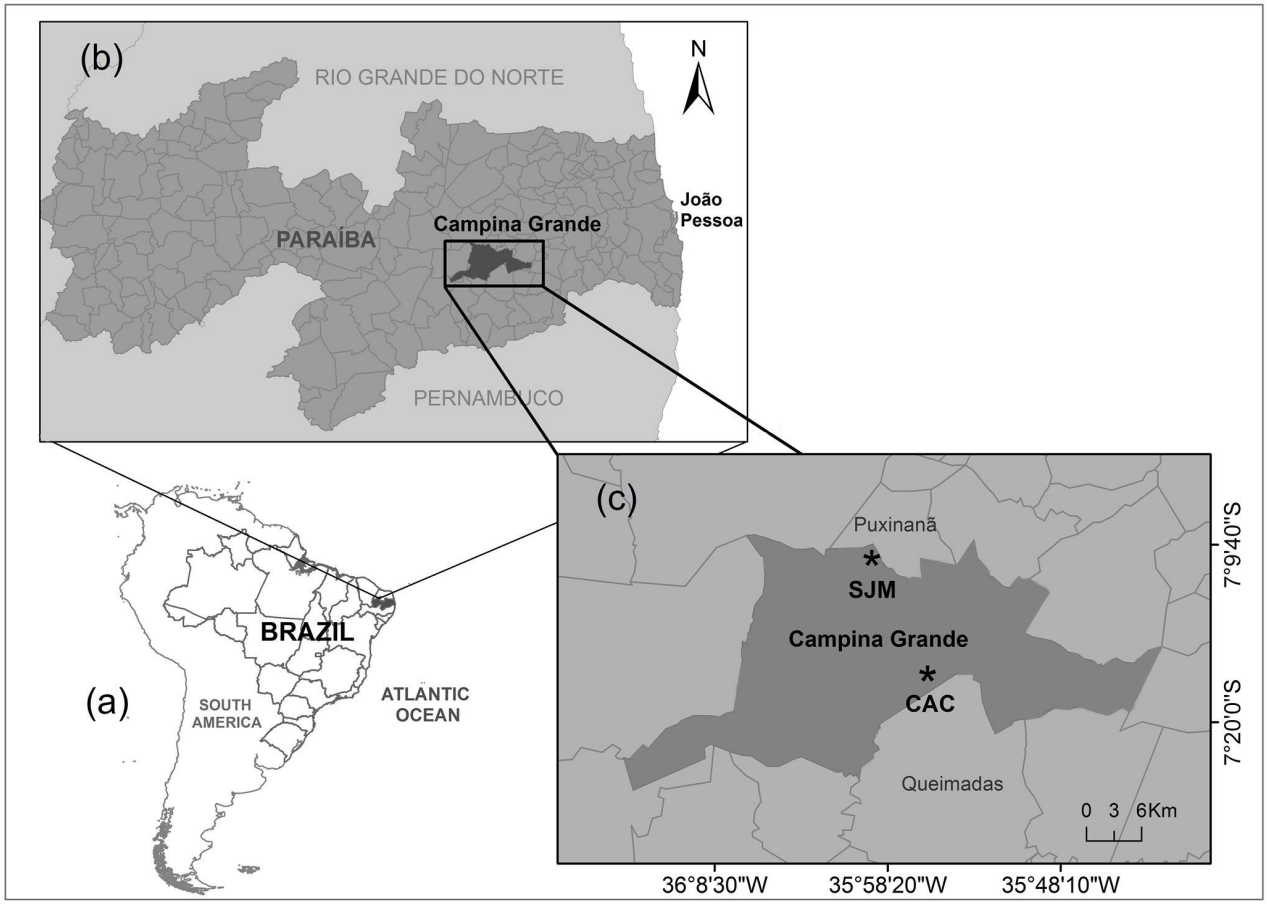

Figure 2. Collecting sites in Paraíba State (a) in North-east Brazil, Campina Grande municipality; (b) Study areas; (c) Forest Fragment at São José da Mata (SJM) and Forest Park Complexo Aluízio Campos (CAC).

autecology of population of the lizard *A. vanzolinia* from the Caatinga Region in north-east Brazil. Specifically, we describe the diet, reproductive biology and morphological aspects of this species. We also tested the hypothesis that dietary composition is affected by seasonality and ontogetic differences. Lastly, we looked for sexual dimorphism in body size and shape.

MATERIALS AND METHODS

Study area and collecting procedures

We collected the lizards from the "Parque Florestal Complexo Aluízio Campos" and from a forest fragment in São José da Mata district, both in Paraiba State, north-eastern Brazil (Fig. 2), about 20 km away from each other. The "Parque Florestal Complexo Aluízio Campos" (7°16'34"S, 35°53'7"W) is a Caatinga area at an altitude of approximately 500 m, with shrubby vegetation represented mainly by Bromeliaceae and Cactaceae, and a large number of rock outcrops and accumulated leaf litter (Alves et al., 2010; Silva et al., 2010). São José da Mata district (7°11'2.85"S, 35°59'6.17"W), with an altitude of approximately 700 m, is located between the arboreal formation locally known as Brejo and consists of a typical Caatinga vegetation. It is probably one of the last remaining transitional arboreal vegetation in Paraíba State, containing typical plant species from both Caatinga and Atlantic Forest (MMA, 2003).

This region has a tropical climate with a mean temperature of 22.9 ˚C; January being the hottest (about 24.5 ˚C) and July the coldest (about 20.7 ˚C) months of the year. The highest precipitation rates occur in April (around 115 mm) and the lowest in October (about 13 mm) and November (about 7 mm) (Climate-Date, 2016). Here, we consider the wet season from March to August and the dry season from September to February, based on monthly rainfall indices (Climate-Date, 2016).

Sampling from "Parque Florestal Complexo Aluízio Campos" occurred once a month from July 2013 to June 2014. At these times, we searched for and hand collected animals by revolving the leaf litter, rocks, termite nests, and fallen logs, and by digging in the soil with gardening shovels. Sampling was always carried out during the day (from 0800 h to 1700 h). Sampling from the Forest Fragment at São José da Mata occurred monthly from May 2013 to April 2014. At these times, we used only pitfall-traps installed on six sampling units at the site. Each sampling unit contained one central and three external 20L buckets arranged in a Y-shape and joined by 3 m long plastic fences. The traps remained open for 15 days per month. In addition, we also analysed the lizards collected by Oliveira & Pessanha (2013), from April–May, July–August 2011.

All collected lizards were euthanised with a lethal injection of 2% lidocaine hydrochloride, preserved with 10% formalin and stored in 70% alcohol. All collected specimens were housed in Coleção Herpetológica da Universidade Federal da Paraíba (CHUFPB, João Pessoa, Brazil).

Microhabitat use

We defined three categories to describe the microhabitat used by this species: (1) buried in soil, (2) between leaf litter and (3) under rocks. We did not categorise the microhabitats of lizards collected from pitfall-traps. To describe solar incidence at the collecting site, we used three categories: (1) sunny, (2) shaded, and (3) cloudy.

Diet

We dissected the lizards and analysed their gastrointestinal tracts under a stereomicroscope. We identified prey items to (usually) order level. Prey volume was estimated using the ellipsoid formula:

$$V = \frac{4}{3}\pi\left(\frac{length}{2}\right)^2\left(\frac{width}{2}\right)$$

To determine the relative contribution of each prey category in the lizards diet, we calculated a Relative Importance Index (RII) introduced by Pinkas et al. (1971):

$$RII = F\% \times (N\% + V\%)$$

where *F* represents frequency of prey category, *N* is the total number of prey category and *V* is the total volume of prey category.

We calculated volumetric and numeric niche breadth for individual and pooled stomachs using the inverse of the Simpson's diversity index (Simpson, 1949):

$$B = \frac{1}{\sum_{i=1}^{n} p_i^2}$$

where *i* represents prey category, *p* is the proportion of category *i*, and *n* is the total number of categories.

To investigate dietary overlap between males and females and between adults and juveniles, we calculated numeric and volumetric niche overlap indices using the overlap equation (Pianka, 1973):

$$O_{jk} = \frac{\sum_{i}^{n} p_{ij} p_{ik}}{\sqrt{\sum_{i}^{n} p_{ij}^2 \sum_{i}^{n} p_{ik}^2}}$$

where *p* is the proportion of the prey category *i*, *n* is the number of categories, and *j* and *k* represent the groups being compared (males/females; adult/juveniles). The overlap φ_{jk} ranges from 0 (no overlap) to 1 (complete overlap). To investigate differences among groups, based on the presence of non-random patterns in the niche overlap, we used the Niche Overlap Module of EcoSim.

We compared sexual, ontogenetic and seasonal dietary differences with the nonparametric Wilcoxon test, using the five prey categories with the highest values of Relative Importance Index (*RII*).

Sexual dimorphism

We measured the snout-vent length (SVL), tail length, head length, width and height, body width and height, and forelimb and hindlimb length of each lizard using a digital calliper. We log-transformed (base 10) all morphometric variables prior to analysis to meet the requirements of normality. We considered the body size to be an isometric size variable defined a *priori* by the multiplication of $p^{-0.5}$, with the $n \times p$ matrix of log-transformed data, where *p* is the number of variables and *n* is the number of observations (Jolicoeur, 1963; Somers, 1986). To remove the effects of body size from the log-transformed variables, we used Burnaby's method (Burnaby, 1966) defined as:

$$L = I_p - V(V^T V)^{-1} V^T,$$

where I_p is the identity matrix $p \times p$, *V* is the isometric size eigenvector defined above and V^T is the transpose of matrix *V* (Rohlf & Bookstein, 1987). Hereafter, we refer to the resulting size adjusted variables as shape variables. To test the null hypothesis that there is no morphological difference between males and females, we conducted a separate analysis of variance in body size (ANOVA), and we created an empty model based on logistic regressions and included the significant shape variables with the lowest AIC values to create the best model that explains the variation between shape variables among sexes. *A posteriori*, we performed a discriminant analysis based on 9999 bootstrap replications to determine the misclassification error based on the selected variables from logistic regression models.

Reproduction

The sex of each lizard was determined by direct observation of the gonads. We defined the minimum size at sexual maturity based on the smallest reproductive male and female. Therefore, all lizards with equal or bigger SVL were considered adults.

We described females as reproductive by the presence of vitellogenic follicles and/or oviductal eggs. We considered the simultaneous presence of vitellogenic follicles and oviductal eggs to indicate the production of more than one clutch per reproductive season. We considered males to be reproductively active when they presented enlarged testis and convoluted epididymis.

In females, we measured the width and length of vitellogenic follicles and oviductal eggs when present. In males, we measured the width and length of the testis. We estimated the volume of eggs, follicles and testis using the ellipsoid formula described above. We also conducted regressions between SVL and testis/egg volume. For males, we used an ANCOVA to consider the effect of SVL on individuals and analysed monthly variations in testis volume.

In order to obtain information about hatchling size and incubation time, five clutches were collected and maintained in terrariums under natural temperature and humidity conditions. After hatching, the hatchlings were euthanised, measured and submitted to the same procedures described previously.

We conducted all statistical analyses (except Niche Overlap) using the software R (R Development Core Team 2015), with a significance level of 5% to reject null hypotheses.

Table 1. Dietary composition of *A. vanzolinia* (n= 120) from Caatinga. F = frequency; N = number; V = volume; RII = relative importance index.

Prey Items	Occurance		Pooled stomachs					
	F	F%	N	N%	V	V%	RII	RII%
GASTROPODA								
Pulmonata	1	0.83	1	0.15	8.00	0.40	0.46	0.01
DIPLOPODA	1	0.83	1	0.15	3.00	0.15	0.25	0.01
ARACHNIDA								
Araneae	10	8.33	14	2.12	68.88	3.46	46.47	1.03
MALACOSTRACA								
Isopoda	6	5.00	15	2.27	19.53	0.98	16.25	0.36
INSECTA								
Blattaria	7	5.83	11	1.66	58.99	2.96	26.99	0.60
Coleoptera	30	25.00	45	6.81	189.78	9.53	408.41	9.04
Coleoptera (larvae)	18	15.00	38	5.75	445.43	22.36	421.69	9.34
Diptera	3	2.50	3	0.45	3.00	0.15	1.51	0.03
Eggs	11	9.17	121	18.31	140.07	7.03	232.27	5.14
Hemiptera	1	0.83	3	0.45	37.43	1.88	1.94	0.04
Hymenoptera (Formicidae)	59	49.17	158	23.90	213.18	10.70	1701.48	37.67
Hymenoptera (non-Formicidae)	2	1.67	2	0.30	14.38	0.72	1.71	0.04
Isoptera	32	26.67	196	29.65	505.64	25.39	1467.72	32.50
Larvae	15	12.50	26	3.93	152.36	7.65	144.73	3.20
Lepidoptera (larvae)	1	0.83	2	0.30	56.00	2.81	2.60	0.06
Orthoptera	3	2.50	3	0.45	21.17	1.06	3.79	0.08
PLANT MATERIAL	6	5.00	7	1.06	8.49	0.43	7.43	0.16
NON-IDENTIFIED	8	6.67	15	2.27	46.47	2.33	30.68	0.68
Total	-	-	**661**	-	**1991.8**	-	-	-
Niche breadth	-	-	**5.26**	-	**6.68**	-	-	-

Table 2. Morphological variables of *A. vanzolinia* from Caatinga. SVL = snout-vent length, TL = tail length, HL = head length, HW = head width, HH = head height, BW = body width, BH = body height, FLL = forelimb length and HLL = hindlimb length. Size-adjusted values are in parentheses. Values in millimetres. *Isometric size variable, see methods.

