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Contents

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Front cover: The broad-snouted caiman *Caiman latirostris*. There is an article about this species on page 237.
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Full papers

Genetic study of an isolated population of adders <i>Vipera berus</i> founded by historic translocation: implications for conservation <i>Sarah Ball, Silviu Petrovan, Esme Ashe-Jepson, Claire Dobson, Becki Lawson, Liz Morrison & Trent Garner</i>	197–210
Diversity and distribution of reed frogs (<i>Hyperolius</i> spp.) on Bioko Island, Equatorial Guinea <i>John V. Nguyen, Patrick J. McLaughlin, Christian Irian, Lauren A. Scheinberg & Rayna C. Bell</i>	211–220
Predation of anurans in southern England by <i>Batrachobdella algira</i> , a leech previously unknown in the UK <i>Katharina Seilern-Macpherson, Becki Lawson, Craig R. Macadam, Pete West, Neil Reed, Louise Gibson, Piotr Świątek, Łukasz Gajda, Andrew A. Cunningham, Joseph Heaver & Angela M. Julian</i>	221–227
Small-scale geographic factors promote a high diversity of amphibians and reptiles in Colombia <i>Elson Meneses-Pelayo, Víctor Hugo Serrano Cardozo & Martha Patricia Ramírez-Pinilla</i>	244–255
Differential longevity in free-living smooth snakes <i>Coronella austriaca</i> <i>Christopher J. Reading</i>	266–271

Reviews

Methods used in sea turtle genetic and molecular studies <i>Cibele Castro Monteiro, Jhonatas Teixeira Viana, Karen Karoline Abreu da Silva, Rafael dos Santos Rocha, Vicente Vieira Faria & Rodrigo Maggioni</i>	228–236
Scorpion mud turtle breeding in the Amazon: zootechnical and environmental activity for the species conservation <i>Palmira Francisca Gonçalves Ferreira, Ednaldo da Silva Filho, Larissa Coelho Marques, Relionan Pimentel Leal & José Ribamar Felipe Marques</i>	256–265

Short note

GPS-telemetry as a method to assess nest attendance by a female broad-snouted caiman <i>Caiman latirostris</i> <i>Paulo B. Mascarenhas-Junior, Rafael S.L. Barboza, Malu Caminha, Gabriela Lucena, Carlos F. Rodrigues, Pedro Ivo Simões & Jozelia Maria de Sousa Correia</i>	237–243
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Genetic study of an isolated population of adders *Vipera berus* founded by historic translocation: implications for conservation

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Adder *Vipera berus* populations in Great Britain have undergone substantial declines in recent decades. Isolation due to habitat fragmentation particularly threatens the demographic and genetic health of small populations. Despite the potential benefit of population supplementation in the conservation management of affected populations, there are currently no consensus guidelines for conservation-motivated translocations of this species. Translocations of adders for ecological mitigation of land development are more frequently undertaken, although these are typically poorly documented and insufficiently monitored, representing a wasted opportunity for strategic learning and improvements. We studied an isolated adder population on a protected site in eastern England, founded in 1999 by the translocation of adders from a development site. With known numbers, age and sex of released individuals, this represented an opportunity to improve our understanding of the genetic outcome of a newly created population. Although apparently thriving despite a low founder number, the finding in 2015 of a stillborn clutch of adders raised the possibility of inbreeding in the population. We sampled adders from the translocated population in 2017, and two individuals from the donor site in 2018. Although we found no increase in homozygosity, relatedness and maximum likelihood sibship analysis revealed high levels of consanguinity, especially within the subgroup of adults. Demographic modelling with Approximate Bayesian Computation supported the known origin of the population, but also a subsequent, undocumented adder release to the site, accounting for the observed healthy proportion of young adders with lower levels of consanguinity. Despite the protected habitat site, the population remains isolated, and thus demographically and genetically vulnerable. We highlight the importance of careful post-translocation monitoring including targeted genetic analyses. Strategic data gathering coupled with careful management of translocations, whether for ecological mitigation or conservation rescue, could support significant improvements to the conservation management of this species, including reintroduction initiatives.

Keywords: relatedness, inbreeding, stillbirth, foundation bottleneck, population supplementation

INTRODUCTION

Populations of the adder *Vipera berus* (Linnaeus, 1758) are declining in parts of the species' native range across Western Europe (Reading et al., 2010; Guiller et al., 2022), including Great Britain (Arnold, 1995; Baker et al., 2004). Small, spatially confined populations are at risk of becoming demographically non-viable (Lande, 1988; Ball et al., 2020), notably threatened by habitat fragmentation, predation and human disturbance (Gleed-Owen & Langham, 2012; Gardner et al., 2019). Adders, in common with other temperate snakes, are viviparous with low fecundity, low vagility and high philopatry (Madsen & Shine, 1992; Bauwens & Claus, 2019). They are especially vulnerable to stochastic environmental threats (Traill et al., 2010; Wootton & Pfister, 2013), including severe weather events (Reading et al., 2010; Maxwell et al., 2019) and disease (Lorch et al., 2016; Franklinos et al., 2017). Inbreeding depression is another important potential mechanism for the collapse of isolated populations. This

is exemplified by a long-isolated adder population in Sweden, in which a fall in numbers was associated with a high rate of stillbirths and deformities (Madsen et al., 1996). Similar findings have been reported in fragmented populations of the congeneric Hungarian meadow viper *Vipera ursinii rakosiensis* (Ujvári et al., 2002). Breeding between closely related individuals increases the chance of homozygosity and thus the expression of rare recessive deleterious alleles, contributing to inbreeding depression (Morton et al., 1956; Lynch et al., 1995; Keller & Waller, 2002; Charlesworth & Willis., 2009). A loss of genetic diversity may additionally reduce the adaptive potential of a population, and thus the ability to respond to stress and environmental change (Willi et al., 2006; Kardos et al., 2021), underscoring the importance of active genetic management of small populations (Ralls et al., 2018).

The decline of the well-documented inbred adder population in Sweden was reversed by the introduction of twenty male adders, translocated from a large, outbred population (Madsen et al., 1999; 2004). In another

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example of translocations in adder conservation, the species has been successfully reintroduced onto a site in Greater London which had previously supported an adder population (Atkins, 2016; Worthington-Hill, 2016). However, an attempted reintroduction of captive-bred adders in Bedfordshire, eastern England, did not result in a sustained population, possibly because of predation by a high density of common pheasant *Phasianus colchicus*, a widely introduced gamebird (Worthington-Hill, 2016). These contrasting outcomes highlight important considerations in planning translocations, including the origin and demographic composition of the translocated individuals (Willoughby & Christie, 2019), and the identification of exogenous threats to the introduced population (Pérez et al., 2012; Converse et al., 2013; Berger-Tal et al., 2020).

In practice, translocations of adders are more often motivated by mitigation than conservation, with the immediate aim of removing wildlife from a development site, rather than ensuring the long-term survival and genetic health of the translocated individuals and their progeny (Nash et al., 2020; Hunter et al., 2021). In the UK, adders are legally protected under the Wildlife and Countryside Act (1981), but this protection does not encompass their habitat, and individuals may be moved off-site to an alternative area in case of development (Defra, 2021; Natural England, 2022). In addition, UK legislation does not require translocations of adders from development sites to be reported to national recording schemes (Defra, 2021), nor does it include specific provisions for post-translocation monitoring (Nash et al., 2020), thereby losing a potentially valuable source of information for improving the outcome of adder translocations whatever the motivation.

This study was instigated in response to the finding of five dead adder neonates on a nature reserve bordering a large urban site in eastern England in August 2015. The adder population on the reserve is known to have originated from the introduction of seven adders sixteen years previously, from a nearby location in response to planned development at the donor site. The population thus provided a valuable opportunity to study the genetic status of a translocated adder population after its known origin from a small number of founders. While the nature reserve supports an apparently healthy population of adders, it is isolated, being partly bounded by large roads and by progressive housing and commercial development, and with no known adder populations in the vicinity. Our initial aim was to investigate the demography and genetic health of the resident adder population using microsatellite markers, with particular focus on the possibility of inbreeding depression. The dead neonates were found as a group still coiled within the amniotic sac, allowing them to be identified as a stillborn clutch, born to the same female parent. This obligate maternal sibship was a useful tool to inform sibship reconstruction and parentage analysis. The clutch was potentially derived from different fathers, as polyandry is widespread in viviparous snake taxa (Wusterbath et al., 2010), including the adder (Stille et al., 1986; Höggren & Tegelström, 1995;

Ursenbacher et al., 2009a). Our ultimate objective was to inform the effective conservation of small, isolated adder populations, especially those originating from mitigation translocations or reintroductions.

MATERIALS & METHODS

Study site

The study site is a 146 hectare Special Site of Scientific Interest nature reserve in eastern England (exact details withheld as its adder population is already under pressure). An industrial site until the 1990s, it now consists of scrub, grassland, mature woodland, and multiple ponds, bounded by major roads and development. For nearly 20 years it has been actively managed by the wildlife charity Froglife (<https://www.froglife.org/>). Despite repeated standardised reptile surveys, adders were not detected on the site until after 1999, when there had been a documented introduction of seven adders, comprising three adults (1 male, 2 female), and four subadults (2 male, 2 female), removed as part of development mitigation from a location within 20 km (details withheld to protect residual adders on site). This was the only documented release of adders on to the reserve.

Samples

Tail tip samples were taken from five dead newborn adders comprising a stillborn clutch collected from the nature reserve in August 2015, as part of a national wildlife disease surveillance scheme (www.gardenwildlifehealth.org). There was no macroscopic evidence of deformities or trauma. Samples were obtained from 30 live adders on the nature reserve between March and May 2017, avoiding resampling the same spot between visits to reduce repeated captures. Two juvenile adders found at the original translocation donor site were sampled in 2017. Juveniles and subadults were distinguished from adults on the basis of colouring and size (Prestit, 1971; Arnold, 1995; Bauwens & Claus, 2018). Cloacal swabs were collected from adults and subadults, and buccal swabs from juveniles (Miller, 2006). Handling was kept to a minimum to reduce capture stress, with the use of transparent plastic tubes for partial immobilisation to safely access the cloaca. Capture of adult females was avoided from the start of May to reduce disturbance to potentially gravid snakes (Phelps, 2004). Adders were released where they had been found, usually within three minutes of capture. DNA was extracted using a QIAmp DNA Minikit (Qiagen), according to manufacturer’s protocols.

Mitochondrial DNA (mtDNA) sequencing

A 265-bp portion of mtDNA cytochrome B (Cytb) was amplified using primers L14724Vb and H15914Vb (Ursenbacher et al., 2006). PCR products were cleaned using QIAquick PCR purification kit (Qiagen). Sanger sequencing was undertaken commercially by GATC Biotech (Zurich), using the same primers as for amplification. Results were confirmed by bidirectional resequencing.

Genetic determination of sex

Sex was determined by PCR amplification of the female-specific W-homologue of the snake gametologous gene CTNNB1 (Matsubara et al., 2016; Laopichienpong et al., 2017), as detailed in the Supplementary Material.

Microsatellite genotyping

We used eight microsatellite primer sets, of which six had been developed for *V. berus* (Carlsson et al., 2003; Ursenbacher et al., 2009b), and two for the meadow viper *V. ursinii* (Metzger et al., 2011), previously evaluated for their cross-genus applicability to *V. berus* (Ball et al., 2020). Protocol details are given in the Supplementary Material. Amplified products were resolved by capillary electrophoresis on a 3130xl Genetic Analyser with a LIZ-500 size standard (Applied Biosystems). Replicates and template negative controls were included in each PCR plate to confirm reproducibility of results. Alleles were scored and binned manually, using PeakScanner 1.0 software (Applied Biosystems), without knowledge of the identity of individual samples to avoid subjective bias.

Microsatellite analysis

We used FSTAT v2.9.3.2 (Goudet, 2001) and pegas (Paradis, 2010), implemented in R v3.4.0 (R Core Team, 2017), to test for linkage and Hardy-Weinberg equilibrium (HWE), and to estimate allele richness and F statistics (Weir & Cockerham, 1984), using F_{IS} as an indicator of homozygosity (Wright, 1922; 1965), and expected heterozygosity (H_S) as a measure of gene diversity. Life history stage groups were compared in FSTAT with respect to allele richness and F-statistics, using 1000 permutations. Confidence intervals for F_{IS} were calculated using the boot.ppfis function of HIERFSTAT v0.04-22 in R (Goudet, 2005). Pairwise relatedness (R_{xy}) was estimated using a maximum likelihood (ML) method in ML-Relate (Kalinowski et al., 2006). A genetic estimate of the pedigree inbreeding coefficient F (Frankham et al., 2010) was derived using the inbreeding function of adegenet, version 2.0.1 (Jombart, 2008) implemented in R. A Wilcoxon rank sum test, implemented in R, was used to compare estimated values of F and R_{xy} between subgroups.

To estimate effective population size (N_e) we used two single sample methods. The linkage disequilibrium method (Hill, 1981) was implemented in NeEstimator ver 2.1 (Do et al., 2014), assuming random mating, deriving confidence intervals by jack-knifing (1000 iterations). In the sibship assignment method (Wang, 2009), the frequencies of full- and half-sib dyads were used to estimate the current effective breeding size of the population, implemented in COLONY 2.0.6.3 (Jones & Wang, 2010), using the same input parameters as for sibling and parentage analysis. Confidence intervals were obtained by bootstrapping.

For the detection of population bottlenecks we used the BOTTLENECK v 1.2.02, test for significant heterozygosity excess (Piry & Luikart, 1999), applying a one-tailed Wilcoxon test with 1000 iterations, using the two-phase model (90% stepwise mutations, variance=10) (Piry & Luikart, 1999). A mode-shift test for distortion of

the allele frequency distribution (Luikart et al., 1998) was also implemented in BOTTLENECK.

For parentage and sibship analysis we used a full-likelihood method, implemented in COLONY 2.0.6.3 (Jones & Wang, 2010). Both male and female polygamy were assumed, with error rate 0.0001, and three medium runs based on a medium sibship prior, not updating allele frequency. The outputs of three independent replicate runs were examined to confirm convergence to the same configuration and log likelihood. The best ML configuration was used to infer parentage and sibship dyads for each offspring.

We used STRUCTURE v2.3 (Pritchard et al., 2000; Falush et al., 2003) to infer genetic clustering, using correlated allele frequencies and admixture models, with or without the locprior option (Hubisz et al., 2009). Results were uploaded to Structure Harvester (Earl & von Holdt, 2012) to derive mean log likelihood and delta-K as a function of K, detecting hierarchical levels of structure (Evanno et al., 2005). Cluster membership coefficients from replicate runs were permuted in CLUMPP (Jakobsson & Rosenberg, 2007). Genetic clustering was further investigated using discriminant analysis of principal components (DAPC) (Jombart et al., 2010) in adegenet version 2.0.1 (Jombart, 2008). The find.clusters function was applied to determine the optimal number of clusters (k) in each population, according to curves of Bayesian Information Criterion (BIC) values as a function of k. The dapc function was applied to the same groupings of sites, using cross-validation and α -score functions to determine the optimum number of principal components to retain in each analysis. The probabilities of assignment of individuals to the different DAPC clusters were visualised using the compoplot function of adegenet.

Demographic modelling

We used Approximate Bayesian Computation (ABC) (Beaumont et al., 2002; Cornuet et al., 2008), implemented in DIY ABC ver 2.1.0 (Cornuet et al., 2014) to investigate the demographic history of the sampled population. ABC is a coalescence-based approach which compares datasets simulated under different competing scenarios, drawing parameters from prior distributions based on available ecological information, and results from genetic testing. The model scenario that best fits the data is identified as that with summary statistics closest to those of the observed dataset. A minimum of 100,000 simulations was performed per scenario. Scenarios were compared using linear discriminant analysis of summary statistics with logistic regression analysis (Beaumont, 2010; Fagundes et al., 2007) for estimation of posterior probability with 95% confidence intervals (Cornuet et al., 2008). Models were evaluated for goodness of fit and potential discrepancies (Gelman et al., 1995), with the inclusion of a range of summary statistics not used for the original simulation process. Parameters were estimated from the posterior parameter distributions of the 1% simulated datasets closest to the observed, using logit transformation. Details of model scenarios, prior settings, summary statistics and model checking are presented in Supplementary Material.

RESULTS

mtDNA haplotypes

All adders sampled on the nature reserve, including the dead neonates, were found to share an identical mtDNA cytb haplotype. The cytb sequence of the two individuals sampled at the donor site differed from this at a single nucleotide, corresponding to position 15828 of the *V. berus* mtDNA sequence accession MF945570 in Genbank (Gao et al., 2017).

Genetic determination of sex

Field-assigned sex of snakes was confirmed, and sex was determined for previously unsexed individuals, including the stillborn clutch (detailed in the Supplementary Material).

Microsatellite genotyping

Twenty eight of the samples collected from adders at the nature reserve site were genotyped at eight loci, and two at seven loci. Both donor site samples and all five samples from the stillborn clutch were genotyped at eight loci. There was consistency across PCR replicates. There was no evidence for null alleles, linkage disequilibrium, nor deviation from HWE. Three samples derived from adult males were shown to have an identical 8-locus microsatellite genotype. Another 8-locus genotype was seen in two samples from juvenile males. In both examples of repeated genotypes, samples had been collected on different site visits, which could reflect repeat sampling of the same individual. Records of individual head scale pattern (Bauwens et al., 2018) were not available to exclude replicate sampling. Only one example of each genotype (one adult male and one juvenile male) was therefore included for further analysis. Two other samples with an identical genotype were derived from one adult and one juvenile, evidently different individuals, and both were retained for analysis.