Variable	Mean ± Standard Deviation	
	Males (n=39)	Females (n=41)
Body size*	2.60±0.08	2.65 ± 0.07
SVL	38.56±2.21 (0.72±0.02)	42.61±2.21 (0.75±0.03)
TL	49.33±12.87 (0.81±0.11)	47.71±15.87 (0.76±0.16)
HL	5.91±0.40 (-0.30±0.05)	6.05±0.35 (-0.27±0.06)
HW	3.97±0.28 (-0.41±0.03)	4.02±0.31 (-0.40±0.04)
HH	2.88±0.26 (-0.24±0.04)	3.02±0.27 (-0.22±0.04)
BW	4.21±0.54 (-0.09±0.03)	4.62±0.45 (-0.10±0.03)
BH	3.69±0.55 (-0.27±0.03)	4.16±0.59 (-0.28±0.03)
FLL	4.10±0.37 (-0.25±0.04)	4.08±0.40 (-0.27±0.04)
HLL	8.12±0.39 (0.04±0.03)	8.28±0.59 (0.04±0.04)

RESULTS

Microhabitat use

A total of 154 specimens of *A. vanzolinia* were identified, of which 66% (101) were buried in soil, 21% (33) were present in leaf litter and 3% (5) were found under rocks. Considering solar incidence, 103 specimens (74%) were located in shaded environments and only seven (5%) in sunny environments. Twenty one percent (29) of lizards were found on cloudy days, when it was not possible to characterise solar incidence on the lizard.

Diet

Seventeen prey categories were identified (Table 1). The most frequent prey categories were Formicidae (ants), Isoptera (termites) and Coleoptera (beetles). Numerically, Isoptera was the most commonly consumed, followed by Formicidae and insect eggs. Volumetrically, Isoptera dominated the diet, followed by Coleoptera (larvae) and Formicidae. The five most important prey items (based on *RII*) were Formicidae, Isoptera, Coleoptera (larvae), Coleoptera (adult) and insect eggs. The diet niche breadth for pooled stomachs was 5.26 based on prey number and 6.68 based on prey volume.

Adult males and females exhibited a relatively high dietary overlap based on number (φ_{jk}=0.77) and volume (φ_{jk}=0.64). Adults and juveniles also showed a similar overlap based on number (φ_{jk}=0.59) and volume (φ_{jk}=0.68).

We did not find sexual differences in the five most important prey in adults. Both sexes exhibited highest *RII* values for Isoptera and Formicidae (Appendix 1). However, juveniles showed larger values than adults for Formicidae and Coleoptera (larvae) (Appendix 2). In the dry season, Formicidae and Coleoptera (larvae) were the most important prey, and in the wet season, Isoptera and Formicidae were the most important (Appendix 3).

We found significant differences in the numeric and volumetric consumption of Isoptera (W_n=2167, p=0.008; W_v=2194.5, p=0.004) and insect eggs (W_n=2067, p=0.002;

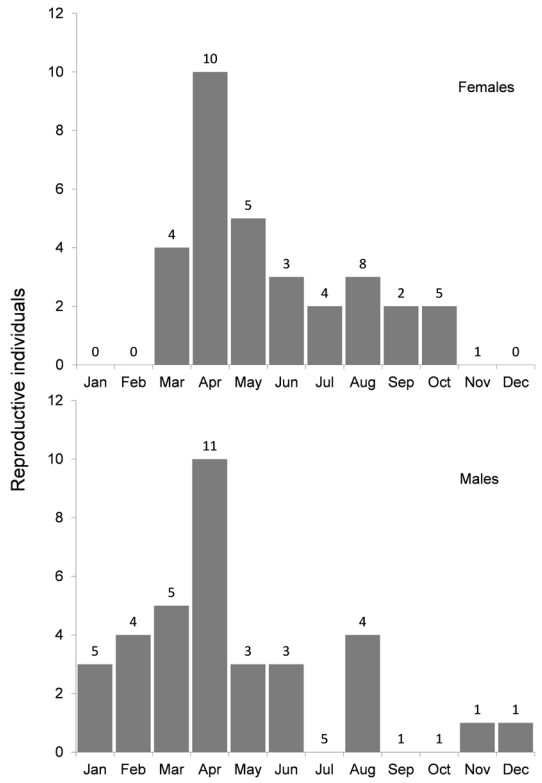

Figure 3. Monthly distribution of reproductive adult males and females of *A. vanzolinia* from Caatinga. Numbers on top of bars indicate sample sizes.

W_v=2067, *p*=0.002) between adults and juveniles; and Isoptera (W_n=2185, p=0.002; W_v=2206.5, *p*=0.001) and insect eggs (W_n=2009, *p*=0.004; W_v=2009, *p*=0.004) between seasons.

Sexual dimorphism

In total, 44 adult males, 41 adult females and 69 juveniles were analysed. The SVL of the smallest adult male was 34.9 mm and of the smallest adult female was 38.4 mm. The SVL of the largest adult male was 42.9 mm and of the largest adult female was 47.7 mm.

We found a significant difference in the body size between males and females (ANOVA $F_{1,78}$=9.08; *p*=0.003), with females being larger than males (Table 2). The model selection analysis indicated that snout-vent length, body height and hindlimb length presented the best discrimination index between sexes, with males being taller and having longer hindlimbs, while females had longer bodies (Model = sex ~ SVL + BH + HLL, AIC = 75.960, *p*=0.003). The linear discriminant function using the three selected variables had a misclassification error of 0.20.

Reproduction

In this species, reproduction occurred during the wet season, from March to August, with reproductive activity declining in September and October (Fig. 3) and hatchlings emerging mainly in April and August (Fig. 4). Females had the highest volume of vitellogenic follicles in March and April (Fig. 5), and egg presence from March to August.

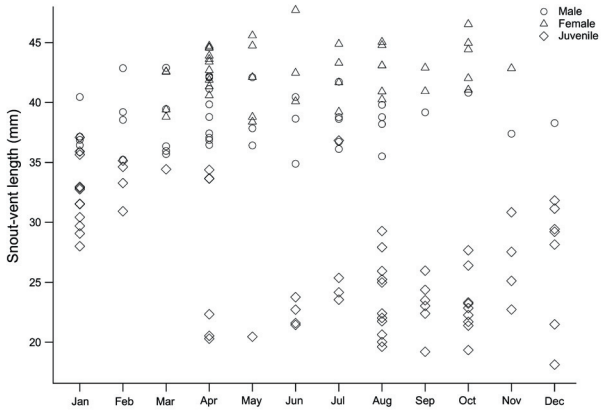

Figure 4. Monthly distribution of individuals of *A. vanzolinia* from Caatinga according to snout–vent length

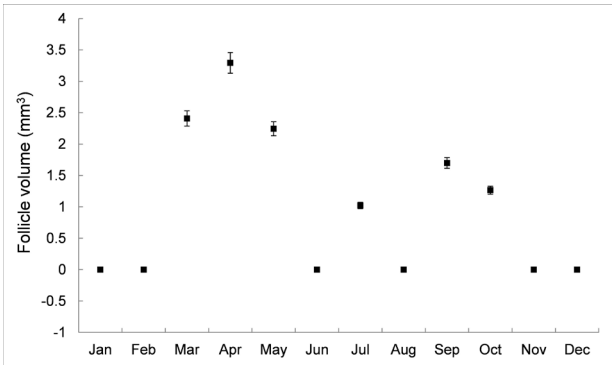

Figure 5. Monthly variation (mean + SE) in follicle volume of *A. vanzolinia* from Caatinga

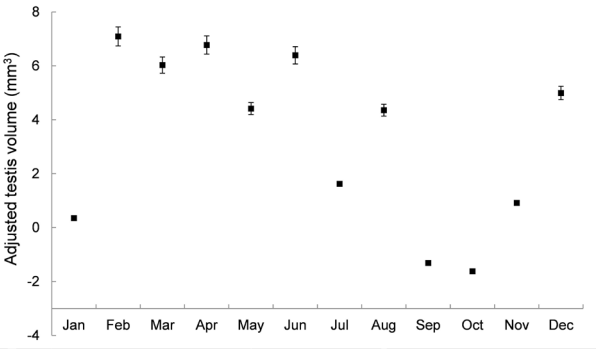

Figure 6. Monthly variation (mean + SE) in adjusted testis volume of *A. vanzolinia* from Caatinga. The adjusted volume was calculated by summing the mean volume of the testis with regression residues between CRC and testis volume.

The mean egg length and width were 7.17±2.19 mm and 3.72±0.66 mm, respectively, and the average egg volume was 55.68±26.09 mm^3. We did not find relationship between average egg volume with female SVL (R^2=0.003; *p*=0.840). Gravid females had a fixed clutch size of two eggs. Five females contained vitellogenic follicles and oviduct eggs simultaneously, indicating the presence of more than one clutch during the reproductive season.

The reproductive activity of males showed peaks in March, April and August, coinciding with the period of highest female reproductive activity. However,

reproductive males could be identified in almost all months of the year (Fig. 3). In males, testis volume was positively correlated with male SVL (R^2=0.268; p<0.001). We did not find significant differences between monthly testis volume (ANCOVA $F_{11,39}$=1.911; p=0.068) (Fig. 6).

We collected five egg clutches of *A. vanzolinia*, one in July 2013, two in September 2013 and more two in April 2014. The eggs were collected from under leaf litter, always in pairs. Two eggs hatched in seven and nine days, respectively. The incubation period for the other eggs was from 43 to 49 days. The mean size of hatchlings was 18.85±0.91 mm (Appendix 4).

DISCUSSION

Anotosaura vanzolinia is a lizard species that inhabits semi-arid Caatinga areas, but it seems to be strictly located in the milder microhabitats within the biome, characterised by large amounts of leaf litter and organic matter with low direct solar incidence. The use of milder microhabitats, such as forest fragments within the Caatinga, or sites with accumulated leaf litter, was previously described for this species (e.g. Rodrigues, 1986; Delfim & Freire, 2007; Gonçalves et al., 2012). Rodrigues (1986) suggested that this species have relictual distribution, and here we show that it was found only within these specific microhabitats. In open sites in same study area, with high solar incidence and little leaf litter, we did not find the species.

Besides that, it is a wide foraging lizard that feeds on a variety of arthropods, both mobile and sedentary preys, such as ants and termites. The consumption of termites by active foragers is very common (Huey & Pianka, 1981). On the other hand, the high consumption of ants, not only by *A. vanzolinia* but also by other active foragers, is still uncertain, since ants and other Hymenopterans can possess chemical compounds that are harmful to lizards (Vitt & Pianka, 1994; Vitt et al., 2003). Since these lizards, like other autarchoglossans, can chemically discriminate their prey, they should avoid them (Vitt & Pianka, 1994; Vitt et al., 2003). Despite this, *A. vanzolinia* seems not to avoid eating ants, reflected in the high rates of ants in our data. Another interesting point is that juveniles also eat lots of ants, which indicate that the resistance from ant toxins seems to be something innate.

Apparently, some autarchoglossans could have developed a way to get rid of the effects of the toxins produced by ants, because ants also were described as important prey for other gymnophthalmids, like *Dryadosaura nordestina*, *Ecpleopus gaudichaudii* and *Leposoma scincoides*, from Atlantic forest, and Andean lizards of genus *Proctoporus* (Teixeira & Fonseca, 2003; Doan, 2008; Maia et al., 2011; Garda et al., 2014). From this reason, we suggest that in some gymnophthalmids (e.g. Ecpleopodini), the feeding ecology can be influenced by their phylogenetic history (i.e. niche conservatism).

Furthermore these data shows that ant consumption not occurs only or more within Iguania, as previously stated (e.g. Vitt & Pianka, 1994; Vitt et al., 2003; Sites et al., 2011).

Adults feed on more termites than juveniles, which could be explained by the size incompatibility between prey and predator (Costa et al., 2008), since termites are larger than ants, or simply that for juveniles may be easier to catch ants than other prey types. Vitt (2000) suggested that ontogenetic differences in lizard diets are important for population balance and juvenile survivorship, because juveniles can compete with adults if they continue feeding on small prey.

We observed differences in the consumption of termites and insect eggs during different seasons. The difference on prey availability may result from changes in resource availability for season (Van Sluys, 1995; Wiederhecker et al., 2002). For example, insect eggs are absent during the dry season and termites are more abundant during the wet season (Vasconcellos et al., 2007; Araujo et al., 2010).

The diet niche breadth of *A. vanzolinia* is similar to that of other gymnophthalmids, such as *Micrablepharus maximiliani* and *Colobosaura modesta* from Cerrado, which show niche breadth values of 5.0 and 4.0, respectively (Mesquita et al., 2006; Dal Vechio et al., 2014), or Amazonian species, like *Alopoglossus* genus, which show values around 7.0 (Vitt et al., 2007). In the present study, we also observed that females have a larger niche breadth than males, which is likely a result of different energetic requirements between the sexes, due to the high costs of egg production and maintenance by females (Shine, 1980; Shine & Schwarzkopf, 1992).

The greater body size and snout-vent length observed in females of *A. vanzolinia* can be likely related to sexual differences in reproductive success, since they possess a larger space in the peritoneal cavity for the development of eggs (Olsson et al., 2002; Cox et al., 2003). Most female gymnophthalmids show a larger body size than males, including the close related species *A. collaris* (Rodrigues et al., 2013). Therefore, this trend is probably a general pattern within the family (Vitt, 1982; Balestrin et al., 2010; Rodrigues et al., 2013). Furthermore, males *A. vanzolinia* reached sexual maturity at a smaller body size than females, which may simply reflect the largest female size, similar to *Cercosaura schreibersii* (Balestrin et al., 2010).

The reproduction of *A. vanzolinia* in the Caatinga occurs during the wet season, with gravid females appearing from March to August. Both males and females present higher reproductive activity in April – the month with the highest precipitation values (Climate-Date, 2016) – with reproductive activity decreasing in September and October. The amount of rainfall appears to be an important regulatory factor in reproductive seasonality of tropical lizards (Rocha, 1994), which may be related to large prey abundance during the wet season or their low levels in the dry season (Van Sluys, 1995), or to avoid desiccation of eggs in dry season (Overall, 1994). Andrews & Sexton (1981) stated that dampness in the wet season can produce more appropriate conditions for egg deposition and embryo development.

Anotosaura vanzolinia has a fixed clutch of two eggs – a recurrent pattern in Gymnophthalmidae which is considered a synapomorphy of the family (Fitch, 1970; Vitt, 1992) – and produces at least two clutches per

reproductive season. Lizards with fixed clutch size can increase their reproductive effort by investing in multiple clutches per reproductive season (Vitt, 1986; Selcer, 1990), similar to other gymnophthalmids, such as *C. schreibersii* (Balestrin et al., 2010), *D. nordestina* (Garda et al., 2014), *M. maximiliani* (Dal Vechio et al., 2014) and *Vanzosaura multiscutata* (Vitt, 1982).

In summary, *A. vanzolinia* is a small semifossorial lizard that inhabits forest fragments in semi-arid Caatingas, foraging in the leaf litter and eating small arthropods, especially ants and termites. They are small elongate lizards with reduced eyes and limbs, a long tail and no ears. Female *A. vanzolinia* are larger than males. They reproduce in the wet season, when females deposit their eggs, two eggs per reproductive event, twice a year.

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The influence of visual cues of conspecifics based on density and habitat features on the growth of *Bufo gargarizans minshanicus* larvae: an experimental approach

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Anuran larvae may use chemical, visual and tactile cues to assess habitat features, and subsequently mediate their growth and development. Of the three cues, chemical ones have been analysed the most, but little is known about the role of visual cues and the extent to which tadpoles rely on their vision for intraspecific social assessment. In this study, we investigated whether conspecific visual cues affect development and growth of Bufo gargarizans minshanicus tadpoles, and analysed whether they use visual cues as indicators of density. The tadpoles did not significantly alter their growth and development in response to low visual stimulation. However, tadpoles under high visual stimulation were significantly smaller than single tadpoles without visual cues. Therefore, we suggest that B. g. minshanicus tadpoles are susceptible to high visual stimulation when the environment changes (little vegetation and clear water), allowing for decreased growth in the presence of high-density conspecifics.

Key words: body mass, visual cues, density, intraspecific competition, habitat features, *Bufo gargarizans minshanicus*

INTRODUCTION

Visual signals play important roles in foraging, mate choice, anti-predator defence and social interactions in many vertebrates (Liao & Lu, 2009; Møller and Erritzøe, 2010; Liao & Lu, 2011; Yu et al., 2009). For instance, eye size in birds has evolved as a means of predator avoidance since larger eyes allow early detection of an approaching predator (Møller & Erritzøe, 2010). In toads, males prefer larger females as mates using visual signals (Liao & Lu, 2009; Liao & Lu, 2011; Yu et al., 2009). In fish, while experiencing visual social stressors, the smaller male uses an opportunistic strategy, acting like a subordinate male while maintaining the physiology of a dominant male (Chen & Fernald, 2011). In amphibian species, larvae may use chemical, visual, and tactile cues to assess features of their habitats (reviewed by Rot-Nikcevic et al., 2006). Many previous studies demonstrated that chemical cues are associated with predator detection (Petranka et al., 1987; Kats et al., 1988; Semlitsch & Reyer 1992; Kiesecker, Chivers & Blaustein, 1996; Laurila, 2000; Benard, 2006), kin recognition (Waldman, 1985, 1986; Blaustein & Walls, 1995; Gramapurohit et al., 2006; Eluvathingal et al., 2009), growth and survival (Crossland & Shine, 2012). Anuran larvae use visual cues to find their prey, competitors, and predators when water is clear (Hettyey et al., 2012). Moreover, Rot-Nikcevic et al. (2006) suggested that visual stimuli were perceived as stressful ones, therefore inducing tadpoles to become more active in response to enhanced visual stimuli, resulting in less energy available for growth.

Tadpoles forming aggregations with kin or non-kin may gain many of the advantages of group living (reviewed by Blaustein & Waldman, 1992). For example, group-living animals forage more efficiently and detect or avoid predators more effectively (reviewed by Blaustein & Waldman, 1992). Although visual cues are not sufficient for discriminating between kin and non-kin (Wassersug, 1973; Wassersug & Hessler, 1971; Wassersug et al., 1981; O'Hara, 1981), they are important in schooling with conspecifics (Blaustein & O'Hara, 1982). Conversely, crowding may reduce growth, development rate, and survival of metamorphosis in most amphibians (Smith-Gill & Berven, 1979; Semlitsch & Caldwell, 1982; Smith, 1990; Hokit & Blaustein, 1994, 1997; Girish & Saidapur, 1999; Relyea & Hoverman, 2003).

We tested whether visual cues affect fitness-related metamorphic traits (e.g., age and size at metamorphosis, growth rates, and body condition) in a laboratory experiment with *Bufo gargarizans minshanicus*. This animal is a typical explosive breeder (Wells, 2007), and is distributed at high elevations on the eastern Tibetan plateau (Fei & Ye, 2001). The breeding season is extremely short (7-15 days), and once the eggs hatch, the larvae typically group together as they grow and mature in permanent wetlands (although it is not known whether

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these groups are composed of kin or non-kin). Our aim was to test whether visual cues would lead to reduced larval growth and development in response to habitat change.

MATERIALS AND METHODS

Study area

Our field study was carried out at two altitudes in Gahai-Zecha National Nature Reserve of Luqu County, Gansu, China in April of 2014 and 2015. The length of *B. g. minshanicus* breeding season at individual sites is as follows: (1) Site A, Gaihai Lake (34°12'N, 102°19'E, altitude 3477 m), from mid- to late April; and (2) Site B, Guomao Lake (34°18'N, 102°18'E, altitude 3449 m), from early to mid-April. At site A, water quality is usually clear because fencing generally excludes yaks from drinking at this water source. On the contrary, at site B, water quality is often muddy because yaks often frequent this water source for drinking and/or feeding.

Data collection

During peak reproductive periods, we captured a total of 60 amplectant pairs of *B. g. minshanicus* by hand at site A and site B. We then placed amplectant pairs into individual 5-liter plastic containers filled with 12 cm of water until females oviposited. We randomly selected 20 fertilised eggs from each clutch for our experiment, and placed each egg mass into separate opaque plastic vessels containing 3000 ml of water. Adult toads and the remaining eggs were released back into the wetlands. We carried out this experiment in the laboratory, which allowed us to avoid confounding environmental effects and predation pressure.

Experiment design

Once the tadpoles reached Gosner stage 25 (absorption of external gills and fully formed spiracle; Gosner, 1960), we created two treatments designed to provide tadpoles with different visual cues. In the first set of treatments (in 2014), each tadpole was kept in an individual transparent glass beaker (250 ml). Then, two tadpoles, still housed individually, were placed adjacent to one another and wrapped together with an opaque paper. In this case, the tadpoles from the two adjacent containers could see one another. In 2015, all containers with single tadpole were arranged into a 7 row × 8 column grid. In this arrangement, 4 containers (i.e., the containers in the corners of the grid) were placed adjacent to 2 other containers, 22 containers (i.e., the outside rows excluding the corners) were immediately adjacent to 3 other containers, and 30 containers (i.e., the inner rows) were placed adjacent to 4 other containers. Therefore, visual signals varied between 2 and 4 other tadpoles being visible.

In the second set of treatments (2014 and 2015), tadpoles were housed individually in opaque glass beakers (250ml, n = 30). Each tadpole was on a single shelf by itself. The tadpoles in each treatment were placed on the same food regimen (50 mg of commercial fish food per tadpole per day at the beginning, and two pieces of water weed, *Potamogeton crispu*). As the larval period progressed, ration levels were increased based on tadpole mass to keep up with the normal demands of growth and development. Tadpoles were exposed to an 11L:13D photoperiod throughout the study period, and room temperature was kept at 25.80 ± 1.39°C (2014) and 27.72 ± 1.12 (2015). The water in the containers was changed weekly.

After the first metamorph (defined as the emergence of the first forelimb, stage 42) was discovered, the 60 glass beakers were checked daily in both treatments. We excluded 4 or 5 cases in visual or non-visual treatments (all from site B) because they indicated a developmental abnormality, and therefore were not a suitable candidate for the experiment. Several variables were measured: (1) development time (number of days from the beginning of the experiment until metamorphosis, Gosner stage 42); (2) body mass (weighed with an electric balance to the nearest 0.001g); (3) snout-vent length (SVL, using digital callipers to 0.01 mm); (4) growth rate (measured as the mass at metamorphosis divided by the age at metamorphosis); (5) body condition at the start or the end metamorphosis (defined as body condition = (mass/SVL^3) × 1000), and (6) the overall metamorphosis survival rate (the percentage of surviving tadpoles that metamorphosed).

Data analysis

All data were log-transformed to meet the assumptions of parametric analysis of variance. Difference between treatments in development time, body mass, body length, body condition at the start of metamorphosis and growth rate were analysed using Student's t-tests for each site. The Pearson Chi-square test was employed to test the metamorphosis survival rate. We did not analyse differences in metamorphic traits between years because of different rearing temperatures. All data were analysed with SPSS 19.0, SPSS Inc., 2004, Chicago, IL, USA. All p-values given are two-tailed, with values presented as means ± standard error.

RESULTS

The development time was not significantly different between the two treatments for both sites (Student's t-test: site A, $t_{41} = 1.17$, $p = 0.25$; site B, $t_{43} = -0.12$, $p = 0.90$). Although tadpoles in both treatments had similar body mass at site A ($t_{41} = 0.104$, $p = 0.92$), tadpoles from site B raised in non-visual treatments were significantly larger than those raised with visual cues ($t_{43} = 2.06$, $p = 0.045$). Compared with tadpoles raised in visual treatments, the tadpoles raised in non-visual conditions showed two opposing tendencies for body length, but the differences were not significant in either site (site A: $t_{41} = 0.72$, $p = 0.48$; site B: $t_{43} = -1.50$, $p = 0.14$).

The tadpoles raised in non-visual treatments did not have significantly larger mean growth rate or body condition at metamorphosis than that of tadpoles raised in individual containers with visual cues (growth rate, site A, $t_{41} = 0.09$, $p = 0.93$; site B, $t_{43} = 1.44$, $p = 0.16$; body condition, site A, $t_{41} = 0.80$, $p = 0.43$; site B, $t_{43} = 0.14$, $p = 0.89$). Finally, the tadpoles raised in both treatments had similar survival rate at site A (Pearson Chi-Square test: $\chi_1^2 = 0.12$, $p = 0.73$) and site B ($\chi_1^2 = 0.00$, $p = 1.00$; Table 1).