After censoring of possible replicates, the 2017 sample from the nature reserve comprised 27 individuals, including 19 adults (16 male, 3 female), and 8 young (juvenile or subadult snakes) (5 male, 3 female). The stillborn clutch comprised 1 male and 4 females (Table 1). Figure 1 compares the demographics of the study population, after censoring for possible replicate sampling, with published results of adder populations of an equivalent size in Great Britain (Ball et al., 2020).

Table 1. Sample details

	LHS	Number	Male	Female	Analysed
NRS 2017	Total	30	24	6	27
	Adult	21	18 (16*)	3	19
	Young	9	6 (5*)	3	8
NRS 2015	Stillborn	5	1	4	5
DS 2017	Young	2	2	0	2

NRS: nature reserve site; DS: donor site; LHS: life history stage
Young: juveniles and subadults
*: after censoring potential replicates

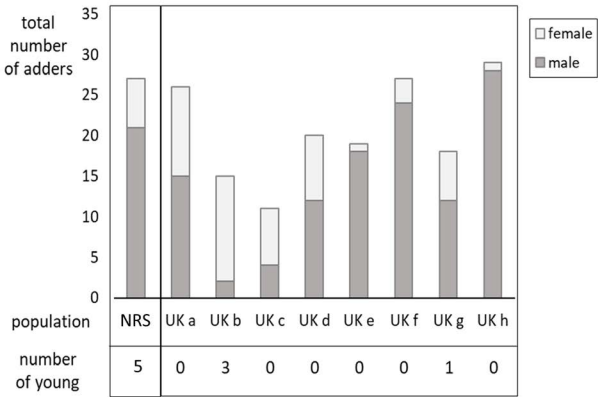


Figure 1. Age and sex composition of study population in comparison with other sites in Great Britain. Bar charts illustrating the relative proportions of male (dark fill) and female (light fill) adders at the Nature reserve site after censoring for possible replicate sampling, in comparison with published data from other sites in mainland Britain, and the number of young individuals included within the total number. The number of young adders does not include subadults, as this category was not specified in other sites. On the y-axis, total number of adders refers to the sample size analysed. NRS: Nature reserve site; UK a-h: sites with >10 samples from Ball et al. (2020).

Comparable population size was inferred from sample size, equivalent to peak adder counts in the Make the Adder Count (MTAC) study of Gardner et al. (2019). Subadults were excluded from the number of young in the nature reserve study site, as this category was not specified in the published study (Ball et al., 2020).

Microsatellite analysis

Genetic diversity, relatedness and inbreeding (summarised in Table 2). Estimates of allele richness and gene diversity were lower in the sample from the nature reserve than in previously studied sites (Ball et al., 2020). The significance of this could not be formally tested, as the panels of microsatellite loci were only partially overlapping between the two studies. There was no

Table 2. Genetic diversity and inbreeding: comparison between life history stages and other UK sites

Sample	No	Ar	H _o	H _s	F _{is}	F _{st}	R _{xy}	F _{DNA}
NRS 2017	27	2.14	0.73	0.56	0	0.005	0.2	0.17
Adult	19	2.1	0.73	0.56	0	-0.001	0.22	0.18
Young	8	2.17	0.68	0.5	0	-0.07	0.19	0.17
Stillborn 2015	5	2.17	0.72	0.57	0	0	0.3	0.17
UKAGP mean	15.89	2.66	0.7	0.71	0.02	0.01	0.19	0.23
SE	1.74	0.04	0.02	0.01	0.02	0.01	0.02	0.01

Measures of genetic diversity: Ar : allele richness; H_s: expected heterozygosity
Measures of relatedness and inbreeding: H_o: observed heterozygosity; F_{is} negative value shown as zero; R_{xy}: mean pairwise relatedness; F_{DNA} : mean estimate of pedigree inbreeding coefficient F
NRS: nature reserve site; young: juvenile and subadult
UKAGP: published UK data from Ball et al. (2020), mean and standard error (SE) derived from eight UK sites where >10 adders

Table 3. Estimates of effective population size (Ne) and testing for bottlenecks

		Bottleneck		Effective population size mean (95% CI)	
	n	TPM	AFM	COLONY	LDNe
NRS all	27	ns	normal	26 (15–30)	62.5 (8.9 – ∞)
NRS adult	19	ns	shift	15 (7–34)	59.4 (7.4 – ∞)

NRS: Nature reserve site; TPM: two-phase model; AFM: allele frequency mode-shift test; ns: not significant (p>0.05)
Shift: distortion of allele frequency distribution (positive result); LDNe: linkage disequilibrium estimate of Ne

significant difference in allele richness and gene diversity between adult (n=19) and young adders (here defined as juvenile and subadult; n=8) in the nature reserve sample.

F_{is} did not differ significantly from zero in the 2017 sample of adders from the nature reserve, nor in the stillborn clutch analysed separately. There was no significant difference in the mean values of the estimated inbreeding coefficient F between adults, young and stillborn. Mean pairwise relatedness (R_{xy}) was lower in young adders (0.197) than adults (0.219), but this was not statistically significant (p>0.05). R_{xy} was higher (0.301) in the stillborn clutch, consistent with a combination of half and full siblings (predicted R_{xy} 0.25 and 0.5 respectively) as expected in a polyandrous mating system (Stille et al., 1986; Höggren & Tegelstrom, 1995; Ursenbacher et al., 2009a).

Effective population size Ne and bottlenecks (summarised in Table 3). The single sample LDNe method (Waples & Do, 2008) failed to deliver finite confidence limits, probably the result of high levels of relatedness (Wang, 2018). COLONY results mirrored the number of inferred parents (see below). In bottleneck testing, the 2017 sample from the nature reserve, including both adults and young (n=27), was negative for both heterozygosity excess and for modal shift methods. The subgroup of adults (n=19) was positive for modal shift, but negative for heterozygosity excess. The sample size was too small to allow separate analysis of the young adder subgroup.

Family structure and parentage analysis in COLONY. We used the best ML configuration in COLONY to infer half- and full-sibship dyads. Replicate runs converged to equivalent log likelihoods, generating very similar patterns of inferred sibship dyads and assignment to hypothetical parents (not shown). In COLONY each individual is assigned to two inferred parents according to the best ML configuration. Figure 2a shows inferred parentage charts for the individuals sampled at the nature reserve. In the absence of pedigree data, it is not possible to distinguish between maternal and paternal genotypes, and inferred parents are given an arbitrary number on the x and y axes on parentage charts. Adults (n=19) analysed separately were inferred to all be related at a minimum half-sib level, with a total of five inferred parents of one sex (parent x) and seven inferred parents of the opposite sex (parent y), including nine individuals sharing a single inferred parent. When adults and young

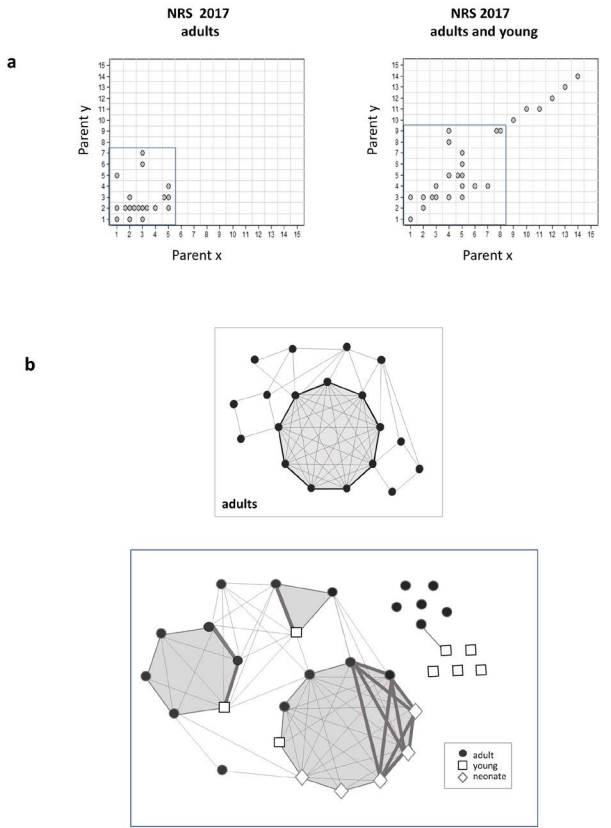
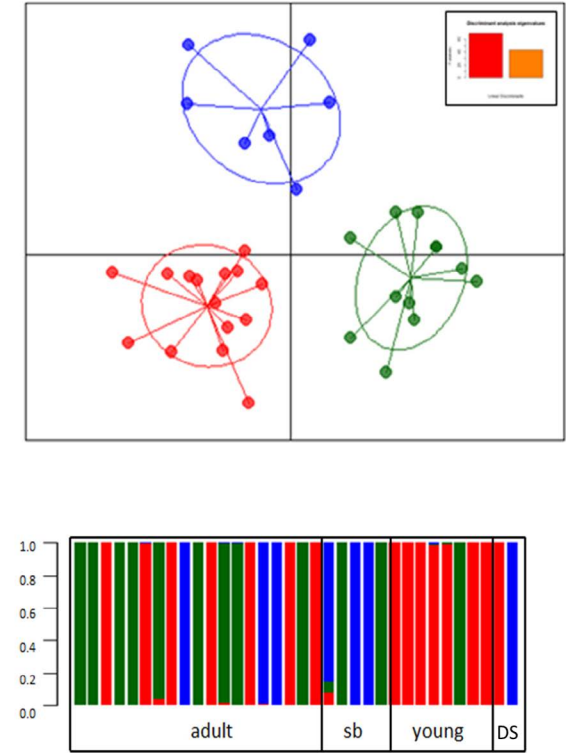


Figure 2. Sibships and parentage inferred in COLONY. **a.** Parentage plots: Each individual is represented as an open circle in a grid according to its two inferred parents. The inferred parents on each axis are given arbitrary numbers, as it is not possible to differentiate which is maternal or paternal in the absence of pedigree data. The adults when analysed separately (left) are inferred to be related at a minimum half-sib level, and half are inferred to share a single parent (y2). This contrasts with the tail of the parentage plot when juveniles and subadults are included (right). **b.** Networks of inferred sibships: Network of inferred sibships according to the best maximum likelihood configuration on COLONY. Inferred half sib dyads are shown as a single thin line, inferred full sib dyads by thick grey lines. Top: adults from 2017 sample analysed separately. The network is dominated by a single large inferred sibship. All adults within the sample are inferred to be connected at a minimum half-sib level. Bottom: adults (filled circles) and young (open squares) from 2017 sample, plus members of the stillborn clutch (open diamonds). The sibship of the clutch is within a dominant cluster with a shared parent, identified by the obligate maternal sibship as being female in origin. The young adders are divided between the main cluster and the group without inferred sibships.

(n=27) were analysed together, there was again a group of individuals (n=21) inferred to be related at the half or full sib level, but with a looser parentage plot, including four inferred singletons.

We reanalysed the nature reserve site samples to include the stillborn clutch (Fig. 2b). The network of the best ML sibship dyads again revealed a large cluster of individuals inferred to be related at a minimum of half-sib level, with three dominant sibships, one including the



in Figure 3 for all samples, including the stillborn clutch and the two individuals from the donor site. The scatterplot shows distinct inertia ellipses, with individual group membership on the barplots. The stillborn clutch is assigned across two clusters, one of which also contains one of the two individuals from the donor site, while the other donor site individual is assigned to a cluster composed predominantly of young adders. This apparent difference in group membership of young and adults was not statistically significant ($p>0.05$ on Fisher's exact test in R).

ABC modelling of demographic history

Rationale for model scenarios. We first tested the assumption that the documented translocation in 1999 had been the founding event for the population. We then queried whether the documented release of seven adders in 1999 would have been sufficient to account for the apparently thriving population present in 2017, including a high number of young adders relative to previously studied sites of similar abundance (Ball et al., 2020) (Fig. 1). We reasoned that the difference in COLONY results between adults and young was indicative of a temporal pattern (Balloux & Lugon-Moulin, 2002). These observations could be explained by a later additional influx of adders, likely to have been from the same donor site to account for the lack of admixture in STRUCTURE.

Comparison of possible scenarios of demographic history with ABC modelling. A scenario in which the nature reserve site 2017 population originated from the donor site population with a founder bottleneck was supported by genetic data (posterior probability 0.6338 [95% CI 0.5990,0.6686]), in comparison with competing models in which the nature reserve site and donor site populations derived independently from a shared ancestral population, with or without a foundation bottleneck (0.1504 [0.1293,0.1716]; 0.2158 [0.1872,0.2444] respectively) (detailed in the Supplementary Material). We then used the supported model as the baseline scenario against which to investigate the possibility of an additional later influx of adders from the donor site. A scenario in which the young on the nature reserve site derive from a later admixture between the nature reserve site and the donor site populations was compared with an otherwise identical model with a fixed prior of zero for further input from the donor site after the foundation event. The admixture model was supported (0.6682 [0.6314,0.7050]).

Estimates of founder size and timing of events. The supported model comprising the original foundation event and subsequent additional influx of adders is illustrated in Figure 4. Model testing of the supported scenarios confirmed goodness-of-fit of the summary statistics of the observed dataset relative to the corresponding posterior predictive distribution (Supplementary Material). In the foundation bottleneck scenario, the posterior distribution modal value for the founder bottleneck size was 7.82, and the modal value of the time of the founder event was 10.4 generations

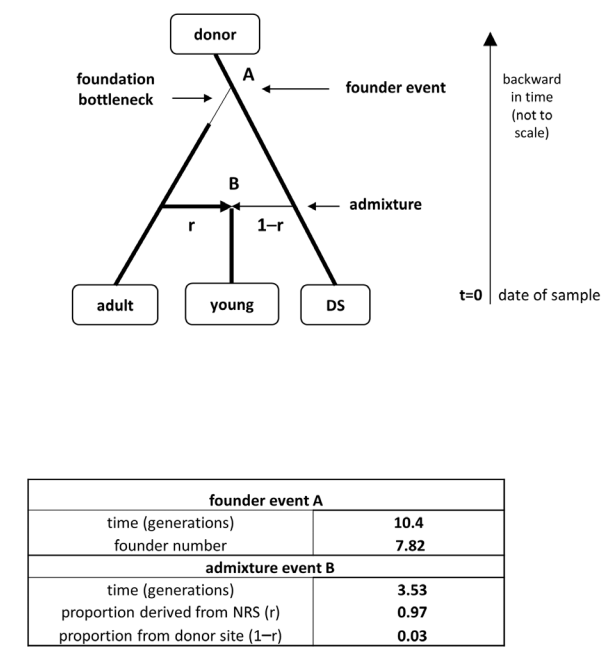


Figure 4. Supported model scenario in ABC. Schematic representation of the supported scenarios, showing the derivation of the nature reserve site (NRS) population from the donor site (DS) population with a foundation bottleneck lasting an arbitrary 5 generations (depicted as a thin line), and the subsequent admixture between adults from the NRS site and a further input from the donor site. The table shows posterior estimates in the supported models for the timing and founder population size at the foundation bottleneck (Nf), and for timing and relative proportions in the admixture event. Results are shown as modal point estimates. Full details of the derivation of the model parameters and testing for goodness of fit are provided in Supplementary Materials.

before the time of sampling. The posterior distribution modal value for the timing of the admixture was 3.53 generations before 2017. In the admixture model, the posterior distribution modal value for the proportional contribution of the original nature reserve site population to the admixture was 0.97, with 0.03 from the donor site. Using the recorded release date of 1999 to calibrate the posterior distribution modal value of 10.4 generations pre-2017 for the 1999 foundation event, the subsequent release in the admixture model (posterior modal value 3.53 generations pre-2017) can be estimated to have occurred around 2011.

DISCUSSION

Levels of homozygosity have long been considered the “most natural coefficient of inbreeding” (Wright, 1922). However, identity by descent may not be associated with an increase in homozygosity for every breeding system (Wright, 1922). The observed pattern of retained heterozygosity in the presence of high levels of relatedness in our study population resembles that described in wild adder populations in Great Britain (Ball et al., 2020), and is likely to reflect the polyandrous mating system in the

adder (Stille et al., 1986; Höggren & Tegelstrom, 1995; Ursenbacher et al., 2009a).

In addition, mean heterozygosity, whether based on a small panel of neutral genetic markers as in our study, or genome-wide (Schmidt et al., 2021), may not provide a true representation of the extent and patterns of identity by descent. In a recent study, Pozzi et al. demonstrated that the genome-wide pattern of heterozygosity in adders is skewed, with most regions showing low heterozygosity, despite high mean genome-wide heterozygosity. Modelling showed the pattern to be consistent with recent severe bottlenecks (Pozzi et al., 2023), of particular relevance to our study population.

High resolution scans of the distribution of homozygosity within the genome may provide further information on the demographic history of adders. On a finer scale, genomic stretches of consecutive homozygous markers (runs of homozygosity, ROH) may be identified. These represent identical chromosomal segments inherited from a common ancestor, their lengths being determined by the occurrence of recombination events. Longer ROHs are expected where breeding between closely related individuals has occurred within past ten generations, including recently bottlenecked populations, while shorter ROHs reflect historical inbreeding (McQuillan et al., 2008; Kirin et al., 2010; Keller et al., 2011; Palamara et al., 2012; Ceballos et al., 2018).

Significance of the stillborn clutch

The above discussion indicates that retained heterozygosity does not preclude inbreeding depression as the cause of the stillborn clutch. However, it is unlikely that the loss of the entire clutch can be attributed to the homozygous expression of a recessive deleterious allele, as the clutch was inferred to have multiple paternity. This contrasts with the occurrence of deformities affecting four out of seven offspring from a single brood in an inbred population of *V. ursinii rakosiensis* (Ujvári et al., 2002). It is more likely that the clutch on the nature reserve site was stillborn as a result of maternal fitness, which could be related to inbreeding, or an extrinsic factor. No information was available on the condition of the mother of the clutch.