Table 1. Summary of laboratory experiment data for Bufo gargarizans minshanicus following treatments

		Single, visual	Single, non-visual
Variables	**Year**	**Mean(SE)**	**Mean(SE)**
Development time	2014	26.30(0.21)	25.91(0.26)
	2015	10.78(0.31)	10.77(0.20)
Body mass	2014	0.147(0.006)	0.148(0.008)
	2015	**0.128(0.005)**	**0.144(0.005)**
Body length	2014	9.47(0.15)	9.32(0.116)
	2015	9.64(0.16)	9.99(0.17)
Growth rate	2014	5.60(0.25)	5.73(0.32)
	2015	12.24(0.65)	13.48(0.63)
Body condition	2014	0.14(0.005)	0.18(0.008)
	2015	0.14(0.006)	0.15(0.007)
Survival rate	2014	66.67	76.67
	2015	90	90

Significant effects have P-values in bold

DISCUSSION

Rot-Nikcevic et al. (2006) found that *Bufo americanus* tadpoles did not respond to visually stimulated increases in conspecific density by altering either their development or growth. Our results showed that visual cues did not affect any fitness-related metamorphic traits of *B. g. minshanicus* tadpoles under low visual stimulation. Interestingly, we found tadpoles with high visual stimulation were significantly smaller than tadpoles without visual cues. Thus, our results support the findings of other authors (e.g., Rana sylvatica, Rot-Nikcevic et al., 2005, 2006; *Rana kukunoris*, Yu & Lambert, 2015) that high visual stimulation, better perceived by compound eyes, results in detrimental effects on tadpole growth and development (Land, 1997).

Previous studies showed that increased competition promotes decreased growth rate in anuran larvae (e.g. Wilbur & Collins, 1973; Smith-Gill & Berven, 1979; Berven & Chadra, 1988; Scott, 1990). We found that growth rate was not statistically different between the tadpoles raised in non-visual treatments and that of tadpoles raised with visual cues. Moreover, our results also showed that the tadpoles with visual stimuli had similar development times with those of tadpoles raised without visual cues. Importantly, body mass of the tadpoles with high visual stimuli were smaller than tadpoles in non-visual treatments. Thus, this may suggest that *B. g. minshanicus* tadpoles may use their vision for environment assessment, and that they are able to modify their growth and development in response to sensory enrichment. Generally, *Bufo* larvae may be composed of tens of thousands of individuals from numerous sibships (Wassersug, 1973; Waldman, 1982). Rot-Nikcevic et al. (2005) found that visual cues do not play a role in affecting growth and development in *Bufo americanus* tadpoles because these cues were redundant in their natural habitats. This result was not consistent with our study. In this study, we speculated that the effect of visual cues may be related to features from the original habitat that impact differences in visual stimulation. If their habitat consisted of turbid water and dense vegetation, vision is often dismissed as unimportant (reviewed by Hettyey et al., 2012). On the contrary, if *Bufo* larvae aggregated in habitats with little vegetation and clear water, visual information may be perceived as stressful. In site B, water quality is often muddy because yaks go there to drink water or feed on aquatic plants. In this case, the tadpoles usually form denser aggregates and rely on olfactory cues; visual cues are redundant in their natural habitats. However, *B. g. minshanicus* tadpoles were raised in two treatments with clean water, which was different from their original habitat. Therefore, we suggested that high visual stimulation with altered habitat features (e.g., water clarity) may induce negative effects on tadpole growth and development.

In conclusion, *B. g. minshanicus* tadpoles that typically form high-density schools are susceptible to visual stimulation, when the environment changes, especially if water quality becomes clear. In this case, vision of conspecifics is a source of stress for tadpoles and may have detrimental effects on tadpole growth and development.

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Effectiveness of the field identification of individual natterjack toads (*Epidalea calamita*) using comparisons of dorsal features through citizen science

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Citizen science is now making an important contribution, both in the collection of large amounts of data over wide geographical areas and in promoting environmental awareness and engagement communities. However, as there are many participating observers, the reliability of the data collected needs to be assessed. This study used a citizen science approach to investigate whether dorsal features, when photographed, can be used in the identification of individual natterjack toads (*Epidalea calamita*). *Epidalea calamita* individuals from a population located at Prestatyn, North Wales, were captured, photographed and released in a legally compliant manner. Forty human participants each completed a timed exercise to match photographs of individual toads that had been taken from different angles. Sixty-five percent of the participants accurately matched photographs on their first attempt. The effect of training on the accuracy and speed at which participants could identify individuals from photographs was then assessed. Twenty of the participants received basic training on recognising the key features of dorsal patterns before carrying out the exercise again. Following training, average accuracy increased to 90% and participants were 41.5% quicker in completing the exercise than those that were untrained. The study revealed that basic training of participants who are involved in citizen science projects was beneficial by having a significant impact on accuracy and speed. In addition, we demonstrate that the dorsal features of tubercles and scarring are useful in identifying individuals of *E. calamita* in the field.

Key words: Epidalea calamita, natterjack toad, photo-identification technique, training, mark recapture, citizen science

INTRODUCTION

Citizen science makes use of volunteers to collect scientific data (Ratruieks et al., 2016). It has the dual advantages of allowing a large number of people to be involved in collecting substantial amounts of data and of raising scientific awareness amongst those people involved. It is also termed participatory or community-based monitoring. Although the reliability and accuracy of such data have been questioned, it is a method that is increasingly accepted and used in a wide range of research across many habitats and species (Aceves-Bueno et al., 2017). Studies using a citizen science approach have tended to focus on species that are relatively straightforward to find and identify, e.g. birds, Lepidoptera and plants (Chandler et al., 2016).

This study investigated the citizen science approach specifically to data collection requiring the accurate identification of individual small animals in the field. The study used *Epidalea calamita* (Laurenti, 1768), the natterjack toad. Other studies have also used amphibians. For example, Casula et al. (2017) analysed data from many observers, collected simultaneously, on individuals of the Sardinian Mountain Newt, *Euproctos platycephalus*. They found that the reliability of observation differed widely amongst observers and training did not remove the variability.

Epidalea calamita has a range that extends from the Iberian Peninsula to the Baltic States. The United Kingdom and Ireland have isolated populations (McInerny & Minting, 2016). This study was carried out in Wales where the natterjack toad is protected under the laws of England and Wales (Wildlife and Countryside Act 1981 (as amended) (Schedule 5)) and European law (Regulation 39 of the Conservation (Natural Habitats &c) Regulations 1994 (as amended) (Schedule 2) as European Protected Species). *E. calamita* is thought to have suffered a 70% population decline in Britain over the last 100 years (Beebee & Denton, 1996). A reintroduction programme along the Denbighshire coast began in 2000, following the local extinction of the species around the 1950's and the population is thought to be gradually increasing (Buckley, 2006). In 2009, there were roughly 60 populations in the United Kingdom with an estimated total of 3,000 breeding females (Amphibian and Reptile Conservation, 2009).

The importance of reliably identifying individuals in populations to investigate species' ecology and behaviour is well documented (Wiirsig & Jefferson, 1990). However, the negative effects that accompany some methods of identification have received attention (Phillott et al., 2008;

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Perry et al., 2011; Corrêa, 2013). For instance, when mark-recapture surveys are carried out, artificial marking methods have often involved physical additions to the animal such as paint or tracking devices, which may affect behaviour and survival (Ferner, 1979; Lemckert, 1996; Schmidt & Schwarzkopf, 2010). In the case of amphibians, previously employed marking techniques have included chemical branding (Wolf & Hedrick, 1971), tattooing and cold branding (Nace et al., 1974), skin dyeing (Gittins et al., 1980; Brown, 1997), Passive Integrated Transponder tagging (Brown, 1997), and the commonly used method of toe-clipping (Denton & Beebee, 1993; Heyer et al., 1994; Retallick et al., 2004). Toe-clipping, which involves the systematic removal of toes in unique combinations, is a low-cost method of marking anurans (Luddecke & Amezquita, 1999). However, studies suggest that it may reduce the mobility of marked individuals of some species (McCarthy & Parris, 2004). Marking often creates a wound, which is a potential site of infection (Bradfield, 2004) and ethical considerations of these practices must be undertaken when planning studies (May, 2004). Therefore, alternative methods for identifying individuals should be developed and tested.

One such alternative is to employ photography for the non-invasive identification of individuals. Digital cameras are readily available to investigators and provide instant photographs which are easily downloaded, copied and distributed. For example, photography has been used extensively for identifying cetaceans (Karczmarski & Cockcroft, 1998; Friday et al., 2000; Calambokidis et al., 2004). Individual cetaceans have physical characteristics and unique markings that distinguish them from other individuals (Perrin & Wursig, 2009). The body parts used commonly for cetacean photo-identification are the dorsal fin or tail fluke (Hebridean Whale and Dolphin Trust, 2008). Photographs of these markings are retained and used repeatedly for identification by different investigators. For terrestrial mammals (Kelly, 2001; Perera and Perez-Mellado, 2004; Jackson et al., 2006; Anderson et al., 2010), reptiles (Knox et al., 2013) and amphibians (Bradfield, 2004; Kenyon et al., 2009; Lama et al., 2011), this method has been used less frequently.

Photo-identification has the potential to be useful for any anuran species that exhibits variability of natural markings on at least one region of their body (Bradfield, 2004). These markings can be photographed in the field and used for identification by visually matching them to photographs already taken during previous surveys, either by eye (manual matching) or by using pattern-recognition software. It is a non-invasive method and permits the permanent identification of an individual (McConkey, 1999; Perera and Perez-Mellado, 2004). Pinya and Perez-Mellado (2009) recognised that individual Majorcan midwife toads (*Alytes muletensis*) had different patterns of spots on their backs and they identified five groupings of patterns for the dorsal spots. In North America, Morrison et al. (2016) used the patterns of "wart-like glands" on the backs of Wyoming toads (*Anaxyrus baxteri*) for "naïve" observers to identify individuals by eye with high reliability. This method of observation gave more accurate results than computer matching. In South America, Caorsi et al. (2012) used patterns on the ventral surfaces of the southern red-bellied toad *Melanophryniscus camabaraensis* to compare the accuracy of the identification of individual toads by the investigators using visual methods as opposed to toe clipping. They found that visual identification was more accurate.

Here we examined the use and reliability of photographic identification for *E. calamita* in the context of citizen science and whether training affects this reliability. *Epidalea calamita* toads can reach 70 mm in length (Beebee & Denton, 1996). They are brown, grey or green in colour, with eyes that have golden irises and horizontal pupils. They have tubercles on their dorsal surfaces and these dorsa are also characterised by a yellow or cream stripe traversing from snout to vent (Natural England, 2007). Mainly a lowland species with strict habitat requirements, natterjack toads are normally only found in coastal dunes, upper saltmarshes and lowland heaths (Beebee & Denton, 1996).

Epidalea calamita have variable, natural, throat spot markings in females (Denton, 1991), but these are difficult to photograph. However, all individuals have distinctive dorsal stripes and wart patterns (McInerny & Minting, 2016; citing Arak, 1983). Photographic identification using the dorsal region for either sex has not been documented. Some individuals possess obvious scarring on their dorsum, most likely a result from bird attacks, and this could also have a use in individual identification (Buckley, pers. comm., 6 September 2015).

The objectives of this study were to:

1. establish whether photo-identification of *E. calamita* is feasible in citizen science using the natural markings on the dorsum of individuals
2. ascertain whether the training of non-specialist observers improves the accuracy of identifying individuals by dorsal markings
3. ascertain whether training improves the speed at which individual toads can be identified.

METHODS

Location and photography

Data collection was carried out at Presthaven Sands, Talacre Warren Special Site of Scientific Interest (OS reference: SJ 10320 84717), in Prestatyn, North Wales. A team of two people conducted four evening surveys under the statutory survey licence for *E. calamita* (Conservation of Habitats and Species Regulations 2010 and the Wildlife and Countryside Act 1981). Surveys commenced later than 2000 on the following dates: 1, 18 and 24 June and 1 July 2015 when weather conditions gave warm and wet evenings above 8 °C (Beebee, 1977). The surveys were carried out at a set of three breeding scrapes (artificial breeding ponds), where males were known to congregate and call for females (Fig. 1).

The following camera settings were established as optimal: shutter speed of 1/100 s, aperture of f/6.3 and 600 ISO, using a Nikon D7000 16 megapixel DSLR camera with a Nikkor 35mm f/1.8 lens. Flash photography was not used in case it promoted stress. Instead, individuals were illuminated by white torchlight.

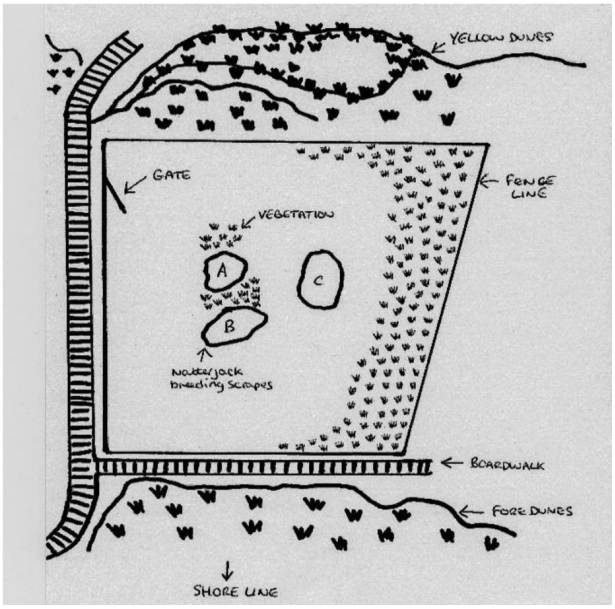

Figure 1. Location of breeding scrapes. The individuals were collected from scrapes A and B.

Survey methods

Each survey lasted no longer than two hours in order to keep disturbance to a minimum. Surveys involved locating individuals by torchlight in and around the breeding scrapes. Those sighted more than three feet into a scrape were ignored, as wading in order to collect was likely to disturb and cause them to swim away. Any individuals seen in amplexus were left undisturbed. Individuals were collected in a large bucket and transported 100 metres to the site warden's vehicle for photographing. Toads were removed individually from the bucket and sequentially placed into a perspex container. A minimum of five photographs of the dorsal region were taken from above each individual, in quick succession, taking no longer than 15 seconds to photograph each individual. The potential effects of handling individuals were not examined in this study. After photographing, individuals were placed in a different bucket to avoid re-photographing them. Once all individuals had been photographed, they were returned to the perimeter of the breeding scrapes.

An estimated 200-300 individuals are thought to be present at the study site (Evans, pers. comm., 14 April 2015). Forty-five individuals were photographed.

Testing the feasibility of photo-identification

The characteristics that were used to identify individual toads were the dorsal stripes, tubercles and scarring.

The accuracy and speed at which human participants could recognise individuals from photographs was investigated using a photo-matching exercise, based on the method developed by Knox et al. (2013). Participants declared that they had no prior experience of photo-matching or of individual toad recognition. Two photographs were arbitrarily selected of each of 5 toads that were also selected arbitrarily. Each of the 40 participants was asked to match one printed photograph of each of 5 individual toads to the other on-screen, photograph of the same toad, from amongst 20 photographs shown on a computer screen. The photographs used on-screen, and those printed, were chosen arbitrarily but checked to make sure identifiable characteristics were visible. Participants were divided into Group One and Group Two, each group comprising 20 individuals. Irrespective of group all the participants worked alone and were thus individually tested. The same sets of photographs were used for both Group One and Group Two. The number of correct matches out of 5 was recorded for all participants. In addition, the time taken to select what the participant considered a match for each of the five photographs was recorded for those in Group One before and after training.

Effect of training on accuracy and speed of photo-identification

Once each participant in Group One had finished the first photo-matching exercise, they immediately received individual training. This training involved describing to the participants the main characteristics of each of the five individual toads in the printed photographs. Training lasted no longer than five minutes and was immediately followed by the participant's second photo-matching exercise.

Participants were advised not to use colour as an identifiable characteristic as colour differed between photographs owing to the angles of torchlight. However, colour photographs were used as they helped to contrast the yellow dorsal stripe against the background colour of the dorsum. Familiarity with identification through using colour images would also assist the observer if, later, working directly with toads. Participants were also told not to rely on the shape of an individual, as the position in which the toads were sitting when photographed changed their apparent body shape.

Once trained, the second photo-matching exercise used an amended photographic database that included the same 20 photographs but with different numerical codes and in a different sequence. The five photographs that they used to match against the database were changed to be different from those in the first exercise. A similar method was used by Knox et al. (2013) in which participants who had no previous experience of photo-matching were compared against the accuracy and timings to a different group of participants who had previous experience and training. Following training, for the second photo-matching exercise, participants were timed once again in matching the 5 photographs. The number of correct matches, out of 5, and the time taken to complete the test, was recorded.

Participants in Group Two did not receive any training and instead repeated the photo-matching exercise in an identical way to their first exercise, after photograph numbers were re-assigned in the same way as for Group 1, thereby acting as a control group. A period of 5 minutes was left between the exercises, equivalent to the time of training for Group One. Their accuracy in terms of correct matches out of 5 was recorded.

RESULTS

Over the 4 surveys, a total of 45 individual toads were captured, photographed and released. Two of these individuals were captured four times, two were captured three times and ten were captured twice.

Differentiating between individual *E. calamita* toads
The following characteristics were used to identify individuals.

Dorsal stripe
Within each individual, the lengths and thicknesses of stripes were unique. As examples, in one individual the stripe formed a large blotch on its right side towards the lower dorsal region (Fig. 2) and in another the majority of the stripe was missing (Fig. 3).

Figure 2. *E. calamita* with dorsal stripe blotch (circled red)

Figure 3. *E. calamita* showing incomplete dorsal stripe

Scarring
Where scarring occurred on the dorsal stripe, there was an obvious grey-translucent area emphasised by the brightness of the dorsal stripe (Fig. 4). Some individuals showed much larger areas of scarring around the snout region (Fig. 5), and this was sometimes more obvious than the dorsal stripe.

Tubercles (warts)
They were generally quite difficult to use as identifiable features as there are so many covering the complete dorsal region. Their better use was when they broke the dorsal stripe (Fig. 6).

Figure 4. *E. calamita* with scarring across dorsal stripe (circled red)

Figure 5. *E. calamita* with scarring on snout (circled red)

Figure 6. *E. calamita* with two warts breaking the dorsal stripe (circled red)

Is photographic identification of *E. calamita* possible?
Of the 40 untrained participants that completed the first photo-matching exercise in two separate groups (Group One Median=4, n=20; Group Two Median=2.5, n=20) a Mann Whitney U Test revealed no significant difference in their levels of accuracy (z=-1.32, p=0.19). Thirty-five percent of those in Group One and 30% of those in Group Two matched all five photographs correctly, and 20% of Group One and 15% of Group Two had only one photographic match incorrect. The remaining 45% of Group One and 55% of Group Two had three or more photographs matched incorrectly during the initial test. This suggested photo-matching of *E. calamita* to be a possible method of identification and that participants had similar ability levels between the two test groups prior to the next stage of the study.

Does training improve the accuracy of photographic identification of *E. calamita*?
Figure 7 illustrates levels of Group One participants' accuracy in photo-matching *E. calamita* toads before and after they received their training. An increased level of accuracy, as measured by scores out of 5, was noted following basic training in their main characteristics. The median score before training was 4 and after training the median increased to 5. This increase in accuracy level following training was significant (Wilcoxon Signed-Rank Test: Z = -2.31, P=0.02).

Before training, 10 cases of misidentification (30% of the total misidentifications) related to one particular photograph. After training, misidentification

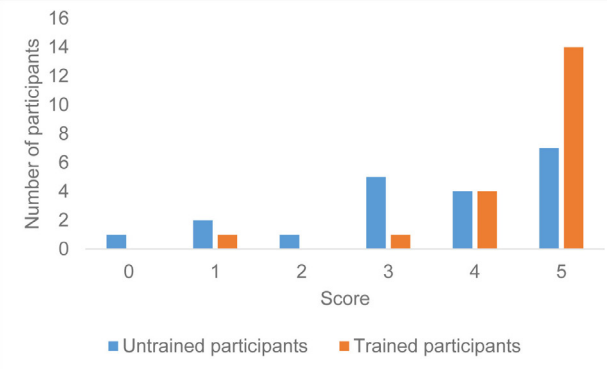

Figure 7. Group one participant's scores gained in an *E. calamita* toad photo-matching exercise, before and after training (n=20).

of this photograph decreased to five occasions, but this represented 56% of the total number of misidentifications in the exercise.

Participants in Group Two (Figure 8) did not receive training prior to their second attempt at photo-matching. Their median score of accuracy at attempt one was 2.5, but this dropped to 1.5 on re-testing. A Wilcoxon Signed-Rank Test revealed a statistically significant reduction in accuracy levels between the two attempts at photo-matching (Wilcoxon Signed-Rank Test: Z = -2.23, P=0.03).

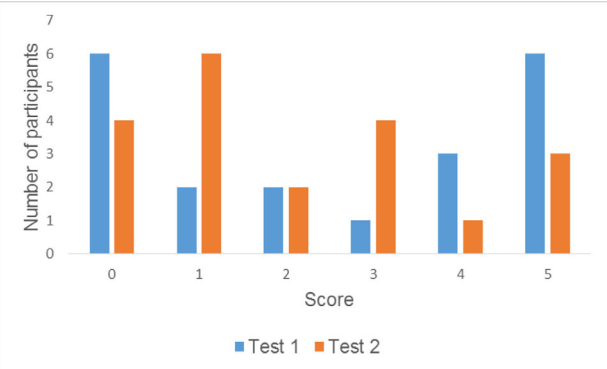

Figure 8. Group two participant's scores gained in an *E.calamita* toad photo-matching exercise, both tests without training (n=20)

Does training increase the speed of photographic identification of *E. calamita* toads?

When the speed of photo-matching was compared before training with Group One participants, the median speed achieved by participants was 9 minutes 1 second and the inter-quartile range was [6 minutes 26 seconds, 14 minutes 52 seconds]. After training the median was 5 minutes 17 seconds and the inter-quartile range was [4 minutes 11 seconds, 9 minutes 23 seconds]. A significant decrease in the time taken in photo matching was seen following training (Fig. 9), (Wilcoxon Signed-Rank Test: Z = -3.25, P=0.001). This demonstrates an increase in speed of correct identification after training.

It was noted that the angle from which the photograph was taken may contribute to the ability to identify an individual toad. Forty-five percent of participants failed to recognise the most obvious feature in one photograph,

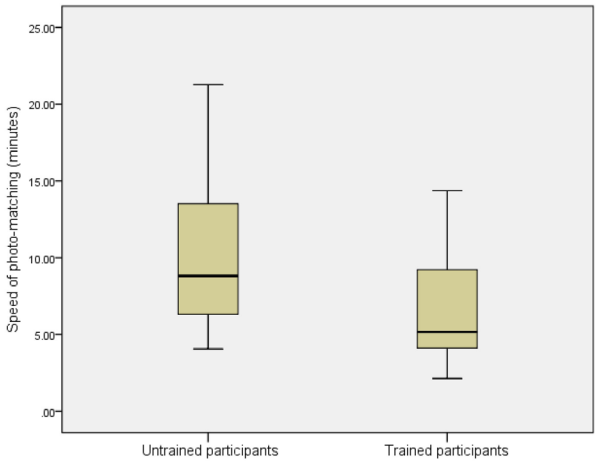

Figure 9. The median time taken to match photographs of *E.calamita* by trained and untrained participants (n=20) in Group One.

which was a straight line of four tubercles to the left of and following the dorsal stripe, and a matching line of four tubercles to the right of the dorsal stripe, although not in a straight line (Fig. 10). The dorsal stripe in this photograph was less noticeable than in photographs of other individuals because of the angle at which it was taken, and the tubercles were less noticeable as features. Once the main feature (line of four tubercles) had been pointed out during training, the percentage of participants incorrectly identifying the toad dropped from 45% to 30%, increasing correct identification to 70%.

Figure 10. Pattern of four tubercles either side of dorsal stripe (circled red). Note the dorsal stripe abnormality (circled green).

Another photograph (Fig. 11) received a 35% misidentification rate before training. The individual's dorsal stripe was very clear with an abnormality. Behind the eyes, the stripe begins to fork off to the left side of the dorsum and then stops. The reason for this frequent misidentification is not clear. One participant mistook a piece of vegetation which was stuck to the mid-dorsum of the toad as being part of its body (Fig. 11). In the matching photograph the vegetation was not there and so this negatively affected the participants answer as they were actively searching for it.

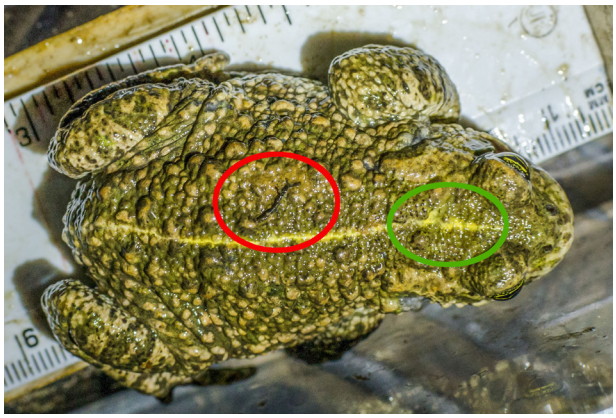

Figure 11. Dorsal stripe abnormality behind the eyes (circled green). Note the vegetation stuck to its body (circled red).

Figure 12. *E. calamita* showing obvious scarring on snout

DISCUSSION

The first objective of this study was to establish whether photo-identification of *E. calamita* is feasible, using the natural markings on the dorsum of individuals, for citizen scientists. This would build on the success and uses of photo and visual identification in mammal ecology and reduce the need for invasive treatments when examining populations of toads. The study demonstrates that *E. calamita* toads at the study site indeed had sufficient variability of natural markings on their dorsum for photo-identification to be effective.

The second objective was to ascertain whether the training of citizen scientists improves the accuracy of identifying individuals by dorsal markings. Training the participants had a significant effect on the results, increasing accuracy of correct identification. Consequently, training significantly improved photo-identification of toads within this study. It may be considered that repetition of the photo-matching exercise may also have had an influence in improving accuracy. However, in the group that received no training there was a decrease in correct photo-identification in the second exercise. This decrease may have occurred because of the changed sequence of photographs in the two exercises they undertook. They were given their scores in between tests and so their confidence may have diminished. This would further demonstrate the effectiveness of the training.