The background level of stillbirths in healthy adder populations is currently unknown. In a study of 15 adder clutches over three years, Ursenbacher et al. (2009a) recorded complete loss of one clutch, and partial mortality in a further eight clutches, although this may have been influenced by the gravid females having been handled (Ursenbacher et al., 2009a). Disturbance of gravid females may contribute to the reported negative effect on adder populations of public disturbance, including dog walking, mountain biking and trampling of vegetation (Gardner et al., 2019). While being generally protected from public access, our study population is likely to be facing pressure from disturbance and human-linked mortality, due to its proximity to housing developments. Three adult adders were recorded dead on the site in 2014–2017, of which two were likely due to predator attack, based on the type of traumatic injuries observed on post-mortem examination (www.gardenwildlifehealth.org).

Weather conditions may also be important; for example, the combination of excessive heat and prolonged drought results in both reduced maternal fitness and increased embryonic mortality in the adder (Dezetter et al., 2021), although these factors are unlikely to apply to the stillborn clutch in our study. The inclusion of adults and juveniles in the same obligate maternal sibship as the stillborn clutch in COLONY does not indicate *de facto* that they represent a minimum of three clutches from the same mother, as inferred sibships are likelihood-based rather than being derived from a true pedigree. Indeed, none of the adult females sampled in 2017 could have been the mother of the 2015 stillborn clutch, on the basis of microsatellite haplotypes. The probability of an inferred parent-offspring dyad will be influenced by the presence of other potentially compatible genotypes, which is especially likely where there is limited diversity and high relatedness.

Irrespective of the cause, the loss of a complete clutch has potentially important genetic and demographic consequences (Stojanovic et al., 2022), especially in a species with low female fecundity. Half the female adders in a Swedish study had only one litter per lifetime (Madsen et al., 1992). Similarly, in a longitudinal study of a large population of adders in Belgium, there was an average of only 1.3 litters per female reproductive lifetime, with 70% of females breeding only once (Bauwens & Claus, 2019). The loss of a single clutch may therefore represent the loss of the entire reproductive output of a female adder, and with it a significant genetic component of the population, especially important in an isolated population with already low genetic diversity. In addition, the reproductive ecology of adders predicts vulnerability to a reduction in breeding females (Madsen & Shine, 1992). The loss of four females, as in the dead clutch in our study, may therefore have demographic implications for a population already showing a relatively low proportion of females.

Inferred demography

We found the study population to have low genetic diversity in comparison with published data from adder populations of equivalent size (Ball et al., 2020), with the caveat of only partially overlapping microsatellite panels. The observed pattern of low genetic diversity and high relatedness in the nature reserve population is typical for a bottleneck in the demographic history of a population (Nei et al., 1975; Greenbaum et al., 2014; Grossen et al., 2018; Fernández-López et al., 2021). A foundation bottleneck is in keeping with the population on the nature reserve having been founded *de novo* from the documented release of adders 18 years previously. The low genetic diversity is likely to be the result of the limited number of founders (Stewart et al., 2017), which also provides an explanation for the high level of relatedness and the low number of inferred parents for the adult adders sampled 18 years after the documented translocation. A founding propagule of seven adders is below the predicted minimum size to ensure the persistence of a genetically healthy population (Shaffer, 1981; Lacy, 1989).

We used COLONY in our study to generate a probability-based best ML of parentage and sibships in the sample, an approach which can also provide an estimate of the effective number of breeders in a population (Ackerman et al., 2017; Bacles et al., 2018), especially when relatedness limits the application of a linkage disequilibrium method (Hill, 1981; Wang, 2018), as in our study. Eight microsatellite loci, the number in our study, are sufficient to identify sibship dyads at a 95% probability in COLONY (Jones & Wang, 2010). This approach does not identify actual parentage and sibships but provides an illustration of the extent of relatedness. High relatedness and sibship groupings are also the likely explanation for clustering evident with DAPC, despite the lack of genetic differentiation in STRUCTURE (Ball et al., 2020). The DAPC clusters are thus more likely to reflect allele frequency patterns driven by a polygynandrous mating system in a consanguineous population, rather than discrete panmictic subpopulations. An equivalent phenomenon of clustering in DAPC, but not STRUCTURE, has also been observed in wild adder populations in the UK (Ball et al., 2020), and in the Prairie rattlesnake *Crotalus viridis* (Weyer et al., 2014).

The pattern of large clusters in the adult subgroup contrasted with the looser network of inferred sibships in the juveniles and subadults, indicative of a temporal genetic structure (Balloux & Lugon-Moulin, 2002). This raised the possibility that there had been an additional undocumented influx of adders, likely by deliberate introduction, as the isolation and lack of connectivity would have precluded natural migration into the site from wild populations. This would provide an explanation for the apparently high proportion of juvenile adders on the nature reserve in comparison with previously reported wild populations in Great Britain (Fig. 1)(Ball et al., 2020). We investigated this further using coalescent modelling in ABC, comparing simple competing models of equivalent complexity, an approach which in simulation studies was reported to be the most likely to identify the correct scenario (Cabrera & Palsbøll, 2017). While the very small sample size from the donor site in our study clearly necessitates caution, models of the origin of the study population and an additional later influx from the donor site were strongly supported. The accuracy of parameter estimates using approximate computation is lower than for full-likelihood methods (Robert et al., 2011). However, the modal point estimate for the size of the founder bottleneck in our study population was consistent with the documented number of released adders, despite a relatively broad prior range. With respect to estimates of timing, the relation between the inferred number of generations and chronological time is a function of the mutation rate of the genetic markers used (Kimura, 1968). While there are no data on the microsatellite mutation rate in adders, this variable was constant between the different model scenarios. We therefore used the recorded release date of 1999 to calibrate the posterior distribution modal value of the foundation event. By extrapolation, the presumed subsequent release can be calculated to have occurred

around 2011, although this may have been influenced by the age structure of the population, the survival of females up to the age of each breeding event, and the number of offspring produced at each age class over the entire female life span (Jonasson et al., 2022), as well as by the possibility that undocumented releases of adders may have occurred on more than one occasion.

Lessons for translocations of adders

Our study highlights important issues with respect to translocations, including the selection of the recipient site, the size and composition of the release group, and the importance of pre- and post-release monitoring (Armstrong & Seddon, 2008; Perez et al., 2012; IUCN/SSC, 2013; Worthington-Hill, 2016; ARG UK, 2020). While an undocumented translocation may have benefited the founder population in our study site, it is important that all translocations are judiciously planned, and are fully documented at all stages. Genetic monitoring is essential following reintroductions (Marshall et al., 2022), not only for genetic diversity and inbreeding, but also to confirm genetic integration following introductions into sites with a pre-existing population. Our results are consistent with genetic integration between the founding population and adders from a later undocumented introduction, although the extent of this cannot be determined without knowledge of the size and composition of the presumed second release. In addition, in our study mtDNA markers were non-informative, precluding information on sex-specific integration. Genotyping resident and translocated individuals prior to release can provide useful information in this respect. In a study of the parentage of desert tortoise hatchlings *Gopherus agassizii* four years post-translocation, hatchlings from both resident and translocated females were all sired by residents (Mulder et al., 2017). Tagging of male adders provides important information on movement patterns of resident and translocated snakes (Reinert & Rupert, 1999; Nash & Griffiths, 2018; Hand, 2018), but is insufficient to quantify paternity in a polyandrous mating system, especially where there may be high variance in individual reproductive success.

Germano et al. (2015), noting that economically motivated mitigation translocations have been less successful than conservation-driven, concluded that “the application of scientific principles and best practices would probably improve the success rate”. Undocumented or poorly monitored translocations represent a wasted opportunity to study the process in detail, and thus to inform a systematic, evidence-driven approach to translocations, irrespective of the motivation. Data deficiency in this respect is exemplified by a large-scale mitigation translocation of herpetofauna from a port development site in eastern England in 2011. While legally protected great crested newts had to be relocated locally, the same constraint did not apply to the adders on the site. As a result, a very substantial population of nearly 300 adders were translocated 140 miles away to a nature reserve with a resident adder population (Williams, 2011; BBC, 2011).

Despite the stated intent to monitor the translocated population for 5 years (Williams, 2011), no outcome information is available, whether undocumented, or impenetrably buried within “grey” literature (Bradley et al., 2022). A valuable opportunity to systematically address an evidence shortfall in ecological mitigation practice and guidance (Hunter et al., 2021) was thus lost, especially important given the scale of the translocation. Some potentially useful information may still be available; a genetic survey of adders currently inhabiting the recipient sites should show the extent of integration of the translocated adders into the original resident population (Stewart et al., 2017), which would be predicted to have divergent mtDNA haplotypes given their original geographic separation (Ball et al., 2020). If there is no residual genetic trace of the translocated animals, it would add to the concern that too many mitigation translocations of snakes simply result in their death (Cornelis et al., 2021).

Future management of study population

Although apparently flourishing, and expanding across the site, with a limited number of founders and no prospect of recruitment from the wild, the adder population on our study site remains demographically and genetically vulnerable. As well as inbreeding, there is the risk of continuing genetic erosion on the background of already limited genetic diversity, such as that described in the pink pigeon *Nesoenas mayeri* in Mauritius, where declining populations supplemented by release of captive-bred individuals after a severe population decline showed loss of genetic variation despite a population rebound (Jackson et al., 2022). Lack of interpopulation movement also results in loss of protection against stochastic events, such as skewed mortality affecting individuals of breeding potential, especially where the number of breeding females is limited. The nature reserve population in our study is thus likely to be dependent on active conservation management, including genetic monitoring and consideration of supplementary introductions, either at the same site or a nearby site with opportunities to generate habitat corridors. Information gained on the trajectory of this population will be relevant not only to the long term demographic and genetic health of reintroductions (Marshall et al., 2022), but also to captive populations (Witzenberger & Hochkirch, 2011), and small populations of adders in the wild, isolated as the result of habitat fragmentation and disturbance, and thus at high risk of extirpation (Gardner et al., 2019).

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Author Contributions

The study was conceived by SP and TG. All authors contributed to the study design. Reptile surveys were performed by SP and LM. Post-mortem analysis was performed by BL. Samples were collected by SP, LM, CD and EA-J. Material preparation, genetic laboratory tests and analysis were performed by CD, EA-J and SB. The first draft of the manuscript was written by SB. All authors commented on previous versions of the manuscript, and read and approved the final manuscript.

Ethical Statement

The study was reviewed and approved by ZSL Ethics Committee for Animal Research. Details of measures taken to minimise stress and injury of captured snakes are provided in Materials & Methods.

DATA ACCESSIBILITY

The datasheet of microsatellite genotypes is included in the Supplementary Material.

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Diversity and distribution of reed frogs (*Hyperolius* spp.) on Bioko Island, Equatorial Guinea

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Reed frogs (*Hyperolius* spp.) are the most species-rich genus in the family Hyperoliidae, a group of frogs endemic to sub-Saharan Africa and the Gulf of Guinea islands. Three species are endemic to oceanic islands in the archipelago, but the diversity and distribution of reed frogs on Bioko – a land-bridge island in the archipelago – remains unclear. Two species, *H. tuberculatus* and *H. ocellatus ocellatus*, were formally documented on Bioko in the colonial era, however, two additional reed frog species have been detected in recent surveys. In addition, observations of heterospecific amplexus suggest one of the unidentified species and *H. o. ocellatus* may be hybridising on the island. In this study, we examined mitochondrial DNA (mtDNA), nuclear DNA (nuDNA), morphological and acoustic data to confirm the tentative identifications of the reed frogs and determine if there is any evidence of hybridisation. Our data confirm that the two unidentified species are *H. endjami* and *H. fusciventris*, and that Bioko *H. endjami* have unique genetic diversity not shared with the mainland population. Additionally, our mitochondrial (16S) and nuclear DNA (CMYC) haplotype networks suggest that *H. o. ocellatus* and *H. endjami* are not hybridising on Bioko. Finally, we report colour pattern variation in *H. endjami* and *H. o. ocellatus* that is unique from populations on the continent, and describe male advertisement calls of Bioko *H. endjami* and *H. o. ocellatus*. Our study provides a first look at the diversity and distribution of reed frogs on Bioko Island and sets the stage for future studies of the ecology, evolution, and conservation of this island community.

Keywords: hybridisation, polymorphism, mitochondrial and nuclear DNA, morphology, land-bridge island

Ranas de caña (*Hyperolius* spp.) son el género más rico en especies de la familia Hyperoliidae, un grupo de ranas endémicas del África subsahariana y las islas del Golfo de Guinea. Tres especies son endémicas de las islas oceánicas del archipiélago, pero la diversidad y distribución de las ranas de caña en Bioko, una isla puente terrestre en el archipiélago, sigue sin estar clara. Dos especies, *H. tuberculatus* y *H. ocellatus ocellatus*, fueron documentadas formalmente en Bioko en la época colonial; sin embargo, se han detectado dos especies adicionales de ranas de caña en estudios recientes. Además, las observaciones de amplexo heteroespecífico sugieren que una de las especies no identificadas y *H. o. ocellatus* puede estar hibridando en la isla. En este estudio, examinamos el ADN mitocondrial (mtDNA), el ADN nuclear (nuDNA), los datos morfológicos y acústicos para confirmar las identificaciones tentativas de las ranas de caña y determinar si hay alguna evidencia de hibridación. Nuestros datos confirman que las dos especies no identificadas son *H. endjami* y *H. fusciventris*, y que Bioko *H. endjami* tiene una diversidad genética única que no comparte con la población continental. Además, nuestras redes de haplotipos de ADN mitocondrial (16S) y nuclear (CMYC) sugieren que *H. o. ocellatus* y *H. endjami* no hibridan en Bioko. Finalmente, reportamos la variación del patrón de color en *H. endjami* y *H. o. ocellatus* que es único de las poblaciones del continente, y describe los cantos masculinos de Bioko *H. endjami* y *H. o. ocellatus*. Nuestro estudio proporciona un primer vistazo a la diversidad y distribución de las ranas de caña en la isla de Bioko y sienta las bases para futuros estudios sobre la ecología, la evolución y la conservación de esta comunidad isleña.

INTRODUCTION

Biodiversity in the Gulf of Guinea archipelago reflects the region’s geologic and geographic history (Jones, 1994; Melo et al., 2022a). The archipelago sits along the western terminus of the Cameroon volcanic line, a 1,600 km chain of Cenozoic volcanoes that stretches from the Gulf of Guinea to the interior of Central Africa (Marzoli et al., 2000). During the Miocene, an event of crustal uplift formed four volcanic islands (Bioko, São Tomé, Príncipe

and Annobón) that comprise the archipelago today (Meyers & Rosendahl, 1991). Bioko, a land-bridge island, is separated from continental Africa by <35 km of shallow sea (Deruelle et al., 1991; Bell et al., 2017). Cycles of rising and retreating sea resulted in periods of isolation and connectivity between Bioko and continental Africa with the last period of connectivity taking place during the Last Glacial Maximum (~21 kya) when sea levels lowered enough to connect Bioko to continental Africa via a broad land bridge (Jones, 1994; Bell et al., 2017). By contrast, São

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Tomé, Príncipe and Annobón are oceanic islands and have never been connected to continental Africa (Ceríaco et al., 2020). Bioko comprises three stratovolcanoes that formed 1–3 mya and, relative to the oceanic islands which formed 5–30 mya, is young in age (Jones, 1994). The combination of oceanic and land-bridge islands in the Gulf of Guinea archipelago provides an excellent framework to compare patterns of species diversity and endemism between islands that differ in geological history.

Biological patterns of species diversity and endemism on the islands indicate that periods of connectivity likely facilitated the dispersal of biodiversity from continental Africa to Bioko (Estrela, 1994; Juste & Ibañez, 1994; Melo et al., 2011; Ceríaco et al., 2016; Bell et al., 2017; Loiseau et al., 2019). Consequently, Bioko exhibits high species richness, but low endemism, reflecting its proximity and short periods of isolation from the continent (Jones, 1994). Conversely, the oceanic islands are less species-rich but have high endemism as a result of millions of years of geographic isolation from the mainland. For example, only two bird species are endemic to Bioko while São Tomé and Príncipe host seventeen and eight single-island endemic birds, respectively (Jones, 1994; Melo et al., 2022b). Intermittent periods of isolation and connectivity may contribute to population-level diversification on Bioko, particularly for species that are poor dispersers across saltwater barriers, such as amphibians. For instance, there is evidence of moderate genetic divergence between mainland and Bioko populations of some reed frogs (*Hyperolius o. ocellatus*, *H. tuberculatus*; Bell et al., 2017), African tree toads (genus *Nectophryne*; Liedtke et al., 2021), and leaf-folding frogs (*Afraxalus paradorsalis*; Charles et al., 2018). However, for larger-bodied foam nesting frogs (*Chiromantis rufescens*; Leaché et al., 2019), torrent frogs (genus *Petropedetes*; Barej et al., 2014), and giant tree frogs (*Leptopelis rufus*; Jaynes et al., 2022), genetic divergence between mainland and island populations is seemingly less pronounced. Investigating the genetic history of island populations relative to their mainland counterparts can reveal how amphibians are responding to periods of geographic separation during glacial cycles and whether these cycles are generating unique diversity on Bioko.