The third objective was to ascertain whether training improves the speed at which individual toads can be identified. The time taken to complete photo-matching was reduced following training of the participants. When the technique is used in practice, care must be taken not to reduce accuracy in photo-identification in an attempt to increase speed. However, it will also be very important, in practice, to minimise the time handling the toad for photographing in order to reduce possible stress to the toad.

It is possible that participants naturally found scarring easier to use for identification as opposed to the dorsal stripe. Individual identification using natural scarring is not uncommon in animals and is described by Gilkinson et al. (2007), in which sea otters were identified from their nose scars. According to Bertolotti et al. (2013), African clawed frogs fail to maintain regenerative capability past metamorphosis and show a progressive loss of scar-free repair as they develop. While this appears to be inferring that permanent identification of scarred adults could be possible, it may not be the case with *E. calamita* and should not be relied upon without further investigation. A variable that photographs Figure 9 and Figure 10 shared with each other was that the toads had little to no scarring visible. In contrast, the remaining three photographs, which were identified with much more accuracy, had noticeable scarring, for example, Figure 12. One possible explanation as to why the toads shown in Figures 9 and 10 were misidentified more often could be that participants naturally found scarring easier to use for identification as opposed to the dorsal stripe.

Changes in pattern can result in false-positive and false-negative identifications, resulting in faulty estimates of populations (Gebauer, 2009). Other than scarring, Odum and Sonntag (2010) state that once amphibians reach adulthood, their skin pattern is unlikely to change. Denton & Beebee (1993) found that throat-spot patterns in female *E. calamita* did not change considerably during a two-year study. However, the permanence of dorsal markings requires further study.

It was noted that when photographing the toads, the position of the toad needs to be as standard throughout as practicable. In this study, each toad was placed in a Perspex container with ample room to move freely, thus changing orientation and body shape between photographs. Prior to training, some participants searched for a curved dorsal stripe, when in fact the curvature was due to the position that the toad was sitting in. Curvature in the posture of the toad can also result in some features being out of focus in the photograph.

Knox et al. (2013) states manually matching photographs of individual animals is only suitable for small populations, because as the sample base increases, so does the time spent in manually matching photographs. For species living in small and fragmented populations, such as the *E. calamita* at the study site, manual matching appears to be suitable.

In the context of monitoring endangered populations, developing survey methods that minimise the potential handling effects and welfare concerns related to animal capture is vital (McMahon et al., 2005). An important

advantage of the photo-identification method is the ability to identify individual animals without damage, although it still requires the capture and handling of individuals. Kenyon et al. (2009) stated that photographing green-eyed tree frogs *Litoria genimaculata* took longer than toe clipping. However, in this study, which took place over the period of a month, there was no indication that disturbance affected the individuals as some were recaptured on multiple occasions. It should be noted that, in the United Kingdom, photo-identification of *E. calamita* can only be carried out with a licence and having a licence holder present.

CONCLUSION

Citizen science can be a useful way of collecting data intensively and repetitively over wide geographical areas. However, Aceves-Bueno et al. (2017) recommend that the citizen science tasks should be designed with the skills of the citizen scientists in mind. They recommend that in order to do this, initial reference data needs to be collected on the reliability of the data collection. The data for the photo-identification of *E.calamita*, in the study described in this paper, act as reference data and shows that the correct identification occurs in the majority of cases, but there is not complete reliability. This factor requires that statistical adjustment would need to be made when estimating population size from photo-identification and recapture surveys.

Although toe-clipping has been the method of choice in individual recognition of *E. calamita* toads (Boomsma & Arntzen, 1985; Tejedo, 1988; Denton, 1991), this study reveals that the dorsum has sufficient variability of natural markings in order for photo-identification to be an effective alternative. A difference was observed between trained and untrained participants in the accuracy and speed of individual identification.

The method is easily repeatable by investigators with the statutory licence. However, in order to obtain the highest level of accuracy, the quality of photographs and the position of the toad should be taken into account.

Ideally, an identification method used within a survey by citizen scientists should adhere to all of the following criteria (Lewke & Stroud, 1974; Ferner, 1979; Reaser, 1995): (1) does not affect the animal's survivorship or behaviour, (2) allows the animal to be as free from stress and pain as possible, (3) identifies the animal as a particular individual, (4) is reliable over the duration of the study, (5) is easily read or observable, (6) is adaptable to organisms of different sizes, (7) is easy to use in both laboratory and field conditions, and (8) utilises materials that are easy to obtain.

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SHORT NOTE

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Colouration in male blue-throated keeled lizards (*Algyroides nigropunctatus*): Evidence for ultraviolet reflectance of throat and lateral patches

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The blue-throated keeled lizard, *Algyroides nigropunctatus*, is distributed along the Adriatic coast from Italy to Greece and is sexually dichromatic. Males display a striking blue on their throat, an orange ventrum, and a dark brown dorsal colouration, but their colouration has never been objectively assessed. Here, we describe the colouration of 13 male blue-throated keeled lizards from Cres Island (Croatia) using spectrophotometry and ultraviolet (UV) photography, and show that the blue throat and the blue spots located on the flanks reflect in the UV part of the spectrum. We discuss the potential role of UV-blue colouration in social signalling.

Key words: Chromatic signal, Ultraviolet, Spectrophotometry, Lizards, Squamates

The lizard genus *Algyroides* comprises four species with disjoint distributions in southern Europe (Arnold & Ovenden, 2010). Compared to other lacertid species, relatively little is known about the behaviour, ecology, or evolution of these lizards, although morphological and molecular analyses have confirmed the monophyly of the genus (Harris et al., 1999). *Algyroides nigropunctatus* Duméril & Bibron (1839), commonly known as the blue-throated keeled lizard or the Dalmatian *Algyroides*, has the largest distribution range of the genus, and is found along the coast of the Balkan peninsula from Italy to Greece (Böhme, 1981). *Algyroides nigropunctatus* inhabits open and semi-open habitats (e.g. open woodland, bushy areas, stonewalls), usually favouring shady areas (Haxhui, 1991; Bressi, 2004; Arnold & Ovenden, 2010). This species exhibits a marked sexual dimorphism in size and shape with males having relatively larger heads than females, which allows the packing of jaw musculature and a compressed braincase (Ljubisavljevic et al., 2011). They are also sexually dichromatic with adult males displaying an orange ventral colouration and a striking blue colouration on their throat (Arnold & Ovenden, 2010; Carlino & Pauwels, 2016), while females are ventrally white to yellow and have no or less bright blue colouration on their throat (Arnold & Ovenden, 2010). Dorsally, males and females are dark brown with black spots present on some of their dorsal scales, to which the species owes its name. Geographical variation in colour pattern has been described, particularly in some insular populations. In the Ionian islands of Lefkada, Kephalliana, and Itaka (Greece) male *A. n. kephallithaticus* are ventrally yellow and their throat shifts from blue to green after the mating season (Arnold & Ovenden, 2010). In other Greek islands like Corfu and Erikoussa, males may exhibit a flashy orange colouration on their throat (unpublished information). However, to the best of our knowledge, no study to date has examined the colour pattern of *A. nigropunctatus* using objective (i.e. independent of the human visual system) methods. Here, we use standard reflectance spectrophotometry along with UV and human-visible photography to provide the first objective description of the colour pattern of males of this species.

In April 2016, we captured 13 adult males (SVL > 59 mm) of *A. nigropunctatus* by noosing in Cres Island (Croatia) in their typical habitat (e.g. shady areas, stone walls with vegetation, open woodland). Upon capture, we took ultraviolet (UV) pictures of every individual using a digital camera (Olympus PEN Mini) converted for UV photography by replacing the standard internal hot mirror filter with a Spectrosil 2000 fused silica filter, which transmits light wavelengths down to 170 nm. The camera was fitted with a UV-transmitting macro lens (Noflexar Novoflex 1:3,5/35mm) and a Baader U-filter with peak transmission between 320 and 380 nm. UV photographs were taken outdoors in the shade using natural illumination. For comparison, we also took pictures identical to the UV ones but in the human-visible range using standard digital cameras.

Lizards were transported in cloth bags to a darkened room where we obtained reflectance spectra of the ventrum, throat, and dorsum using a JAZ spectrophotometer with a R200-7-VIS-NIR reading-illumination probe and a PX-2 xenon strobe light source (Ocean Optics Inc.) for full spectral illumination. We averaged reflectance readings over 5 nm using a kernel

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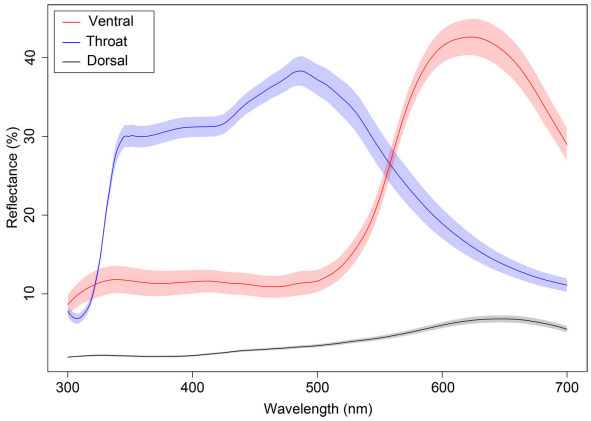

Figure 1. Non-normalised reflectance spectra of the throat (blue), the ventrum (red) and the dorsum (black) of 13 adult males of *A. nigropunctatus*. Reflectance is expressed as mean ± SEM (coloured area surrounding the line).

smoothing function. We set integration time to 30 ms, scans to average to 10, and boxcar width to 10. For data acquisition, we hand-held the probe over the centre of the throat, ventrum and dorsum of the animals, perpendicular to the surface (i.e. illumination and readings angles were both 90°). An entomological pin attached to the side of the probe allowed us to maintain a constant distance of 5 mm between the tip of the probe and the target surface (see Badiane et al., 2017 for more details). Colour spots measuring less than 2 mm in diameter were ignored as they are beyond the resolution of our spectrophotometer set-up (Badiane et al., 2017). Reflectance spectra were analysed in R using the PAVO package (Maia et al., 2013). All animals were released at their capture sites after a maximum of 24 hours post-capture.

The throat of male *A. nigropunctatus* has a spectrum with a reflectance peak in the blue part of the spectrum (485 ± 10 nm; mean ± SEM), a relatively flat plateau extending into the UV range, and then a sharp drop-off at approximately 340 nm (Fig. 1). As expected, spectra of the orange ventrum show a pronounced reflectance peak in the orange-red (621 ± 3 nm). In contrast to the throat and ventrum, reflectance spectra of the dorsum are characteristically flat with little reflectance across the spectrum and show no obvious reflectance peaks (Fig.1). UV photographs confirm that the throat colouration reflects in the UV range and is therefore best characterised as UV-blue (Fig. 2A). The UV-blue colouration of the throat covers the whole throat, sometimes extending over the chest and the supra-labial scales. The ventral orange colouration covers the ventrum and the limbs (ventrally only) and merges with the dorsal colouration on the flanks and neck. In addition, some of the captured males displayed regularly-spaced blue spots (up to 9 on each side) on some of their outer ventral scales (OVS) (Fig. 2B). Unfortunately, these spots were too small to obtain reliable reflectance spectra. However, the UV photographs revealed that these spots are also UV-reflecting (Fig. 2C).