Reed frogs (*Hyperolius* spp.) are a diverse group of frogs endemic to Africa and widely distributed across the sub-Saharan region (Channing & Rödel, 2019). They are the most species-rich genus of frogs in the family Hyperoliidae, totaling 144 species (Frost, 2023). A number of *Hyperolius* species have been the subject of taxonomic discussion and reclassification, in part due to the reliance of colour patterns as taxonomic characters, which can be highly variable (i.e. polymorphism) within species (Channing, 2022). Sexual dichromatism (differences in colouration between the sexes) further contributes to the challenge of species identification in *Hyperolius* frogs. In some groups of closely related species, differences in female colouration are more pronounced than those in males (Portik et al., 2019), while the males of different species are often similar looking; this can make it difficult to identify species in the field without finding both sexes or hearing

the male advertisement calls. Identifying *Hyperolius* frogs is particularly challenging on the African continent where there can be five or more species at a single breeding site (Drewes & Vindum, 1994). Species identification can still be challenging in less diverse communities, however, due to high regional variation in colour pattern (Channing, 2022). Consequently, an integrative approach that accounts for morphological, acoustic and genetic variation is recommended for making reliable species identifications in this genus.

Two reed frog species, *H. tuberculatus* (Mertens, 1965; Sánchez Vialas et al., 2020) and *H. ocellatus* (Günther, 1858), were formally documented on Bioko in the colonial era. On the mainland, these two species have a distribution stretching from Nigeria and Cameroon (*H. o. ocellatus* and *H. tuberculatus*) to the interior of Central Africa (*H. tuberculatus* and *H. o. purpurescens*). Morphological and genetic studies of *H. ocellatus* indicate that populations on Bioko Island correspond to the subspecies *H. o. ocellatus* (Laurent, 1943; Perret, 1975; Bell et al., 2017). Two additional reed frog species were detected on Bioko in surveys led by the California Academy of Sciences in 1998, but the identification of these species remains tentative (both were identified in the field as *H. cf. ocellatus*). Moreover, we observed one of the unidentified species and *H. o. ocellatus* in heterospecific amplexus at a breeding site where they co-occur. There is evidence of hybridisation in reed frogs on the nearby island of São Tomé (Bell et al., 2015; Gilbert & Bell, 2018; Bell & Irian, 2019) which raises the question of whether reed frogs may also be hybridising on Bioko. In this study, we use multiple lines of evidence to clarify the identification of the two unidentified reed frogs on Bioko and assess whether one of the unidentified species and *H. o. ocellatus* may be hybridising. We describe the species' distributions on the island, estimate genetic diversity, characterise male advertisement calls and report colour pattern variations unique from populations on the mainland.

MATERIALS & METHODS

Field sampling

We conducted amphibian surveys on Bioko Island over five field seasons: 27 September–18 October 1998, 19 August–12 September 2011, 1 January–22 January 2013, 29 August–17 September 2013, 11 April–30 April 2019. Our sampling spanned a range of habitats including lowland forest, agricultural fields, montane forest and Schefflera forest/Mossy forest, spanning elevations from sea level to >1900 m. Access to much of the northernmost volcano (Pico Basile) is restricted, thus the majority of our sampling effort was based in the southern half of the island (Bioko Sur Province; Fig. 1). Animals were euthanised via immersion in MS-222, tissue samples (liver) were preserved in 95% ethanol or RNAlater, and voucher specimens were deposited at the California Academy of Sciences (CAS), Cornell University Museum of Vertebrates (CUMV), and U.S. National Museum of Natural History (USNM). Voucher specimen information is included in Table S1, see Supplementary Material.

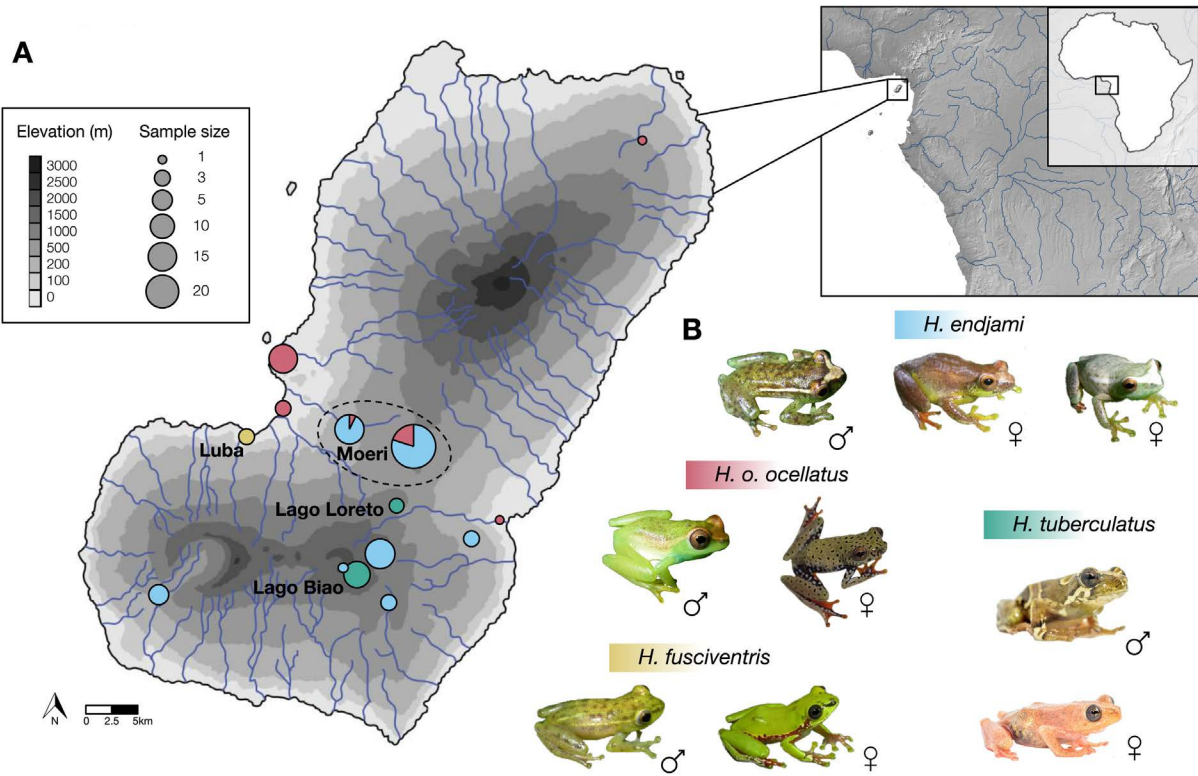


Figure 1. A. Sampling distribution of *Hyperolius endjami*, *Hyperolius ocellatus ocellatus*, *Hyperolius fusciventris* and *Hyperolius tuberculatus* on Bioko Island. Sampling localities are scaled according to sample size and coloured according to the proportion of each species present at the locality. The two crater lakes (Lago Biao and Lago Loreto), port town of Luba, and *H. endjami* and *H. o. ocellatus* zone of sympatry (Moeri) are indicated. **B.** Representative photographs of males and females of each species, all of which exhibit sexual dichromatism. Two colour morphs of female *H. endjami* are shown. Photos of *Hyperolius fusciventris* are of individuals in Cameroon because those from Bioko were not photographed prior to preservation.

Genetic data collection

We extracted genomic DNA from 102 tissue samples using the Qiagen DNEasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). We amplified the 16S mitochondrial and CMYC nuclear genes using polymerase chain reaction (PCR) and the respective primers (16S: 16SA and 16SB (Palumbi et al., 1991), CMYC: CMYC-1U and CMYC-ex2dR (Crawford, 2003; Wiens et al., 2005)). Each PCR reaction was carried out in a volume of 18 µl containing: 1 µl template DNA, 0.18 µl DreamTaq DNA polymerase, 2.4 µl dNTPs, 0.75 µl of each primer, 2 µl 10X DreamTaq Buffer, and 10.92 µl molecular grade water. Amplifications were carried out with initial denaturation for 5 min at 94 °C, followed by 35 cycles consisting of 60s denaturation at 94 °C, 60s annealing at 48 °C, 60s extension at 72 °C and a final extension at 72 °C for 5 min. PCR products were visualised using gel electrophoresis, purified using ExoSAP-IT (USB Corp., Cleveland, OH) and sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI Automated 3730x1 Genetic Analyser (Applied Biosystems). All sequences were aligned and edited using Geneious Prime v2021.1 (<https://www.geneious.com>). Several of the Bioko *H. tuberculatus* and *H. o. ocellatus* specimens had previously been sequenced for a Central African phylogeography study by the authors (Bell et al., 2017) and these sequences were included in our Bioko Island datasets (Table S1).

Species identification and population genetic structure

To assess the identifications of *Hyperolius* sp. 1 and *Hyperolius* sp. 2 from Bioko, we used a recent phylogeny of Hyperoliidae as a reference. Portik et al. (2019) estimated species relationships with >1000 sequence capture nuDNA loci and included 16S because it is a commonly used barcoding gene for African amphibians. We generated a phylogeny based on this 16S dataset with all *Hyperolius* taxa included in the Portik et al. (2019) study (n = 90), one representative sequence of each haplotype for each of the unidentified Bioko reed frog taxa, and *Afraxalus brachycnemis* (also from Portik et al., 2019) as an outgroup. We aligned the sequences with MAFFT using the automatic algorithm selection option (--auto) (Katoh et al., 2002; Katoh & Standley, 2013). To perform maximum likelihood phylogenetic analyses, we used IQ-TREE v1.6.12 on the IQ-TREE server with the built-in model selection tool ModelFinderPlus and 1000 ultrafast bootstraps (Nguyen et al., 2015; Trifinopoulos et al., 2016; Kalyaanamoorthy et al., 2017; Hoang et al., 2018). We calculated the percent sequence divergence between the closest reference samples to the Bioko reed frogs in Geneious. Finally, we examined the physical specimens to confirm the presence of diagnostic characters in species descriptions and field guides.

To estimate genetic diversity and divergence of Bioko reed frogs relative to their continental counterparts, we downloaded GenBank sequences of 16S for the species,

which included samples from Cameroon, Gabon, Ghana and Nigeria (Table S1). For each species, we generated alignments in Geneious and constructed haplotype networks using TCS v1.21 (Clement et al., 2000) and PopART v1.7 (Leigh & Bryant, 2015).

Preliminary investigations of potential hybridisation

We sequenced one mtDNA (16S) and one nuDNA gene (CMYC) for *H. o. ocellatus* and the sympatric *Hyperolius* sp. 1 as a preliminary investigation of hybridisation. In particular, we wanted to assess whether any individual frogs exhibited mismatched mtDNA-nuDNA haplotypes (e.g. an individual exhibiting the mtDNA haplotype of species 1 and the nuDNA haplotype of species 2), or heterozygosity in the nuDNA gene indicative of mixed ancestry (e.g. one phased haplotype attributable to each of the parental species). Our sampling included 113 individuals from Bioko Island with 51 collected from Moeri (3.467317 N, 8.6411 E), the site at which heterospecific amplexus was observed.

Advertisement call analysis

We collected acoustic recordings for *Hyperolius* sp. 1 and *H. o. ocellatus* during the 2011 field season. *Hyperolius* sp. 2 was detected in 1998 and despite searching near the 1998 locality in subsequent field seasons, no additional individuals have been seen or recorded. Calls were recorded in situ at breeding sites using a Marantz PMD-661 recorder paired with a Sennheiser ME-67 microphone (2011) at an approximate distance of 0.5–2.0 m from the males. For each recording, we measured the ambient air temperature, described the male calling site and behavioral context, and prepared the males as voucher

specimens (Table S1). The recordings were archived in the Macaulay Library at the Cornell Lab of Ornithology (Table S1).

We digitised recordings at a resolution of 24-bit 96 000 Hz sampling rate and analysed them in Raven Pro v1.4 (Cornell Lab of Ornithology) with a Fast Fourier transformation window of 512 points, a brightness of 70 points and a contrast of 70 points. The total pulse groups, average dominant frequency (Hz), and average pulses were recorded for each call as these metrics capture much of the variation in *Hyperolius* calls (Amiet & Goutte, 2017). We compared our Bioko recordings to those obtained by Amiet & Goutte (2017) in Cameroon. Oscillograms and spectrograms were made in R using the Seewave package v2.1.8 (Sueur et al., 2008).

Comparisons of colour pattern variation

Most specimens collected in 2011, 2013 and 2019 were photographed immediately following euthanasia to provide a record of colour and pattern, as colouration typically fades in museum specimens over time. We compared colour pattern variation in male *Hyperolius* sp. 1 (n = 38), *Hyperolius* sp. 2 (n = 3) and *H. o. ocellatus* (n = 32) to comprehensive descriptions and photos of each species (Amiet, 2012).

RESULTS

***Hyperolius* diversity and distribution on Bioko Island**

We found that Bioko *Hyperolius* sp. 1 and *Hyperolius* sp. 2 formed strongly supported clades with *H. endjami* and *H. fusciventris* respectively in the Hyperoliidae phylogeny (Fig. S1, see Supplementary Material). Percent sequence

divergence was low (0.0–0.4% and 0.7% for *H. endjami* and *H. fusciventris*, respectively) and the morphological features of the specimens fit these species descriptions; thus, we are confident in these species identifications. Consequently, the island *Hyperolius* community includes *H. endjami*, *H. o. ocellatus*, *H. fusciventris* and *H. tuberculatus* (Fig. 1). We found *H. endjami* at sites ranging from approximately 300–1950 m elevation, *H. o. ocellatus* from sea level to ~550 m, and a narrow zone of sympatry between the species around 450 m. This area of sympatry is where individuals in heterospecific amplexus were observed by the authors. By contrast, *H. tuberculatus* were only found in habitats surrounding Lago Biao and Lago Loretto, two crater lakes on the southern half of the island. Finally, only three *H. fusciventris* were found, and all were collected in 1998 from a single locality in the port town of Luba (3.4613 N, 8.5524 E). Several subspecies of *H. fusciventris* are recognised on the basis of differences in colouration (*H. f. burtoni*, *H. f. fusciventris*, *H. f. lamtoensis*),

however, with only preserved male specimens we were not able to attribute the Bioko samples to a particular subspecies.

Genetic diversity in Bioko reed frogs and divergence from continental populations

We found moderate genetic diversity at 16S for Bioko *H. endjami* with five haplotypes, none of which are shared with individuals from Cameroon (Fig. 2). By contrast, the three individuals of *H. fusciventris* shared one 16S haplotype (Fig. 2). Of the available reference sequences for *H. fusciventris* from West and Central Africa, the Bioko individuals appear most similar to populations in Nigeria. We recovered four 16S haplotypes for Bioko *H. o. ocellatus* that clustered in two groups, both with variation distinct from that in Cameroon. We found three 16S haplotypes for Bioko *H. tuberculatus*, all three of which clustered with samples collected in Gabon but with unique diversity. There was little to no genetic diversity at CMYC for Bioko

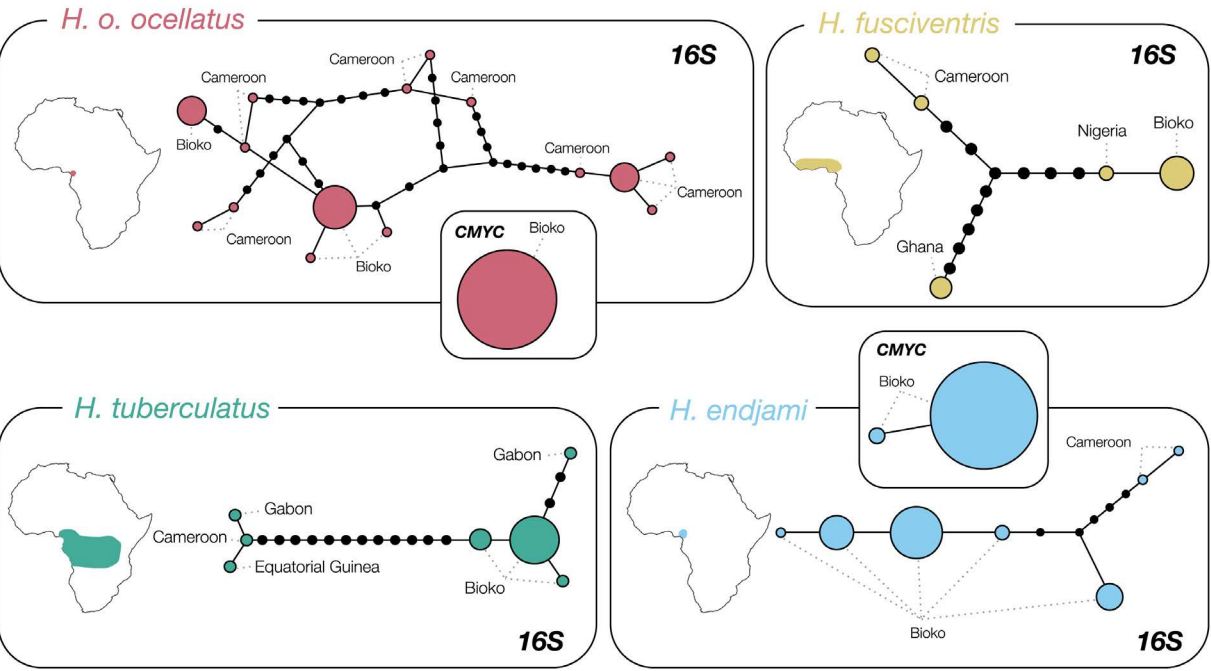


Figure 2. Mitochondrial (16S) and nuclear (CMYC) haplotype networks for *Hyperolius ocellatus ocellatus*, *Hyperolius endjami*, *Hyperolius tuberculatus* and *Hyperolius fusciventris* and distribution of species on mainland Africa. Haplotype networks are scaled according to sample size and coloured according to species. No mtDNA or nuDNA haplotypes were shared between *H. endjami* and *H. o. ocellatus*. Bioko and reference (Cameroon, Ghana, Equatorial Guinea, Gabon, and Nigeria) specimens are indicated.

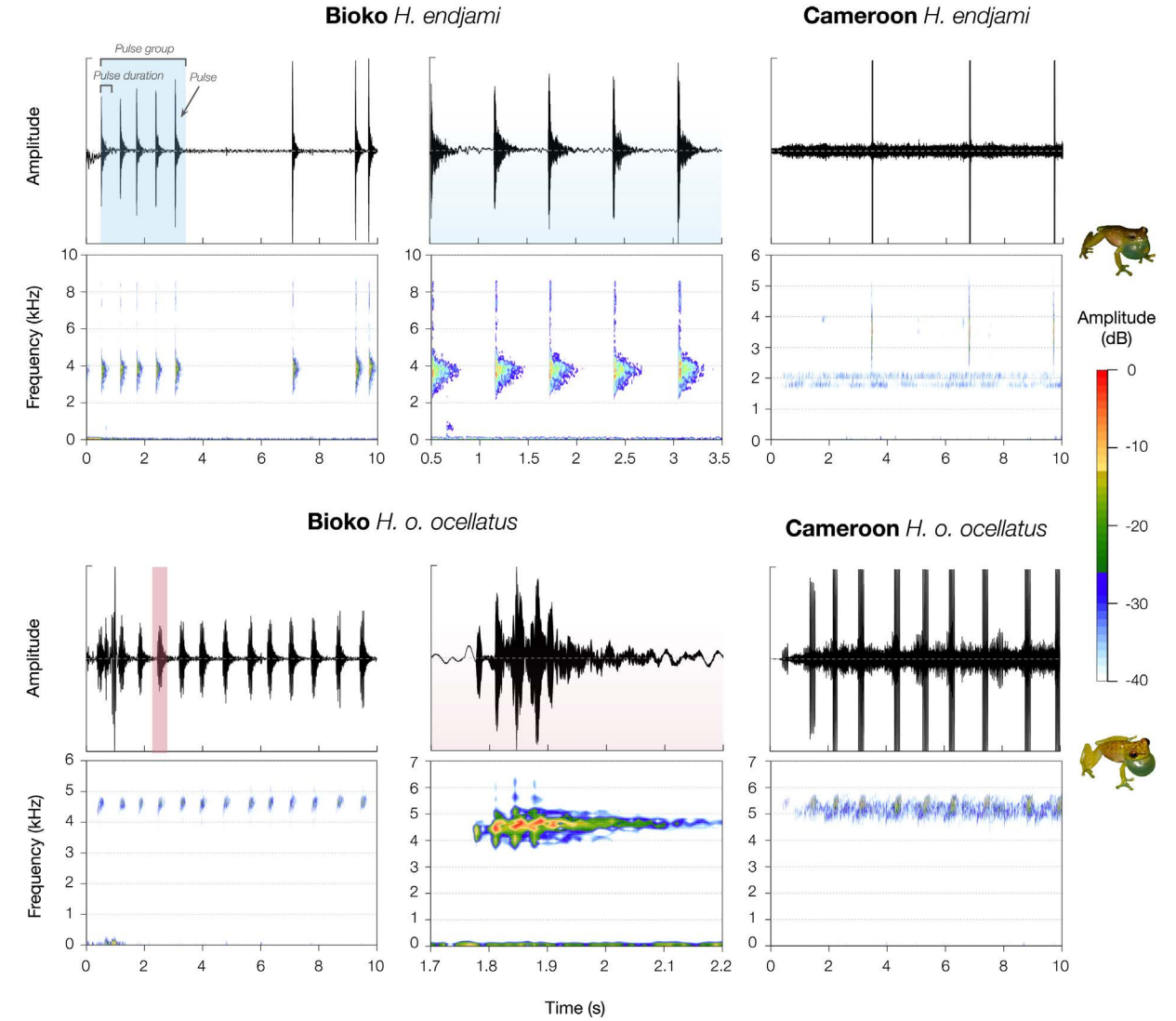


Figure 3. Oscillograms and spectrograms of advertisement calls of *Hyperolius endjami* and *Hyperolius ocellatus ocellatus* from Bioko Island (this study) and Cameroon (Amiet & Goutte, 2017). Amplitude and frequency of calls is shown over a duration of ten seconds and over a duration of a single pulse group for each species' call. Each vertical line represents a single pulse. The pulse group is defined as a set of pulses. The pulse duration is the duration of the pulse. The middle panels show a zoomed-in view of the advertisement calls of Bioko *H. endjami* and Bioko *H. o. ocellatus*.

Table 1. Advertisement calls of *Hyperolius endjami* and *Hyperolius ocellatus ocellatus* from Bioko Island and Cameroon. Snout-vent length (SVL) and ambient temperature are reported for the recordings generated in the present study.

Species	Location	Voucher	SVL (mm)	Temperature (°C)	Recording	Total Pulse Groups	Avg Dominant Freq (Hz)	Avg Pulses (range)
<i>H. endjami</i>	Bioko	CUMV 15162	23.5	18.3	ML 173579	18	3510	2.7 (1 – 6)
<i>H. endjami</i>	Bioko	CUMV 15201	20.7	20.1	ML 173581	4	3795	5.3 (1 – 10)
<i>H. endjami</i>	Cameroon	N/A	--	--	2.9, Amiet & Goutte 2017	9	3732	1.0 (1)
<i>H. endjami</i>	Cameroon	N/A	--	--	2.10, Amiet & Goutte 2017	9	3531	1.1 (1 – 2)
<i>H. o. ocellatus</i>	Bioko	CUMV 15217	22.0	19.4	ML 173582	37	4505	4.4 (3 – 7)
<i>H. o. ocellatus</i>	Cameroon	N/A	--	--	2.37, Amiet & Goutte 2017	15	4272	2.5 (1 – 3)
<i>H. o. ocellatus</i>	Cameroon	N/A	--	--	2.38, Amiet & Goutte 2017	28	5349	5.0 (4 – 5)

H. endjami and Bioko *H. o. ocellatus* (Fig. 2). We recovered one haplotype for Bioko *H. ocellatus* and two haplotypes for Bioko *H. endjami*; none of the CMYC haplotypes were shared.

No evidence of hybridisation between *H. endjami* and *H. o. ocellatus*

In 2011 and 2019 we collected ten amplexant pairs at Moeri. Our updated identifications indicate that we collected two pairs of *H. endjami*, seven pairs of *H. o. ocellatus* and one heterospecific pair. Mitochondrial and nuclear haplotypes showed no individuals with admixed genetic ancestry (Fig. 2, Table S1). Thus, there is no evidence of hybridisation between *H. endjami* and *H. o. ocellatus* despite multiple observations of heterospecific amplexus.

Male advertisement calls

Advertisement calls for *H. endjami* and *H. o. ocellatus* differ in call dominant frequency and pulses per call (Fig. 3, Table 1): *H. o. ocellatus* has a higher average dominant frequency and typically produces more pulses per call than *H. endjami*. Although there are slight genetic differences between *H. endjami* from Bioko and Cameroon, male advertisement calls for both localities are similar in amplitude and share a distinct ‘clicking’ timbre (Fig. 3).

Likewise, advertisement calls of *H. o. ocellatus* from Bioko and Cameroon are similar with males in both populations producing high frequency trills (Fig. 3).

Colour pattern variation in Bioko *H. endjami* and *H. o. ocellatus*

Recorded variants of male *H. endjami* from Cameroon exhibit yellow to light-green mid-dorsal pigmentation and dorsolateral spots. The degree of connectivity of dorsolateral spots and pigmentation give way to a diversity of variants (Figs. 86 & 87 in Amiet (2012)). Male *H. fusciventris* display a range of colour patterns including solid green or green with white dorsolateral lines, with various degrees of dark speckling (Fig. 135 in Amiet (2012)). Male *H. o. ocellatus*, however, are described as possessing a distinctive colour pattern: white dorsolateral lines that join at the snout and form a triangle. Morphological variants of *H. o. ocellatus* differ in ocellation and colour but all retain the above diagnostic colour pattern (Fig. 138 in Amiet (2012)). The three male *H. fusciventris* from Bioko had colour patterns consistent with previous descriptions of the species from Cameroon; however, we observed colour pattern variation in male *H. endjami* and *H. o. ocellatus* from Bioko that have not previously been described for these species (Amiet, 2012).

Three *H. endjami* specimens (CAS 265146, CAS 265245, CAS 265246) exhibited white dorsolateral stripes that join at the snout, forming a triangle. This colour pattern is typically diagnostic for male *H. o. ocellatus* and confounded some of our initial field identifications. By contrast, we observed three colour patterns in *H. o. ocellatus* that lacked the diagnostic white triangle on the snout: ocellated with no white dorsolateral stripes (CAS 265180, CAS 265141, CAS 265240), ocellated with white dorsolateral stripes that do not join at the snout (CAS 265183), and solid green with no white dorsolateral stripes (CAS 265138, CAS 265139, CAS 265140; Fig. 4).

DISCUSSION

Our integrative datasets confirm the presence of a robust population of *H. endjami* on Bioko Island. Many field identifications have confused *H. endjami* with *H. o. ocellatus*, likely due to the shared colour patterns we report in males of the two species. Thus, our study provides a significant range expansion for *H. endjami*, which was described in 1980 (Amiet) and is known from only a few localities in Cameroon. Specimens and other records from Bioko previously attributed to *H. ocellatus* (e.g. MNCN specimens examined in Sánchez-Vialas et al., 2020) should be reassessed in light of this new information. Our modest mtDNA dataset indicates that the Bioko population of *H. endjami* harbours unique genetic diversity not shared with Cameroon. A similar pattern has been observed in Bioko populations of other hyperoliid frogs including *Afrixalus paradorsalis* (Charles et al., 2018), *H. o. ocellatus* (Bell et al., 2017), and *H. tuberculatus* (Bell et al., 2017). Divergence time estimates for Bioko populations of these species indicate they likely became isolated from their continental counterparts during recent Pleistocene glacial cycles (Bell et al., 2017; Charles et al., 2018). The amount of divergence we observed between Bioko and Cameroon populations of *H. endjami* is similar to that in these other taxa, suggesting the populations have been isolated for a comparable period of time. Quantifying population genomic and phenotypic divergence between *H. endjami* on Bioko and in Cameroon would enable more robust, model-based investigations of the demographic history of the island population.

Despite extensive amphibian surveys on the southern half of Bioko Island, *H. fusciventris* are only known on the island from three males collected in the port town of Luba. Subsequent searches at this locality and in adjacent areas indicate there is suitable habitat for amphibians, including breeding choruses of *H. o. ocellatus*, but no other *H. fusciventris* have been found. On continental Africa, this species ranges from Sierra Leone to south-western Cameroon with four subspecies that differ in female (phase F) colouration (Schlötter, 1999; Amiet, 2012). Unfortunately, only males were sampled, therefore the Bioko frogs could not be attributed to a particular subspecies based on distinctive colouration; however, the sequences were most similar to continental populations from Nigeria, which is within the distribution

of *H. f. burtoni* (Boulenger, 1883). A larger genetic dataset and more sampling across the species range could help clarify whether *H. fusciventris* is native to Bioko or was introduced, and further surveys in lowland habitats across Bioko are needed to determine whether they are still extant on the island.

Although *H. endjami* and *H. o. ocellatus* occur at the same breeding sites and can be found in heterospecific amplexus, no evidence was found of successful hybridisation. Hybridisation of *Hyperolius* frogs occurs on the nearby island of São Tomé. In localities where *H. thomensis* and *H. mollerii* are sympatric, Bell et al. (2015) documented extensive genetic admixture between species. However, the São Tomé species are closely related, unlike *H. endjami* and *H. o. ocellatus* (Portik et al., 2019). The call of *H. endjami* is rather distinct from *H. o. ocellatus*: *H. endjami* produces lower frequency clicks, while *H. o. ocellatus* produces higher frequency trills. The pulse rates, which some female frogs use to distinguish between males of their own species and closely related sympatric species (Lemmon, 2009), also differ between *H. endjami* and *H. o. ocellatus*. Despite these differences, the two species were observed in heterospecific amplexus, indicating that advertisement call is an incomplete prezygotic behavioural barrier. Further prezygotic barriers that come into play during courtship, such as species-specific gular gland compounds in reed frogs in particular (Starnberger et al., 2013), and postzygotic barriers like hybrid sterility (Richards & Schlötter, 1977) may prevent successful hybridisation between sympatric species.

Reed frogs are notorious for their sexual dichromatism, and extensive intraspecific and interspecific variation in colouration (Schlötter, 1999). On Bioko, all the colour pattern variations for *H. endjami* and *H. o. ocellatus* detailed by Amiet (2012) were observed, as well as new colour patterns for both species that have not been documented in mainland populations. Polymorphism is common in the family Hyperoliidae, with species such as those in the *H. viridiflavus*, *H. marmoratus* and *H. parallelus* complexes exhibiting extreme diversity in dorsal colour patterns (Channing, 2002). The function of colour polymorphism and sexual dichromatism in *Hyperolius* is still unclear, but may play a role in species and mate recognition (Portik et al., 2018; 2019). Our findings further suggest that colouration is variable in *H. endjami* and *H. o. ocellatus* much like other *Hyperolius* species, though surprisingly this novel variation is in the male colouration, and more work is needed to understand the genetic basis and ecological consequences of these differences.

In summary, we confirmed the presence of *H. endjami* and *H. fusciventris* on Bioko Island and recovered unique genetic diversity that is not shared with mainland populations. We described male advertisement calls of *H. endjami* and *H. o. ocellatus* on Bioko, and found that they are not hybridising on Bioko despite observations of heterospecific amplexus. Finally, we reported new dorsal colour pattern variations in male *H. endjami* and *H. o. ocellatus*, further documenting polymorphism in these two species. Unfortunately, our dataset was limited to frogs collected from the southern half of Bioko Island due

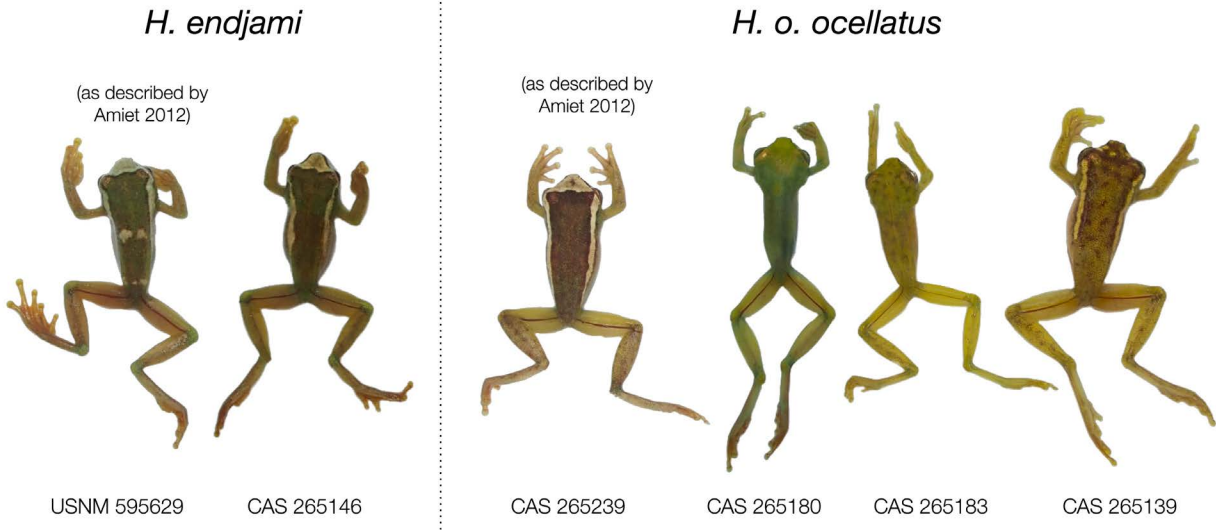


Figure 4. Newly described colour pattern variations of male *Hyperolius endjami* (CAS 265146) and *Hyperolius ocellatus ocellatus* (CAS 265180, CAS 265183, and CAS 265138 from left to right) compared to colour patterns described by Amiet (2012; *H. endjami*: USNM 59629, *H. o. ocellatus*: CAS 265239).

to restricted access in the northern region (particularly for nocturnal surveys). Future studies sampling the northern half of Bioko would provide a more complete account of the distribution of *Hyperolius* frogs on the island and a better understanding of how reed frog species are partitioning geographic and ecological space. For instance, although *H. tuberculatus* are a widespread lowland forest species in their continental distribution (Channing & Rödel, 2019), to date they have only been documented in the vicinity of the two, high elevation crater lakes on the southern half of Bioko Island and it is unclear whether they are present at higher elevations on the northern peak (Pico Basile). Likewise, *H. o. ocellatus* seem to occur at lower elevations and *H. endjami* at higher elevations on the southern half of the island with a narrow range of sympatry, and it is unclear whether this pattern is replicated on the northern peak. Future surveys of *Hyperolius* on Bioko will benefit from careful attention to advertisement calls and/or genetic data to differentiate between male *H. o. ocellatus* and *H. endjami* given the challenges in identifying them based on morphology alone. Our study sets the stage for future studies of the ecology, evolution and conservation of this island community.

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Author contributions

R.C.B. designed the project; R.C.B., P.J.M., L.A.S., and C.I. collected field samples and took photographs; R.C.B. and J.V.N. performed the lab work; R.C.B. and J.V.N. analysed the data; J.V.N. produced the figures; J.V.N led the writing and all authors contributed to paper revision.