Many lizard species, including lacertids, are capable of seeing in the ultraviolet range of the spectrum (Fleishman et al., 2011; Pérez i de Lanuza & Font, 2013; Martin et al., 2015a) and display UV colours that often appear as blue to the human eye (Whiting et al., 2006; Pérez i de Lanuza et al., 2014). Our results show that the throat of adult males of *A. nigropunctatus* is highly reflective in the UV range of the spectrum. Although this is the first report of UV colouration in *A. nigropunctatus* (see Arribas, 2002), many other lacertids display UV colour patches that purportedly function as social signals, including *Gallotia galloti* and *G. atlantica* (Font & Molina-Borja, 2004; Molina-Borja et al., 2006), *Timon lepidus* (Font et al., 2009), *Lacerta agilis* (Pérez i de Lanuza & Font, 2007), *L. viridis* (Bajer et al., 2010), *Zootoca vivipara* (Martin et al., 2015b), and several species of *Podarcis* (e.g. Marshall & Stevens 2014; Pérez i de Lanuza et al., 2014; our own unpublished data). It is possible that the UV-blue colouration also plays a

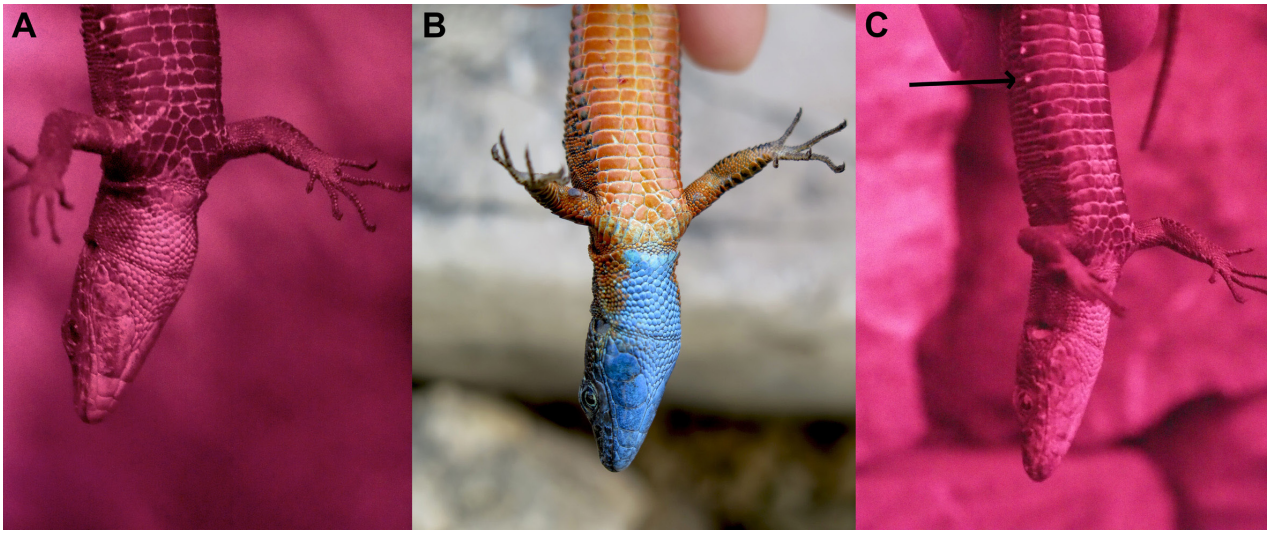

Figure 2. Photographs of a representative male of *A. nigropunctatus* from Cres Island in the UV (A, C, ventrally and laterally respectively) and in the human-visible range (B). The photographs in A and C were taken with a modified digital camera through a UV-transmitting filter that blocks most of the light wavelengths outside the 320-380 nm range. UV-reflecting skin patches in A and C are visible due to their lighter, whitish colouration (UV reflecting spots are indicated by an arrow in C).

role as a social signal in *A. nigropunctatus*, either as an ornament (female choice) or as an armament (male-male competition) because the ventrum and throat of lizards, and particularly of lacertids, are often the target of sexual selection and are used to convey information to conspecifics by means of chromatic signals (Leal & Fleishman, 2004; Bajer et al., 2010; Martin et al., 2015b). For example, males of *Lacerta schreiberi* also exhibit an iridescent UV-blue colouration on their throat (Pérez i de Lanuza & Font, 2014) with a spectral shape similar to the throat of *A. nigropunctatus*, which may function as an indicator of individual quality (Martín & López, 2009). Although we do not have spectral data for the UV-blue OVS, based on their similarity to UV-blue OVS from other lacertids (e.g. *Podarcis*, Pérez i de Lanuza et al., 2013) it is likely that their reflectance peak is also located in the UV range. Interestingly, this is the first lacertid species in which both a UV-blue throat and UV-blue OVS are described as most species studied to date have only one type of UV-blue patch, or they have none. Unfortunately, almost nothing is known about the behaviour of *A. nigropunctatus* and so discussion about the putative functions of these UV-blue patches is speculative.

The body colouration of male *A. nigropunctatus* conforms to the general pattern found in lacertids, with a relatively cryptic dorsum, possibly selected for background-matching, and conspicuous colour patches on the less visible lateral and ventral body surfaces. In many species, the latter are used for signalling and are made more or less visible through a variety of stereotyped movements and/or postural adjustments. The allocation of functionally different colour patches to different body regions (i.e. signal partitioning) allows balancing effective communication with the risks of detection by unintended receivers (Endler, 1992; Marshall & Stevens, 2014). Furthermore, the colours of the throat and ventrum in *A. nigropunctatus* are complementary, each one reflecting in the region of the spectrum where the other does not (see Fig. 1), suggesting that their presence on adjacent patches has been selected to maximise signal conspicuousness (Pérez i de Lanuza & Font, 2016).

Recent technical and methodological advances have expanded the breath of taxa for which spectral data are available (Kemp et al., 2015). However, our knowledge of animal colouration is largely shaped by studies of a handful of model species and very little is known about the colouration of most species alive today. Research on relatively understudied species, such as *A. nigropunctatus*, is a useful complement to studies with model species and essential to uncover general principles of lizard colouration.

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SHORT NOTE

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Effect of fish stocking on alpine populations of European common frog (*Rana temporaria*) in the Pyrénées National Park

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The introduction of non-native species is one of the factors driving the global decline of amphibians. We examined the effect of fish stocking in naturally fishless mountain lakes and ponds on the local populations of the European common frog (*Rana temporaria*). We surveyed 215 mountain lakes and ponds and noted the presence or absence of frogs and signs of frog reproduction (i.e. tadpoles, eggs). We compared these data with fish stocking data from the regional park management (all surveyed lakes and ponds). Our results show a strong negative effect of fish stocking on the presence of *R. temporaria*, and an even stronger effect on its breeding presence, but we found a small number of lakes and ponds where coexistence occurred. In addition, the preferential stocking of large, deep lakes and ponds left smaller ponds as the only remaining habitats, a number of which are likely to become temporary due to increased summer temperatures. We recommend a series of measures to conciliate fish stocking for recreational fishing whilst conserving *R. temporaria* populations, which might be extensible to other high mountain environments.

Key words: Fish stocking, *Rana temporaria*, *Oncorhynchus mykiss*, amphibian conservation, Pyrénées National Park

Amphibians have become a cause of great concern in recent years due to their sharply declining populations worldwide and their high extinction rates (McCallum, 2007). Although the exact numbers are in dispute (Stuart et al., 2004; Pimenta et al., 2005; Stuart et al., 2005), there is no doubt that a very large proportion of amphibian species are under severe threat. Some of the main factors that drive the decline of amphibians include climate change (Corn, 2005; Ryan et al., 2014), habitat fragmentation and loss (Cushman, 2006), emerging amphibian diseases (Daszak et al., 1999; North et al., 2015; Yasumiba et al., 2016) and the introduction of non-native species (Adams, 1999).

For many years the last factor has been the main subject of countless studies (reviewed by Kats & Ferrer, 2003). Specifically, the effects of non-native fish introduction as a potential new predatory pressure on frog populations have received much-needed attention (Knapp & Mathews, 2000; Bosch et al., 2006; Welsh et al., 2006). Introduction of salmonids in lakes and ponds with no native fish fauna is one of the more common ecosystem modifications of mountain lakes (Radomski & Goeman, 1995; Lodge et al., 1998; Rahel, 2000).

Although the introduction of some fish species seems to have low or imperceptible effects on the native communities (Moyle & Light, 1996), other species have been known to produce local extinction of native fauna and substantial changes in ecosystem structure (Anderson, 1980; Herbold & Moyle, 1986; Hrabik et al., 1998). Due to its predatory nature, the introduction of trout in some lakes in the USA has caused a remarkable impact on faunistic groups like Amphibia, zooplankton and benthic macroinvertebrates, leading in some cases to their complete disappearance (Anderson, 1975, 1980; Bradford et. al., 1998; Carlisle & Hawkins, 1998; Knapp & Matthews, 2000), as well as the alteration of the food web and algae production (Leavitt et al., 1994).

Fish species tend to become dominant in the lakes' food chain, because they live for many years and their biomass is usually constant from one year to the next. In the case of cold water fish like trout, warm summers make it possible for the fish born that year to reach a sufficient size to resist the winter conditions, and a lack of predators ensures the success of many consecutive cohorts (Wetzel, 1981).

Amphibians living in mountain lakes and ponds are very sensitive to the introduction of salmonids in their habitats, and their populations show significant decreases in response to fish stocking (Knapp & Matthews, 2000) as well as signs of recovery when the practices are stopped (Knapp et al., 2007). The French National Park of the Pyrénées has for some time stocked lakes and ponds with trout for recreational fishing, but the impact of these practices in the native populations of *Rana temporaria* has not been properly evaluated. Our hypothesis is that trout stocking diminishes frog numbers in the lakes and

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ponds where they are introduced. The present study evaluates the impact of rainbow trout (*Oncorhynchus mykiss*) stocking in the populations of *R. temporaria* inhabiting mountain lakes and ponds within the confines of the French National Park of the Pyrénées (Fig. 1A).

During July of 2001 and 2002 we surveyed 215 lakes and ponds in the core of the French National Park of the Pyrénées within an area of ca. 450 square km located from 0°36'54"W to 0° 9'3"E and 42°42'50"N to 42°52'9"N, at a altitude between 1580 and 2747 m a.s.l. (Fig. 1C). Most of the sampled lakes and ponds (Online Appendix 1) are inaccessible by car, so the survey was conducted in two 20-day treks, one each year in July and August (Fig.1B). In summer, frog adults, tadpoles and egg clutches are mainly located in shallow water near the shore (Bradford, 1989). This allowed us to use visual encounter surveys (Crump & Scott, 1994) along the lakes and ponds' entire shoreline to evaluate the presence or absence of adult *R. temporaria* as well as any evidence of ongoing reproduction (by presence of eggs or tadpoles).

We characterised the lakes and ponds by a number of variables: location (longitude and latitude), altitude (m a.s.l.), total surface (m^2), pH, conductivity (µS/m), geomorphology (drainage basin) as a vector for isolation and presence of rock, and vegetation in the riparian area. We measured water pH and conductivity on site at the surface and bottom of the lake from samples taken with a Teflon bottom water sampler. We used Hach HQ40d portable pH and conductivity probes (IntelliCAL PHC201, accuracy ±0.002 pH and IntelliCAL CDC401, accuracy ±0.5% from 1 µS/cm–200 mS/cm). We analysed the geomorphology to study the isolation of the lakes and ponds. Other studies from the same sampling surveys have analysed in depth the ecology of these lakes (see Zaharescu et al., 2016a; Zaharescu et al., 2016b; Zaharescu et al., 2017).

As part of the official management policy of the Pyrénées National Park, several different lakes and ponds were stocked for recreational fishing. The fish stocking was carried out by helicopter so that when positioned over the selected lakes, a Park employee (using a climbing harness) descends close to the lake surface holding a bucket of water containing the rainbow trout fry, which are carefully deposited in the water. The park administration provided us with the fish stocking data from 1998-1999, which included 68 of the total surveyed lakes and ponds. Due to the strict control over private stocking in the park, these data were considered reliable. In addition, we found no visual evidence of trout or trout larvae on any of the non-stocked lakes and ponds during our surveys.

Data from the lakes and ponds were analysed by pooling all the lakes and ponds together. Each of the lakes and ponds was characterised according to its combination of presence or absence of rainbow trout fry, frog, and evidence of frog breeding. We then built dispersion diagrams using IBM SPSS Statistics 19.0 (SPSS, Inc., Chicago, IL) statistical package for Windows, in order to examine the effect that the presence of rainbow trout fry had in the frog breeding presence. It is worth noting that evidence of frog breeding presence was considered as evidence of frog presence, but presence of adult frogs was not considered evidence of frog breeding presence.

We surveyed a total of 215 ponds and lakes that we grouped into four lakes and ponds types: 63 were fish stocked (type A), 42 were non-fish stocked and we did not find frog breeding presence (type B), 5 were fish stocked and we found frog breeding presence (type C), and 105 were non-fish stocked and we did not find either frog presence nor frog breeding presence (type D). We conducted Kruskal-Wallis and Jonckheere-Terpstra non-parametric tests on pond and lake types A, B and C as described above, with type D omitted as it had no biological meaning for this study.

A Kruskal-Wallis H and Jonckheere-Terpstra tests showed statistically significant differences in lake and pond type between some of the considered variables: Total surface (m^2) ($\chi^2(2) = 55.976$, p = 0; J-T = -6.636, p = 0), Altitude m a.s.l. ($\chi^2(2) = 7.345$, p = 0.025; J-T = -2.292, p = 0.022), Latitude UTM ($\chi^2(2) = 13.19$, p = 0.001; J-T = -3.529, p = 0), and Longitude UTM ($\chi^2(2) = 0.879$, p = 0.644; -T = 0.817, p = 0.414). From a geological point of view, the Pyrénées mountain chain runs from east to west, and thus the latitude (N-S) is obviously linked to altitude and geology. Therefore, it does not have any biological meaning in our study. The pH and Conductivity values among the studied lakes were similar and did not show any significant differences. The total surface is the variable that best discriminates the grouping variables studied (see Online Appendix 3).

In order to see the effect of the four studied variables in the presence of frog breeding and frog adults we ran Kruskal-Wallis H and Jonckheere-Terpstra tests, which did not show any significant differences between the lakes and ponds with frog breeding presence and frog adults (Total surface (m^2): $\chi^2(2) = 2.052$, p = 0.359; J-T = 0.109, p = 0.913; Altitude m a.s.l.: $\chi^2(2) = 0.602$, p = 0.74; J-T = 0.246, p = 0.806; Latitude UTM: $\chi^2(2) = 6.632$, p = 0.036; J-T = -0.772, p = 0.44; Longitude UTM: $\chi^2(2) = 8.622$, p = 0.013; J-T = 1.511, p = 0.131) (see Online Appendix 4).