Ethical Statement

All research was conducted with IACUC approval (Cornell University 1999-0010, Drexel University 18748, Smithsonian Institution National Museum of Natural History 2016-09, California Academy of Sciences 2017-02).

DATA ACCESSIBILITY

GenBank accession numbers (16S: PP431295 - PP431384, CMYC: PP431075 - PP431154), sampling localities, voucher specimen information, and Macaulay Library accession numbers for audio recordings are available in Table S1.

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Predation of anurans in southern England by *Batrachobdella algira*, a leech previously unknown in the UK

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Leech predation of amphibians is known to occur in Europe. Observation of severe leech infestation affecting a common toad *Bufo bufo* in southern England in summer 2020, with leeches covering the toad's eyes, throat and axillae, initiated a collaborative investigation to learn more about the occurrence of such leech predation of anurans in the UK. Soliciting reports from the general public identified leech predation of common toads and common frogs *Rana temporaria* in Devon, Greater London, Hampshire, the Isle of Wight and Somerset in southern England. Through morphological and/or molecular investigation of samples, *Batrachobdella algira*, a leech species not previously reported in the UK, was identified in the majority of cases. The known native *Placobdella costata* was also identified, with the observed feeding behaviour on anuran hosts being indistinguishable from that of *B. algira*. Whether the latter is a previously unrecorded or an introduced species has not yet been established. However, sequence data from multiple gene loci were identical to *B. algira* found in Tunisia, suggesting it more likely to be a non-native species to the UK. Further work is required to elucidate the potential origin and distribution of *B. algira* in the UK and whether it has any impact on amphibian populations.

Keywords: amphibians, *Bufo bufo*, Glossiphoniidae, Hirudinea, *Rana temporaria*

INTRODUCTION

Leeches (Hirudinea) are a small, but ecologically important group of highly specialised segmented worms. Around 680 species have been described worldwide, the majority of which are found in, or beside, freshwater bodies (Sket & Trontelj, 2008). Leeches differ from other segmented worms in having anterior and posterior suckers and typically a firm, muscular, segmented body (Govedich et al., 2019).

There are 17 species of freshwater leeches known in the United Kingdom (UK) (Elliott & Dobson, 2015). They range in size at rest from 7 mm for *Piscicola siddalli*, to up to 160 mm for *Trocheta subviridis*, and when fully extended, larger leeches may reach a length of over 200 mm. Morphologically, these species can be identified using a combination of the number and position of the eyes, the size of the caudal sucker, the width and pattern of the annulation, the presence and pattern of the papillae, and the position of the gonopores. Depending on the species, they have been reported to feed on live fish, amphibians, mammals, or birds, as well

as invertebrates, but may also scavenge on dead animals (Elliott & Dobson, 2015).

Four British leech species have been reported to be sanguivorous (blood-feeding) on live amphibians: the medicinal leech *Hirudo medicinalis* (family Hirudinidae); and three species of Glossiphoniidae – *Glossiphonia paludosa*, *Hemiclepsis marginata* and *Placobdella costata* (Elliott & Dobson, 2015). In addition, the horse leech *Haemopsis sanguisuga* (family Haemopidae) is macrophagous on froglets and toadlets and may also feed on dead or moribund adult amphibians. It is likely that *Erpobdella* spp. and *Dina lineata* (both family Erpobdellidae) may also occasionally feed on dead amphibians (Elliott & Dobson, 2015). Despite increased monitoring of the health of amphibian populations in the UK over recent years, observations of leeches feeding on live amphibians are rarely reported (e.g. Price et al., 2017; Franklinos et al., 2018; Seilern-Moy et al., 2019).

In summer 2020, the sighting of a severe leech infestation affecting a live common toad *Bufo bufo* in southern England, with leeches covering both eyes and the throat of the toad (Fig. 1A), raised public concern on

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Figure 1. **A.** Common toad *Bufo bufo* reported from Devon, England, in August 2020, with leeches covering both eyes and attaching to the throat, and **B.** common frog *Rana temporaria* reported from the Isle of Wight, England, in 2020, with leeches attached to the ventro-lateral body. Leech samples from both cases were molecularly identified as *Batrachobdella algira*.

social media. Here we report the results of a subsequent collaborative investigation to explore the occurrence of such feeding behaviour on anurans and to identify the leech species involved.

MATERIALS & METHODS

Following the initial report of leeches feeding on a common toad in southern England, the Hampshire and Isle of Wight Amphibian and Reptile Group (HIWARG) and the Isle of Wight Reptilium launched a regional social media campaign and citizen science survey appealing

for reports of leeches feeding on amphibians (www.reptilium.org/leech-survey). This was combined with a similar national appeal by the Amphibian and Reptile Groups of the UK (ARG UK) (www.arguk.org/get-involved/news/a-new-report-of-leech-predation-on-amphibians). Additionally, reports of sick or dead amphibians continued to be solicited from the public by the Garden Wildlife Health project (www.gardenwildlifehealth.org), a national disease scanning surveillance scheme that launched in 2013.

For each sighting, the location and species of amphibian involved and, where possible, the number of

Table 1. Reports received from the general public of leeches feeding on common frogs *Rana temporaria* and common toads *Bufo bufo* in England, summer 2020 – January 2023 inclusive. Where possible, leech species were identified using morphological and/or molecular methods.

County	Year	No. of sites	Anuran species (No. of sites)	Method of leech identification (No. of sites)	Leech species (No. of sites)
Devon	2020	1	<i>Bufo bufo</i> (1)	Morph. + molec. ¹ (1)	<i>Batrachobdella algira</i> (1)
	2021	1	<i>B. bufo</i> (1)	Morph. + molec. ² (1)	<i>B. algira</i> (1)
	2022	3	<i>Rana temporaria</i> (3)	Morph. + molec. ² (1) Report only (2)	<i>B. algira</i> (1) Unknown (2)
	2023	1	<i>R. temporaria</i> (1)	Morph. (1)	<i>Placobdella costata</i> (1)
Greater London	2022	1	<i>R. temporaria</i> (1)	Report only (1)	Unknown (1)
Hampshire	2022	3	<i>R. temporaria</i> (3)	Photo (1) Report only (2)	Suspect <i>P. costata</i> (1) Unknown (2)
Isle of Wight	2020	13	<i>B. bufo</i> (1) <i>R. temporaria</i> (12)	Morph. + molec. ¹ (1) Report only (12)	<i>B. algira</i> (1) Unknown (12)
	2021	9	<i>B. bufo</i> (3) <i>R. temporaria</i> (6)	Morph. + molec. ¹ (1) Report only (8)	<i>B. algira</i> (1) Unknown (8)
	2022	7	<i>B. bufo</i> (1) <i>R. temporaria</i> (6)	Morph. + molec. ² (1) Report only (6)	<i>B. algira</i> (1) Unknown (6)
Somerset	2020	1	<i>R. temporaria</i> (1)	Morph. + molec. ² (1)	<i>B. algira</i> (1)
	2022	1	<i>B. bufo</i> (1)	Report only (1)	Unknown (1)
All counties	2020–2023	41	<i>B. bufo</i> (8) <i>R. temporaria</i> (33)	Morph. + molec. (7) Morph. (1) Photo (1) Report only (32)	<i>B. algira</i> (7) <i>P. costata</i> (2) Unknown (32)

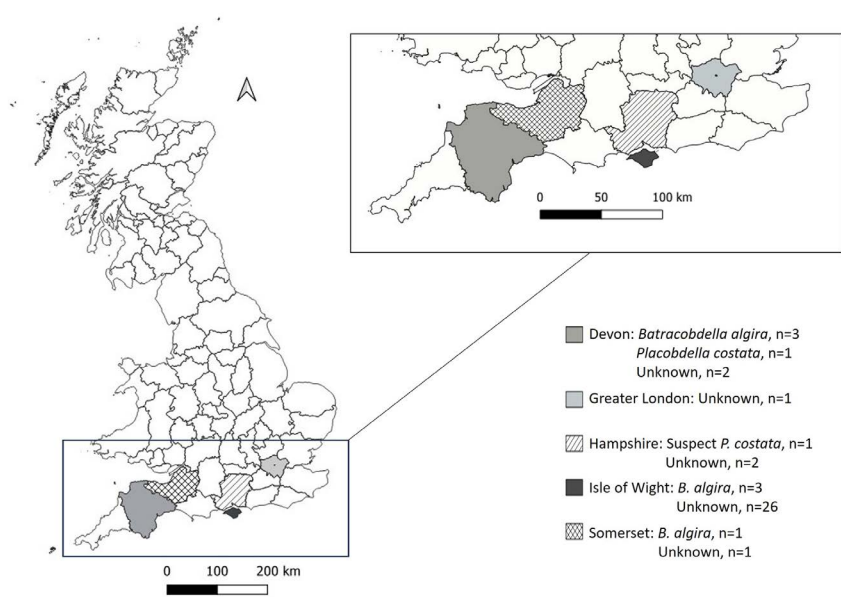


Figure 2. Map of Great Britain highlighting the counties from which reports of leeches feeding on common frogs *Rana temporaria* and common toads *Bufo bufo* were received from the general public (summer 2020–January 2023 inclusive), including information on the number of sites and leech species identified.

amphibians affected and the distribution of the leeches on the body, were recorded. Photographs of affected amphibians accompanied reports when available. In a subset of cases, leech specimens were collected, stored in 70% ethanol, and submitted for species identification.

Morphological identification of 18 leeches from eight sites (Table 1) was conducted by microscopical examination using a stereomicroscope (Olympus SZ40 Stereomicroscope, magnification x 6.25 – 80). Features including the number and position of the eyes, the width and pattern of the annulation, the presence and pattern of papillae, the size of the caudal sucker, and the position of the gonopores were compared with UK and European taxonomic keys and species descriptions (Sládeček & Košel, 1984; van Haaren et al. 2004; Ben Ahmed et al., 2015; Elliott & Dobson, 2015; Govedich et al., 2019). Additionally, high-resolution pictures of leeches attached to a common frog, taken using macro-photography, were submitted from a single site in Hampshire.

For molecular identification of the *Batrachobdella* sp., DNA was extracted from the caudal suckers of 16 leeches from seven sites (Table 1). Each caudal sucker was placed in a microcentrifuge tube and incubated at 37 °C for 30 min to evaporate residual ethanol before using the DNeasy Blood & Tissue Kit (Qiagen) as per manufacturer’s instructions. A PCR protocol targeting the cytochrome c oxidase subunit I (COI) gene was then conducted (Richardson et al., 2010; Martinsson & Erséus, 2014). Additionally, other multi-loci PCR protocols, as described by Świątek et al. (2023), focusing on the 12S rRNA, 28S rRNA and histone H3 genes (Vân Lê et al., 1993; Trontelj & Utevsky, 2005; Martinsson & Erséus, 2014), were carried out on each of three leech samples collected from three separate sites. PCR amplicons were subjected to bidirectional Sanger sequencing and the resulting sequence data were screened against GenBank entries

using BLASTn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Additionally, available COI nucleotide sequences for *Batrachobdella* species were retrieved from GenBank and augmented with the sequence data generated in this study. Sequences were aligned in MEGA7 (Kumar et al., 2016) and trimmed in case they exceeded the 'Folmer region' (excluding primer sequences). A web server version of IQ-TREE (Trifinopoulos et al., 2016) was used to estimate the best-fitting models of nucleotide and to subsequently construct a maximum likelihood tree with 1000 replicates. Bootstrap support values were calculated using the ultrafast algorithm with default settings and the SH-aLRT branch test was used to evaluate the tree branch supports. The tree was rooted at *Haementeria ghilianii* and visualised with Interactive Tree of Life v6 (Letunic & Bork, 2007).

Three common frogs from separate sites, from August 2020 (Isle of Wight), September 2020 (Somerset) and January 2023 (Devon), which were observed by members of the public with leeches feeding on them and were subsequently found dead, were submitted for post-mortem examination (PME). A systematic external and internal examination protocol was followed, including parasitological examination of intestinal contents as well as qPCR testing of liver samples for ranavirus and skin swab samples for *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (Franklinos et al., 2018).

RESULTS

From August 2020 to January 2023 inclusive, reports of leeches feeding on common frogs and common toads were received from 41 sites in the counties of Devon, Greater London, Hampshire, the Isle of Wight and Somerset in southern England (Table 1; Fig. 1). Reports from 37 sites (37/41) involved live anurans, whereas at four

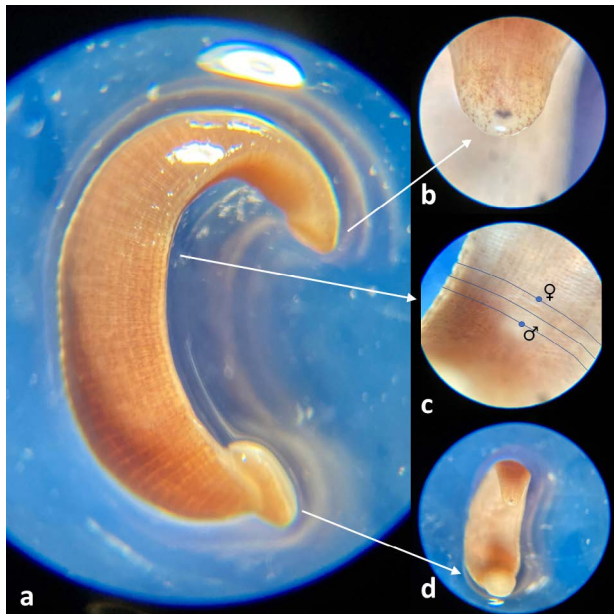


Figure 3. Microscopic images of a leech found feeding on a common frog *Rana temporaria* on the Isle of Wight in 2022, with morphological features of a *Batracobdella* sp. **a.** lateral whole body, **b.** one pair of eyes, **c.** gonopores separated by two annulae, **d.** large posterior sucker. Olympus SZ40 Stereomicroscope. Magnification x 6.25 – 40.

sites (4/41) dead common frogs were observed. Estimates of the number of affected amphibians was typically low (range 1–3 per site), however, this information was only available from a minority of sites. The estimated number of leeches per individual varied (1–≥50). Where details were available, for affected common toads, leeches were typically observed to cover the eyes and periocular region (Fig. 1A) as well as attach to the throat, the axillae, and occasionally the ventral body. In common frogs, leeches were reported to be present over the entire body, on occasion also covering the eyes, although most notably on the underside, in the axillae and inguinal area (Fig. 1B).
Leeches were submitted for species identification from a total of eight sites (eight leeches from three sites in Devon, one from a single site in Hampshire, six from three sites on the Isle of Wight and three from a single site

in Somerset; Fig. 2). All leeches examined microscopically were identified as Glossiphoniidae species. Morphological features, including the number of eyes, the width and patterning of the annulation, the presence of many small papillae, the size of the caudal sucker and the position of the gonopores (Fig. 3), were indicative of *Batracobdella* sp. (Ben Ahmed et al., 2009) in leeches from seven sites (16/18 individual leeches examined), while they were consistent with those of the native freshwater leech *P. costata* from a single site in Devon (2/18 individual leeches examined). Additionally, high-resolution photographs of leeches from a single site in Hampshire where also morphologically consistent with *P. costata*.

Molecular investigation of the 16 putative *Batracobdella* leeches revealed an identical COI gene sequence (GenBank accession number OR381498; 658 bp), which also was identical to a Tunisian *B. algira* COI sequence (GenBank accession number OR366856, 100% query coverage). Furthermore, the obtained COI sequence shares 99% identity with other Tunisian and Algerian specimens of the same leech species (Fig. 4; GenBank accession numbers: OR366855 and OR367453-OR367454). The obtained COI sequence also exhibits a 92% match (86% query coverage) with a previously published sequence from a leech in Spain, reported as *B. algira* sequence (Trajanovski et al., 2010; GenBank accession number HM246609). Identification as a *B. algira* was further supported through multi-loci PCR sequence analyses of leeches from three sites in England. We successfully amplified fragments of three additional commonly used marker genes, which were identical in all three leeches analysed: nuclear 28S rRNA (GenBank accession number OR381568; 329 bp), histone H3 (GenBank accession number OR453923; 328 bp), and mitochondrial 12S rRNA (GenBank accession number OR381567; 508 bp). The mitochondrial 12S rRNA and histone H3 sequences were found to be identical to the Tunisian *B. algira* specimens (GenBank accession numbers OR388126 and OR453924), and the 28S rRNA sequence was identical to those of Tunisian as well as Algerian *B. algira* specimens (GenBank accession numbers OR371771 and OR371772).

Upon PME, numerous dead leeches (subsequently morphologically identified as *P. costata*) were found

loosely attached to the body of a common frog found dead in Devon in January 2023, particularly in the axillae and inguinal area, as well as being free within the plastic bag in which the body was stored. However, no leeches were observed to still be attached to the common frog carcasses submitted from the Isle of Wight and Somerset in August and September 2020 respectively, both of which had been removed from the pond and kept separated from other amphibians prior to death. No macroscopic lesions associated with leech feeding behaviour (e.g. skin abrasions and/or haemorrhages) were observed in these amphibians; however, the compromised state of carcass preservation may have masked subtle changes. All three common frogs that were examined post-mortem were adult males, two in normal and one in thin (August 2020) body condition. No significant abnormalities indicative of disease were observed in two of these common frogs, whereas macroscopic evidence of haemorrhagic enteritis of unknown aetiology was found in the animal submitted from Somerset in September 2020, which might have contributed to ill health and/or death. All three frogs tested negative for ranavirus, *B. dendrobatidis* and *B. salamandrivorans*, and their proximate cause of death remains undetermined.

DISCUSSION

A number of different species of native leeches are known to feed on amphibians in the UK. Here, we describe the detection of a leech species novel to the UK, and the first report of *B. algira* in this country, feeding on anurans in southern England. Like the native *P. costata*, *Batracobdella* spp. belong to the family Glossiphoniidae (order Rhyncho bdellida), which includes leeches which feed on the blood of amphibians (Siddall et al., 2005). The species identified in this study appears to be genetically identical to the Tunisian lineage of *B. algira* studied by Ben Ahmed et al. (2009; 2015; 2021), which strongly suggests that the UK *B. algira* may be an introduced, non-native species.

Generally, *B. algira* is described from freshwater habitats in the Mediterranean rim (e.g. Algeria, Tunisia, the Pyrenean Peninsula, Balearic Islands, Corsica, Sardinia and Sicily) as well as from Bulgaria and Ukraine (Minelli, 1979; Nesemann, 1991; Sorgi et al., 2011; Ben Ahmed et al., 2015), where it is reported to feed on several species of amphibian (Ben Ahmed et al., 2015; Manenti et al., 2016). However, the true geographic range of *B. algira* could be more limited, while the reported broad distribution might be attributed to hidden cryptic diversity of leeches (Raja Ben Ahmed, pers. comm.). Sightings and investigations of *Batracobdella* species remain scarce, and the overall understanding of their distribution and diversity is limited (Nesemann, 1991; Trajanovski et al., 2010).

The apparently restricted southern England distribution of *B. algira* within the UK might suggest this to be the area of arrival in the country, for example through natural routes from continental Europe or anthropogenic movement. The latter route, e.g. through

live animal or plant trade, may offer the more likely explanation given the identical sequences of multiple loci of *B. algira* found in the UK and Tunisia despite their geographical distance. However, natural long-range dispersal, possibly mediated by birds, has also been documented in glossiphoniid leeches such as *P. costata* (Kvist et al., 2022), and therefore remains plausible. Additionally, a reporter bias may have occurred due to the regional emphasis of the HIWARG and Isle of Wight Reptilium social media campaign appealing for sightings of leeches feeding on amphibians, resulting in a perceived localised observation. Potential abiotic environmental features (e.g. temperature, humidity), which influence the regional distribution of leeches, might also restrict the species’ distribution (Lunghi et al., 2018).

Based on public reports, the observed distribution on the body and appearance of leeches feeding on common frogs in the two sites from which *P. costata* was morphologically identified were indistinguishable from the reports involving common frogs affected by *B. algira*. In an additional report of leeches attaching to the eyes and throat of a common toad on the Isle of Wight in 2011, another native widespread freshwater leech *H. marginata* was morphologically identified (Craig Macadam, unpublished data). The explanation that leeches are generally observed to accumulate mostly on the eyes and periocular areas as well as the throat and axillae of common toads is thought to be due to the avoidance of toxic skin secretions and therefore attaching to areas that are unprotected by skin glands (Kutschera et al., 2010). Our findings highlight the importance of citizen science and the morphological and/or molecular identification of the leech species involved to identify new species records and to differentiate potentially introduced species from known native species that form an important part of the natural fauna diversity (Elliott & Dobson, 2015).

Although interactions between leeches and their hosts are poorly characterised to date, it is known that leeches may use amphibians as an opportunistic food source (Elliott & Dobson, 2015), generally without leading to host death (Getz, 2011; Rocha et al., 2012). However, the impairment of vision caused by leeches covering the eyes of their host will likely affect its ability to forage or evade predators. Other potential health impacts, e.g. by affecting host fitness, pathogen transmission (Siddall & Dessler, 1992; Jiménez Sánchez, 1997), or increasing host vulnerability to further infections (Lunghi et al., 2018), have also been previously discussed (Merilä & Sterner, 2002; Stead & Pope, 2010). It has further been proposed that environmental changes (e.g. climate warming or reduced water levels) may enhance negative impacts of leech predation on their hosts (Berven & Boltz, 2001; Ayres & Comesaña Iglesias, 2008).

Although facultative parasitism of the *B. algira* identified in this study seems likely, a phoretic relationship between anurans and this leech species (Khan & Frick, 1997; Maia-Carneiro et al., 2012; Starzecka et al., 2020) remains possible. Molecular investigation of stomach

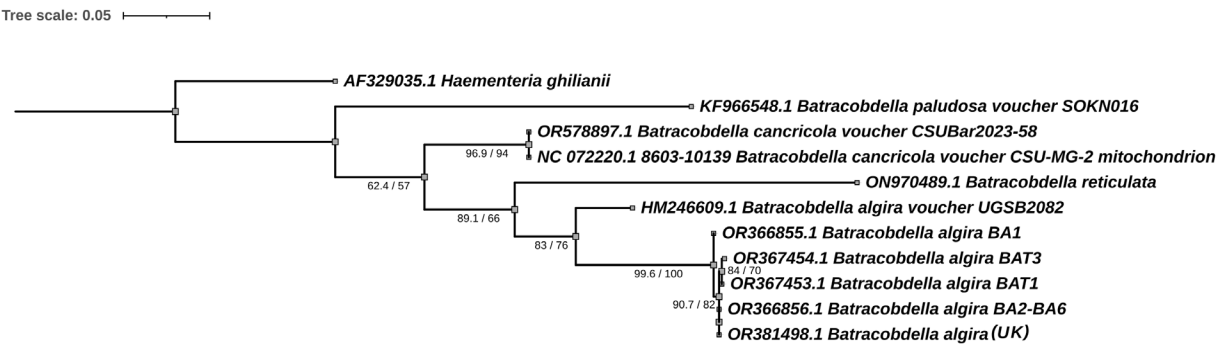


Figure 4. Maximum Likelihood tree resulting from the analysis of COI nucleotide sequences of *Batracobdella* species. SH-aLRT and UFBoot support values are given below the branches. The sequence obtained from the *Batracobdella* species identified from the UK is identical to sequences from *B. algira* BA2-BA6 from Tunisia and is grouped within the Tunisian-Algerian *B. algira* clade. This clade is considered to represent *B. algira* sensu stricto, which inhabits the type locality.

content of leeches could be used to explore the trophic relationship between *B. algira* and anurans in the UK (Marrone et al., 2016). Whilst to date there is no evidence of native leech species affecting amphibians in the UK at a population level, the significance of *B. algira* to amphibian health is yet to be established. In its native habitat, *B. algira* is not known to have negative health or population impacts on amphibians (Ben Ahmed et al., 2014).

Further surveillance across the UK is required to determine the distribution and frequency of occurrence of *B. algira*. Additional genomic sequence analyses across the *Batracobdella* genus might help identify closely related species and determine whether it is a historically unrecorded species in England or if it has originated from elsewhere. Regardless, a risk assessment to elucidate possible incursion pathways of non-native leeches to the UK, combined with a mitigation plan to address anthropogenic routes of introduction, should be developed.

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Author contributions

AMJ, PW and NR ran the social media campaign and collated reports of leeches feeding on amphibians. KSM, BL, AAC and JH co-ordinated the Garden Wildlife Health project. CRM conducted morphological analysis of leech specimen. KSM, JH, LG, PŚ and ŁG conducted various aspects of molecular investigations. KSM, BL, CRM drafted the original manuscript. All authors contributed to, and approved the final manuscript.

DATA ACCESSIBILITY

All new DNA sequences generated in the present study are deposited in GenBank under Accession Numbers: OR381498 (COI), OR381567 (12S rRNA), OR381568 (28S rRNA), and OR453923 (histone H3).

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Methods used in sea turtle genetic and molecular studies

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Sea turtles have a tropical and subtropical distribution and can be found in nearly all seas and oceans. They have been the subject of considerable genetic research. However, it does not yet appear that the molecular techniques used for these genetic studies follow a consensus or universal set of tools to be followed for subsequent studies. This is not desirable since it may preclude data exchange and use among studies. Thus, the aim of this review was to survey the main genetic and molecular methods used for sea turtle research worldwide. To achieve this goal, a total of 95 scientific papers were compiled from online databases. We considered articles that used molecular tools for genetic analysis and provided detailed locality data. The following aspects were assessed: species studied, local of sample collection, type of tissue used for molecular studies and type of genetic material used. The seven known sea turtle species have different distribution patterns, with some overlapping of occurrence. *Chelonia mydas* has been studied genetically along the coasts of all continents. Skin is the most common type of tissue used for molecular analyses. From genetic studies on sea turtles, it is possible to verify the occurrence of hybridism. This phenomenon is relevant to the conservation of the species and was reported in six articles on this topic. Therefore, it is considered that genetic and molecular assays in sea turtles are important tools for biological evaluation and protection. The aim was to survey the main methods used in genetic and molecular research on marine turtles.

Keywords: DNA, Chelonian, Testudines, marine turtles

INTRODUCTION

Sea turtles are highly migratory reptiles that are capable of transoceanic migrations (Boyle et al., 2009). They have a relatively high rate of gene flow, complex population structure and show multiple paternity (Bowen & Karl, 2007). Hybridisation in sea turtles has been reported, indicating that genetic introgression occurs across different species (Vilaça et al., 2012). Population genetics studies of sea turtles are challenging, due to the constant modifications in populations and the long and complex life cycle, including the alteration of habitats during life stages, which alters the behaviour of the group (McClellan & Read, 2007; Arthur et al., 2008). Molecular studies have provided significant insights into sea turtle populations worldwide, including results on genetics, phylogenetics, evolution and conservation (Naro-Maciel et al., 2008; 2010; Barley et al., 2010; Shamblin et al., 2012a; Bradshaw et al., 2018; Camacho-Sánchez et al., 2022). Phylogenetic analyses using nuclear markers reveal a possible pattern of introgression between species of sea turtle populations in Brazil (Vilaça et al., 2012). Furthermore, the combination of nuclear and mitochondrial markers analyses has proven to be effective for evolutionary and genetic studies in the

group (Naro-Maciel et al., 2008; Garofalo et al., 2013; Vilaça et al., 2022) and has allowed the identification of hybrids (Arantes et al., 2020a; 2020b; Brito et al., 2020). In addition, analyses of polymorphic regions and the use of microsatellite markers have provided interesting results that are relevant to the ecology and conservation of the group (Roden & Dutton, 2011; Chow et al., 2019; Zhang et al., 2021; Naguib et al., 2022). The present study aimed to compile the methodologies and results used from genetic and molecular analyses involving sea turtles worldwide. This information should provide a clear picture of the main gaps in the current knowledge about marine turtle genetics and indicate new approaches to study this complex group.

Sea turtles genetic studies in worldwide

We compiled 95 research papers retrieved from scientific online database that covered studies on population genetics and phylogeography of all seven sea turtle species known worldwide. These articles were published between 2007 and 2022, and all of them specified the use of molecular analyses. Only studies in which the information about species and occurrence location are clear were considered for this evaluation. However, some research did not present clear information on specific

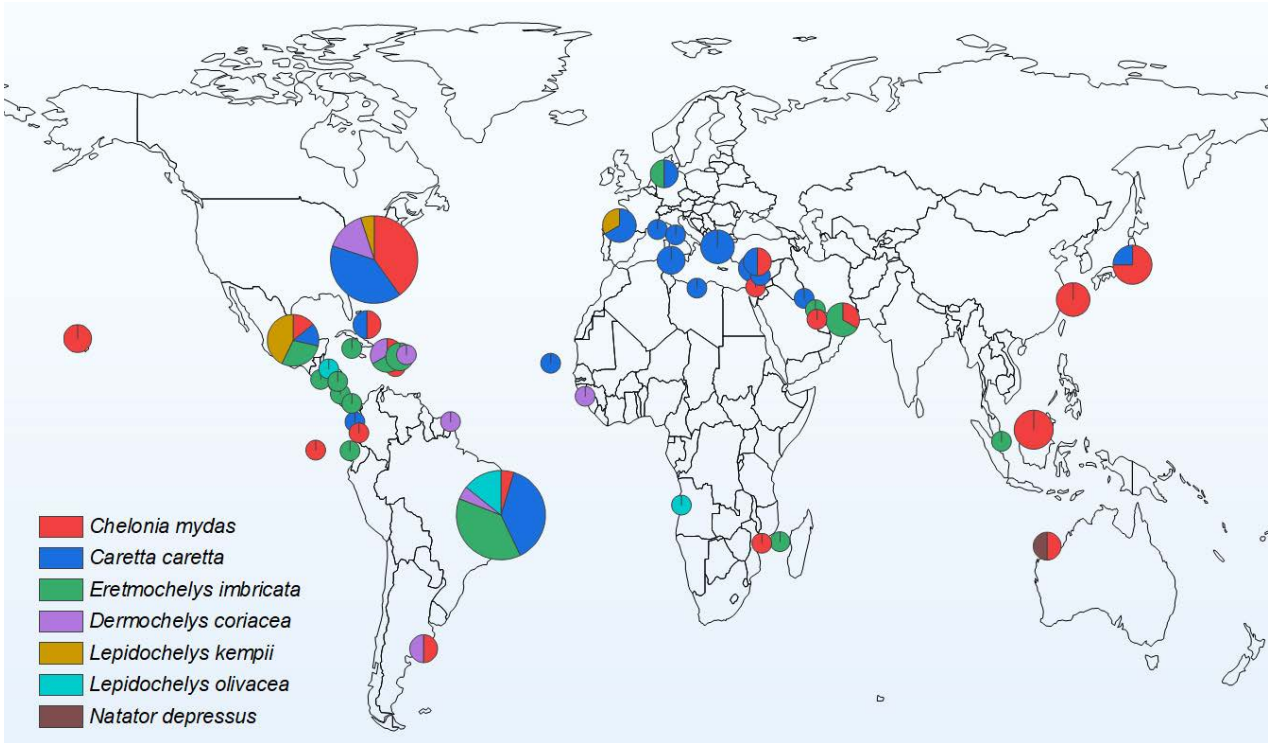


Figure 1. Map of the reporting of sea turtle species, by sampling locations, registered in the literature

points, such as the gene used for the amplification of the genetic material, the exact country in which the samples were collected or the type of tissue used for DNA extraction.

The articles analysed sea turtles recorded in 46 different countries. The continents that were most present in the studies were North America (n = 27) and South America (n = 18). The USA and Brazil were the countries most approached in the surveys. The analyses indicate a predominance of studies about the genetics and phylogeography of sea turtles in the Atlantic Ocean (Fig. 1).

Sea turtles are distributed worldwide in tropical and subtropical waters. The Atlantic Ocean is an area of intense occurrence of species, in different stages of life and foraging behaviour (Hays et al., 2006; Cantor et al., 2020; Colman et al., 2019). The place is also known as a refuge area for hatchlings during the period following emergence (a period known as the "lost years"). At this stage, the hatchlings are adrift at sea, being observed again only when they are young (Carr, 1987; Putman et al., 2020).

Molecular analyses using mitochondrial DNA (mtDNA) indicate that the genetic structure of populations of *Caretta caretta* in the Atlantic Ocean is modified, requiring follow-up studies in the region. For *C. caretta*, the Atlantic Ocean proves to be a place of high complexity for genetic stocks and a complex network of migration paths (Shamblin et al., 2012b; LaCasella et al., 2014; Naro-Maciel et al., 2014). This complexity promotes high interest in investigative studies on the molecular genetics of Atlantic Sea turtle populations.

Our analyses indicate a prevalence of studies that included the species *C. caretta* (n = 38), followed by

Chelonia mydas (n = 34) and *Eretmochelys imbricata* (n = 25) (Fig. 2A). Studies revealed a high complexity of the genetic structure in *C. caretta*, with the occurrence of subpopulations, in addition to a correlation between foraging behaviour and genetic structure (Watanabe et al., 2011).

The population of loggerhead (*C. caretta*) in the French Mediterranean presents a complex diversity, in which different origins have been identified for different groups within this population, obtaining different and complex results with mitochondrial and nuclear analyses (Loisier et al., 2021). Reports indicate hybridisation of the species *C. caretta* and *E. imbricata* (Proietti et al., 2014; Arantes et al., 2020a).

It is possible that the large number of studies on loggerhead (*C. caretta*), green (*C. mydas*) and hawksbill (*E. imbricata*) sea turtles are a consequence of the wide distribution of these species worldwide and of the reports of their hybridisation. There is a well known connection between *C. mydas* foraging areas in Brazil and genetic evidence indicates that turtles from different regions share feeding areas, thus enabling greater connectivity between groups (Naro-Maciel et al., 2007).

The sea turtle species share feeding and nesting areas. Evidence indicates that there is gene flow between species in different locations (Vilaça et al., 2021; Roberts et al., 2004). Genetic analyses of sea turtle populations are essential to fully understanding how gene flow occurs within the group, as well as the occurrence of subpopulations, new haplotypes and hybridisation within and across populations (Vilaça et al., 2012; Roden et al., 2013; Arantes et al., 2020b; Camacho-Sánchez et al., 2022).