To determine if trout stocking effected frog presence in our study area, the degree of isolation between each water body was estimated from the average distance of the focal water body to the nearest three water bodies (in metres) (Cogalniceanu, 2012). Our studied lakes and ponds were gathered in two groups, lakes and ponds in the west with a lower fish stocking rate (7%) and the lakes and ponds in the east with a higher fish stocking rate (34%). The average distance between water bodies groups was 274.5 m (± 175 m) and 342.9 m (± 236 m) respectively, which supported our hypothesis.

We characterised each lake and pond as a function of the percentage of rock and vegetation present in their riparian area and the mean of the total surface in order to find any differences between lake and pond types [ie. fish stocked (type A), non-fish stocked and frog breeding present (type B), fish stocked and frog breeding present (type C)]. In total 63 lakes and ponds were characterised as type A, 42 as type B and 5 as type C. Of those characterised as type A (Online Appendix 2), the mean percentage of rock and vegetation present in the

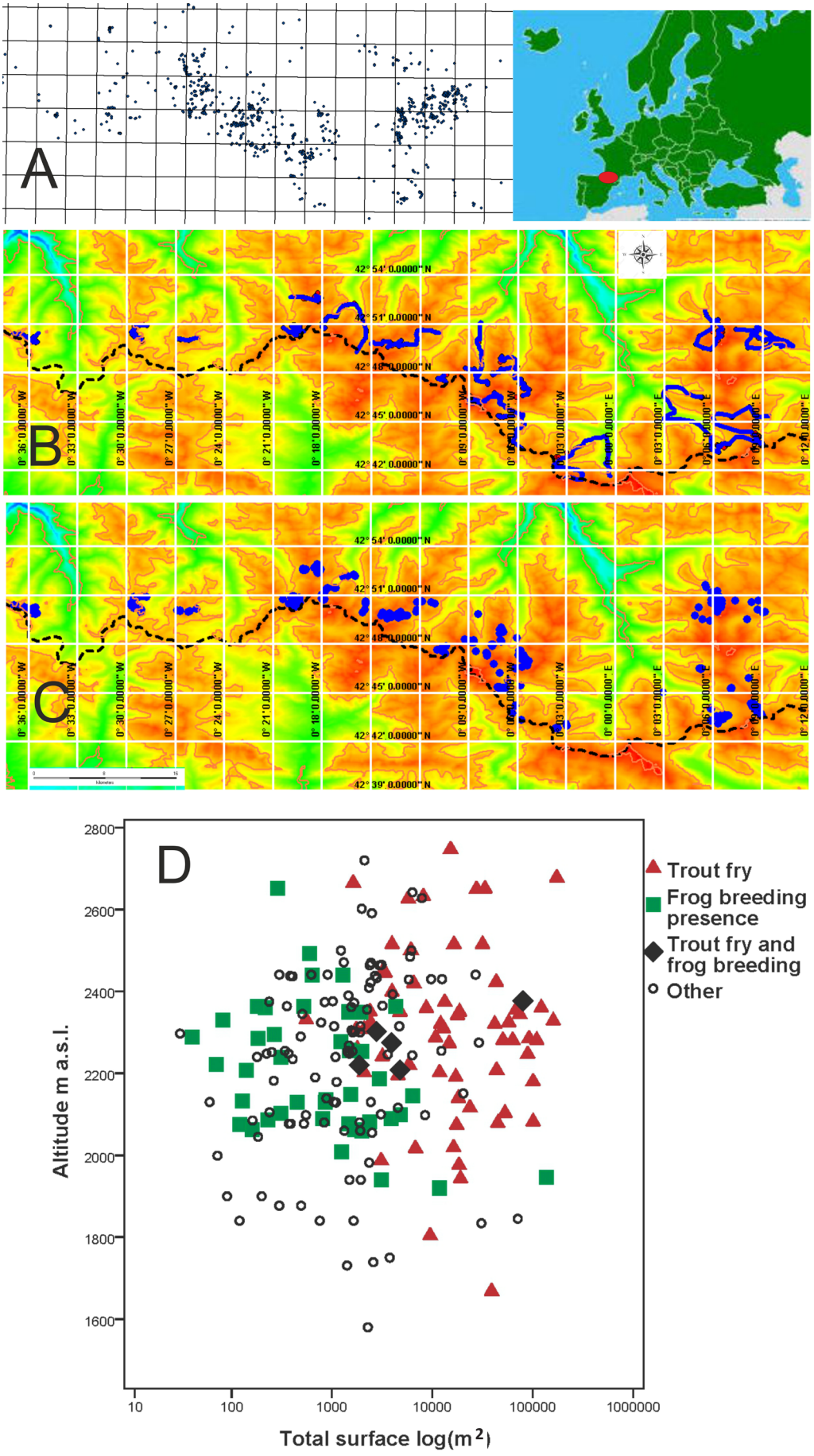

Figure 1. A. Map of the lakes from all the sectors in the French National Park of the Pyrénées. B. Treks surveys carried out during 2001 and 2002 in the core of the Park. C. Sampled lakes. D. Dispersion diagram showing altitude (m a.s.l.) and Total surface log (m^2).

riparian area were 73% and 20% respectively, and the mean total surface area was 31680 m^2. In type B lakes and ponds (Online Appendix 2), the mean percentage of rock and vegetation present in the riparian area were 43% and 44% respectively, and the mean total surface area was 4785 m^2. Finally, in type C lakes and ponds (Online Appendix 2) the mean percentage of rock and vegetation present in the riparian area were 36% and 42%, and the mean total surface area was 18641 m^2. We found that the percentage of rock and vegetation present in the riparian area of lakes type B and C were very similar. More detailed descriptive statistics of these and non-significant variables studied can be found in Online Appendix 3.

Of the 215 lakes and ponds surveyed after fish stocking, *R. temporaria* was present in 97 of them, which represented 45.1% of the total. We also found the presence of *R. temporaria* reproduction in 49 of the lakes and ponds surveyed (22.8%). Fish stocking had occurred in 68 lakes and ponds (31.6%). The altitude range where we conducted our research of *R. temporaria* was between 1580 and 2747 m a.s.l., and we found *R. temporaria* from 1739 m, however we did not find *R. temporaria* or any other amphibian at elevations higher than 2652 m a.s.l. Lakes and ponds with bigger surface areas were found to be stocked more frequently, which is consistent with the fact that stocking was done via helicopter and only the largest lakes were accessible (Fig. 1D). When studying the four types of lake and pond distributions we found that the spatial distribution was homogeneous among six major drainage basins studied (Online Appendix 3, Fig. A), distribution across latitudes and altitudes was uniform due to the north-south drainage basin orientation, the distribution across longitudes and the total surface areas was uniform (Online Appendix 3, Fig. B), and the distribution across altitudes and longitudes was uniform (Online Appendix 3, Fig. C).

Our results showed an adverse impact of rainbow trout fish stocking on the presence of *R. temporaria*, a result consistent with other studies of fish stocking impact on amphibian populations (Bradford, 1989; Braña et al., 1996; Bradford et al., 1998; Tyler et al., 1998; Knapp & Mathews, 2000; Knapp et al., 2001; Martinez-Solano et al., 2003; Pope et al., 2008; Pilliod & Peterson, 2001; Knapp, 2005; Orizaola & Braña, 2006; Dan et al., 2012; Băncilă et al., 2017).

This impact can be seen when evaluating frog presence using individual lakes and ponds. Frogs and trout showed very limited overlap (Fig. 1D and Online Appendix 2C) and as a general rule frog presence declined wherever trout presence increased. Trout stocking within the park was biased towards larger lakes and ponds, which were both more popular among trekkers and easier to stock using helicopters. As a consequence, frogs were more frequently found in smaller, harder to reach ponds (Fig. 1D and Online Appendix 2B) which would become preferred breeding sites (Tiberti & Hardenberg, 2012). Many of these smaller ponds tend to be temporary, which forces new cohorts to adapt to a shorter growing period (Newman, 1989). Populations that become specialised in rapid development and an early metamorphosis at low metamorphic size are known to have a lower degree of phenotypic plasticity (Lind & Johansson, 2007; Miramontes et al., 2018) and lose fitness as a result of their smaller size at metamorphosis (Altwegg & Reyer, 2003). In addition, smaller ponds are likely to see an increase in their temporality with climate change as temperatures in the Central Pyrénées rise (Catalan et al., 2002; El Kenawy et al., 2011; Pérez-Zanón et al., 2016), which results in increasing evaporation in summer and a decrease in the availability of snowmelt water (Lopez-Moreno et al., 2008, 2009). This increase in the temporality of lakes due to climate change has been reported before and has been shown to have deleterious effects on amphibian populations (McMenamin et al., 2008). Such changes can also cause an increase in habitat fragmentation in an alpine habitat that is naturally fragmented, which could also be detrimental to *R. temporaria* populations (Johansson et al., 2005, 2007).

Although it has been shown previously in other protected areas that the only management practice that has a positive effect on the protection and recovery of mountain lakes and ponds and its fauna is the prohibition of fishing (Miró & Ventura, 2013) and other studies have proven that the introduced fish removal has a positive effect in the recovery of the frog populations (Vredenburg, 2004; Knapp, 2007) and other amphibians (Funk & Dunlap, 1999), we recommend considering the lake and pond characteristics before designing the fish stocking strategy (pictures available in Online Appendix 2). Due to recent efforts to study coexistence of predators and preys (Hartman et al., 2014; Kenison et al., 2016; Winandy et al., 2017) and in order to minimise the fish stocking impact, we propose to group lakes and ponds in different classes ordered from minimum to maximum fish stocking impact.

The lakes and ponds susceptible to fish stocking with minimum impact on our studied species are lakes that are used as water reservoirs like Migouelou 2278 m a.s.l.; lakes where the effluent is subterranean and the affluent present a sharp slope making impracticable the displacement of fish (e.g. Remoulis 2017 and 2019 m a.s.l., Badete 2344 m a.s.l., col d'Aratille 2501 m a.s.l., Pouey Laun 2346 m a.s.l. and Nére 2241 m a.s.l.); and lastly lakes with small ponds nearby and interconnected with the main lake and have subterranean effluents (e.g. Batcrabere 2116 and 2180 m a.s.l.).

A second group are the big lakes that are easily accessible (e.g. Suyen 1536 m a.s.l. and Tech 1207 m a.s.l.) and can be fish stocked, as these are appropriate for fish reproduction. These may also be ideal candidates to make neighbouring artificial ponds in order to recuperate amphibian and general fauna, which could be extended to other lakes.

A third group are lakes with a series of nearby and intercommunicated ponds with subterranean effluents (e.g. Houns de Heche 2113 m a.s.l. and lac du Pic Arrouy 2376 m a.s.l.). In this case, to minimise the effect of fish stocking we propose to isolate the lake from the ponds in order to avoid the fish displacement to small neighbouring ponds.

A fourth group would be lakes where the fish could displace through their effluents (e.g. Fache 2332 and 2427 m a.s.l., d'Aratille 2247 m a.s.l.) and also affluent (e.g. Lassiedouat 2200 m a.s.l.). In this case the impact might be higher, thus more effort should be done to avoid and prevent the displacement of the fish through the drainage basin.

A fifth group with the potential to have an extensive impact of fish stocking are small lakes (e.g. Laquets de Micoulaou 2302 and 2333 m a.s.l., Lascarat de Migouelou 2429 m a.s.l., Laquets de Lassiedouat 2220 and 2268 m a.s.l. and Araille 2450 m a.s.l.) where fish would rapidly consume available nutrients; and lakes with flooded lands nearby with a big number of amphibian species (e.g. Plaa de Prat 1656 m a.s.l.). Also, lakes that are physically suitable for amphibians should be conserved to host the most numerous populations of *R. temporaria* in the Park, as in the case of Lake Touest 1955 m a.s.l., or small lakes and ponds along Badete 2344 m a.s.l. and Lacs d'Aratille 2247 and 2315 m a.s.l.

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Supplementary material:

- Appendix 1: pictures of 100 sampled lakes and ponds are available in a pdf file.
- Appendix 2: pictures of the three lakes and ponds types in function of its percentage of rock and vegetation in their riparian area.
- Appendix 3: dispersion diagrams showing different lake types' distribution (longitude (UTM) and latitude (UTM) (Fig. A); longitude (UTM) and total surface log (m2) (Fig. B); longitude (UTM) and altitude (m a.s.l.) (Fig.C).
- Appendix 4: descriptive statistics of the studied variables in the three types of ponds and lakes (A, B and C), one-way ANOVA and non parametric Kruskal-Wallis and Jonckheere-Terpstra tests.
- Appendix 5: non-parametric Kruskal-Wallis and Jonckheere-Terpstra tests to analyse the effect of the total surface area, longitude, latitude and altitude on the distribution of frog breeding and frog adults' presence in the study area.

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