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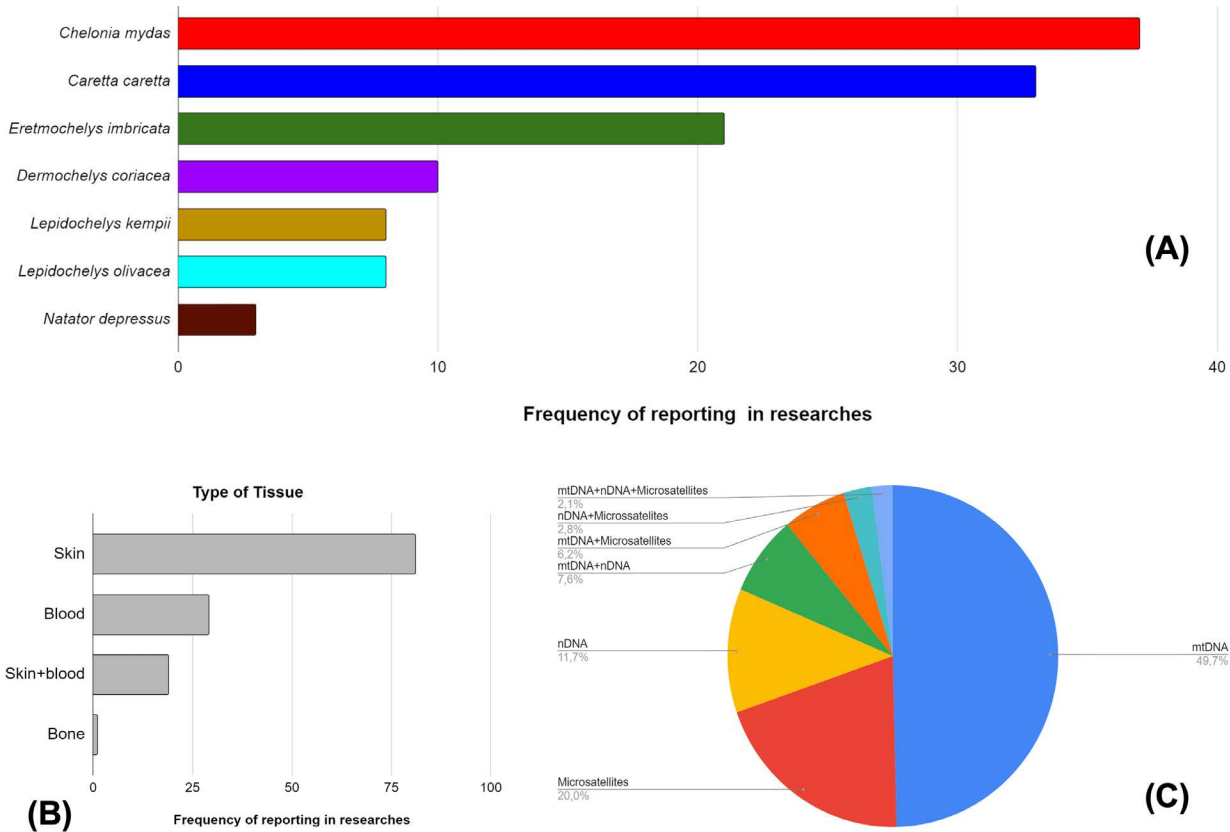


Figure 2. Data summary panel- **(A)** frequency of reporting of sea turtles species according to literature, **(B)** frequency of reporting of type of sea turtle tissue used for molecular analyses, **(C)** percentage of reporting of type of genetic material used on molecular analyses

Methods for molecular analysis

Tissues used for DNA extraction

Four different tissues were reported for the extraction of DNA. Skin was the most frequent, as observed in Alstad et al. (2011), Arantes et al. (2020a), Brito et al. (2020) and Zhang et al. (2021) (Fig. 2B). “Muscle” was not frequently mentioned, but possibly some authors categorised this type of tissue as “skin/tissue”, not making it clear exactly which type of tissue was used. Blood was also used for the analyses, although much less frequently. Blood samples were used for the characterisation of the complete mitochondrial genome, studies on hybridisation impacts and characterisation of molecular markers (Drosopoulou et al., 2012; Dutton et al., 2013; Soares et al., 2018). Blood samples were not generally used for molecular analyses. This may be due to the fact that blood collections require laborious handling of live animals, and that some analyses with blood tissue must be performed promptly after collection to avoid cell degradation. After collection, hematocrits are obtained by centrifuging the blood and are stored at 4 °C (van de Merwe et al., 2010), which is not always possible during field activities.

A study with *Lepidochelys kempii* compared the efficacy of blood samples, cheek swabs, cloacal swabs, tissue and dermal scales. The results showed a very similar quality of DNA obtained from blood and tissue samples, with both techniques indicated for genetic studies (Stephens et al., 2010). Collecting other tissue samples is generally more practical in the field than collecting blood, which requires specific training and more sampling material.

Krestoff et al. (2021) reported the use of bones for extracting DNA. The authors used bones from juvenile *L. kempii* between 1 and 2 years old and obtained high quality mtDNA sequences. This fact suggests an alternative for molecular studies when there is no possibility of obtaining another type of tissue.

Non-invasive sampling has been used in various instances as a source for sea turtle DNA. Lanci et al. (2012) reports the use of swabs with buccal and cloacal content to obtain samples for DNA extraction. Even though the authors obtained good quality DNA, they still recommend extraction from tissues such as blood and skin to obtain high quality DNA. DNA extraction and analysis of microsatellite markers from eggshell samples of non-viable *C. caretta* hatchlings was efficient in terms of quantity and quality of genetic material (Shamblin et al., 2011). Environmental DNA samples from sea turtles can also be extracted from water and sand samples in nesting areas (Farrell et al., 2022). The literature presents a good range of tissue possibilities for DNA extraction from sea turtles, all of them producing reliable results (Roden et al., 2013; Proietti et al., 2014; Bradshaw et al., 2018). However, blood samples require greater storage and collection criteria, while bone samples demand a more complex methodology for cleaning and purifying the DNA after extraction. Skin seems to be the most versatile source of DNA, since it can be obtained from animals, at all life stages, with minimal animal discomfort.

Table 1. Sizes of fragments (bp) generated by PCR amplifications with corresponding mitochondrial targets, primers and molecular analyses

Region	Species*					
	CM	CC	EI	DC	LO	LK
COI						
L-turtCOI/H-turtCOIc	815					
VF/VR						624
CONTROL REGION						
LTCM2/HDCM2	457–481			496		
LTCM1/HDCM1	486–489					
LCM15382/H950	740–818	683–859	740–880	496–832	800–890	800
LCM15382/CC443		241–817				
LCM15382/H599		380				
TCR5/TCR6	380–396	380				
CMD1 F/CMD5R	200					
LTEi9/H950	800		740			
TCR5/TCR6	360					
TCR6/L15926				486		
LTEi3/HDEi1				550		
L71/H599		510				
CMMTF1/TCR	520					
LTCM2/HDCM2	481			496		
HDCM-1/TCR5		306				
LCM15382/H950	740–800	723–830	740–850	740	830–890	
Ei9F/H95R	800					
TCR5/TCR6	360					
16S						
16SAR/16SBR	550					
CYT-B						
MT-NAD4F/MT-NAD4R						371
STR						
CM-D-1/CM-D-5	400					

*CM: *Chelonia mydas*; CC: *Caretta caretta*; EI: *Eretmochelys imbricata*; DC: *Dermochelys coreacea*; LO: *Lepidochelys olivacea*; LK: *Lepidochelys kempii*

mtDNA and nDNA gene analyses

A high percentage of articles (65%) analysed mtDNA (Table 1) as the main methodology for genetic analysis. Of these, combinations of mtDNA and nuclear DNA analyses were used in 7% of the articles, and 6% combined mitochondrial and microsatellite markers, indicating interest in research about polymorphisms found in different species of sea turtles (Fig. 2C).

Mitochondrial sequences provide information on maternal lineages with no recombination and are useful for determining the evolutionary histories of different groups (Otálora et al., 2018). They are also useful for species identification through barcode analysis (Camacho-Sánchez et al., 2021).

The main amplified mitochondrial regions and the size of the respective fragments are shown in the table above. The only region that has been analysed in all species is the Control Region, specifically the fragment amplified by LCM15382/H950, which makes this region a strong candidate for identification and genetic comparison between species. Only one study carried out

the complete sequencing of the mitochondrial genome, corresponding to *C. caretta*.

We observed a smaller variety of regions and primers for nuclear genes (Table 2), with no simultaneity of use between species for any of them. Specific data on the size of the fragment amplified with each gene in relation to different species was not provided. However, the CMOS, R35, RAG1, RAG2 and BDNF genes were used to amplify *C. mydas* (CM), *E. imbricata* (EI), *C. caretta* (CC) and *Lepidochelys olivacea* (LO) samples (Vilaça et al., 2012; Soares et al., 2017; Brito et al., 2020).

Use of highly polymorphic short tandem repeat (STR) markers for *C. mydas* revealed a genetic structure not previously found for the Mediterranean population, in addition to indicating novel migratory routes for the population (Tikochinski et al., 2018). A study of sea turtle phylogenetics based on mitochondrial and nuclear genes shows similar results for both sets of markers (Naro-Maciel et al., 2008). In contrast, a recent study indicated that *E. imbricata* individuals that had been identified both morphologically and by mtDNA analysis, showed

Table 2. Sizes of fragments (bp) generated by PCR amplifications with corresponding nuclear targets, primers and molecular analyses and the respective species used in the studies

Region	Species*						
	CM	CC	EI	DC	LO	LK	ND
MHC	162	162					
CMOS	x	601	601		x		
RAG1	x	x	368		x	624	
RAG2	x	x	620		x		
R35	x	x	439		x		
BDNF	x	x	559		x		
Other techniques							
Microsatellite	x	x	x	x	x	x	x
SNP	x						
RFLP	x						
ddRAD							x

*CM: *Chelonia mydas*; CC: *Caretta caretta*; EI: *Eretmochelys imbricata*; DC: *Dermochelys coreacea*; LO: *Lepidochelys olivacea*; LK: *Lepidochelys kempii*; ND: *Natator depressus*

evidence of hybridisation with *C. caretta* when nuclear sequences were analysed (Brito et al., 2020).

The combination of mtDNA and nDNA was also used to characterise the genetic diversity of different sea turtle populations in the Mediterranean, Turkey and Brazil (Yilmaz et al., 2011; Garofalo et al., 2013; Vilaça & Santos, 2013). This approach has similarly been used to investigate multiple paternity in *L. olivacea* in Honduras (Duran et al., 2015). Thus, in complex genetic structures, with the occurrence of hybridism, gene flow and migratory behaviours, such as those found among sea turtles, it seems highly advisable to use methodologies that encompass both mitochondrial and nuclear regions, ensuring that no relevant genetic information is missing. The combination of both genetic markers has proven important for unravelling phylogenetic relationships and provided new perspectives on relationships between species, for example, on the position of *Dermochelys coriacea* within the genetic relationships of turtles (Deef, 2023).

The use of microsatellite markers was notably prevalent in sea turtle studies. This approach consists of genotyping mainly anonymous nuclear loci characterised by the presence of STR of two, three or four nucleotides. Specific primers that allow amplification of 12 microsatellite loci in *C. mydas* from Hawaii have been described (Dutton & Frey, 2009). Microsatellite markers have also been described for *L. olivacea* (Aggarwal et al., 2008), *E. imbricata* (Lin et al., 2008; Miro-Herrans et al., 2008) and *C. caretta* (Shamblin et al., 2007).

The identification of microsatellite markers enables studies of population, kinship, reproductive and migratory behaviour of sea turtles. Inbreeding identification and cross-amplification tests were successfully performed for *C. mydas* using microsatellite markers (Dolfo et al., 2023).

The genetic structure of populations of *E. imbricata* (Mostafavi et al., 2011), *C. mydas* (Roden et al., 2013) and *D. coriacea* (Dutton et al., 2013), were also studied using microsatellite analyses. Tetranucleotide microsatellite loci have been described for *C. caretta* (Shamblin et al., 2007; 2009) and *D. coriacea* (Alstad et al., 2011). Co-dominant and selectively neutral molecular markers, such as microsatellites, are effective in studies on the level of variability between species in temporal and geographic contexts, including the philopatric behaviour of the group (Bradshaw et al., 2018).

Hybrid identification

Hybridism in sea turtles has not been widely studied yet. On the Brazilian coast, there is a high level of hybridisation between hawksbill (*E. imbricata*) and loggerhead (*C. caretta*) turtles (Lara-Ruiz et al., 2006; Proietti et al., 2014; Arantes et al., 2020a). Crossings between loggerhead and olive ridley (*L. olivacea*) also occur frequently (Soares et al., 2017). It is possible that there is significant gene exchange between *C. caretta* and *E. imbricata* in the region (Arantes et al., 2020b) which is an important migration, foraging and spawning route for five species of sea turtles. The high rates of hybridism observed to date indicate an important complexity of gene flow between species.

Molecular investigations based on mtDNA alone are insufficient to identify hybridisation, given that information on paternal DNA is lacking. The maternal genetic signature may coincide with the morphology of the animal, masking a possible hybrid occurrence. Thus, studies with a combination of mtDNA and nDNA are indicated to obtain a more complete information about the genetics of populations (Brito et al., 2020).

The behaviour of hybrid individuals may be different from their parent species, likely causing some kind of ecological imbalance in the dynamics of groups (Proietti et al., 2014). Hybrid hatchlings have been shown to be viable, with no indication of a decline in eclosion success (Soares et al., 2018). A study identified turtle hybrids in Abrolhos (Brazil), an area where this phenomenon was not previously reported, likely leading to a decrease in the reproductive success of the group (Arantes et al., 2020a).

As these are complex populations that are in constant gene flow and migration, the authors encourage further studies on sea turtle populations, requiring a complete approach with mtDNA and nDNA. Monitoring and government conservation actions are also necessary, in order to instigate investigations of the problem around the world.

Next generation sequencing (NGS) and Restriction site associated DNA sequencing (RADseq)

Phylogeny and characterisation studies of hybrid animals has been carried out using NGS, indicating that the technique brings significant advances in solving genetic problems in the group.

NGS has also made important contributions to the ecological studies of sea turtle populations. NGS was

used to address aspects related to population studies of hybrid animals and investigation into paternity of multiple species, including fitness analyses. One study did not indicate negative impacts when they compared hatchling production rate per nest and viability of hybrid hatchling and parental species, when they investigated only the first generation of hybrids (F1) (Soares et al., 2018).

Arantes et al. (2020b), analysing sea turtles population structure, identified polymorphic sites and inter and intraspecific relationships. This study indicates that there is a male-mediated gene flow that connects populations of loggerhead and hawksbill turtles on the Brazilian coast, with the occurrence of second-generation hybrids (F2). However, the authors conclude that F2 hatchling female hybrids may not survive to adulthood or be less fertile.

The ddRAD-seq technique is a powerful tool that does not require a reference genome and provides sequences from a sample of loci from across the genome. Microsatellites were identified in ddRAD sequences from *L. kempii*, which provides additional material to be used in genetics and conservation research of the group (Hurtado et al., 2016). Furthermore, ddRAD has been used in assays investigating hybridisation and introgression patterns, in which the results indicate cytonuclear incompatibility, resulting in a population decline in future generations (Vilaça et al., 2023).

Arantes et al. (2020a) used the 3RAD (triple-enzyme RADseq) protocol to investigate sea turtles from the Brazilian coast and identified the backcrossing of F1 hybrids and loggerhead turtles. This protocol works as efficiently as other RADseq analyses. However, a lower concentration of DNA is required, with lower initial costs, and can be pooled with other Illumina libraries (Bayona-Vásquez et al., 2019).

Final considerations

In the past 15 years, studies on the population genetics and phylogeography of sea turtles have provided significant advances in the knowledge of these endangered species, promoting a better understanding of their biology and providing a basis for the implementation of effective conservation measures. Although numerous articles have mostly made use of mtDNA sequences, recent studies highlight the importance of assessing nuclear as well as mitochondrial genetic information. Aside from genomic studies, highly variable co-dominant markers, such as microsatellites, seem able to provide the greatest amount of information. This is a good strategy for various purposes in population genetics, relatively practical and more affordable because it is well established.

Molecular assays to detect the genetic inheritance of two parental species of sea turtles were recently performed using RAD-seq and next generation sequencing (NGS) analysis. The results show a possible reduction in the success of hybrid offspring, caused by nuclear incompatibility (Vilaça et al., 2023). These new molecular techniques will be used to answer more detailed questions about the future of sea turtle species.

The reviewed studies report important discoveries about the genetics of sea turtle populations, especially in the Atlantic Ocean. The high complexity of the genetic composition of the Atlantic populations, the high rates of gene flow and the diversity of migratory routes, point to the need for continued studies in the area, including the combination of different techniques using mitochondrial and nuclear genes. Careful attention should be paid to the Mediterranean, which has been the subject of fewer sea turtle studies.

The occurrence of hybrids must be approached with the highest attention and urgency due to the appearance of hybrid individuals in regions where they were not found before. Efforts need to be made to minimise the impacts suffered by the sea turtles, which are quite vulnerable to the environmental changes that have occurred at a rapid rate in recent years. Countries that register sea turtles and their hybrids must have policies that value the preservation of their coasts and consider strategies to mitigate the problem of hybridism, a problem for which the causes are yet to be identified and fully understood.

Finally, we reinforce the importance of using methodologies that are less invasive for the animals. Knowledge about more effective methodologies in chelonian studies will help foster molecular biology studies aimed at obtaining results that can be directly applied to the conservation of all sea turtles.

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Author Contributions

Cibele Castro Monteiro: Conceptualisation, data curation, writing, original draft, review and editing, supervision. Jhonatas Teixeira Viana, Karen Karoline Abreu da Silva, Rafael dos Santos Rocha: Data curation, writing original draft, review and editing. Vicente Vieira Faria, Rodrigo Maggioni: Data curation, writing original draft, review and editing, supervision.

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GPS-telemetry as a method to assess nest attendance by a female broad-snouted caiman *Caiman latirostris*

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Crocodylians exhibit parental care, guarding their eggs during incubation to ensure offspring survival. We present the first description of nest attendance of a wild broad-snouted caiman using GPS-telemetry. During the reproductive season of 2022, we tracked a 1.8-metre female in the Tapacurá reservoir, a water source bordered by Atlantic Forest remnants and human settlements in north-eastern Brazil. Telemetry data revealed that 75.8% of the female’s position records were near the nest, suggesting parental care and nest protection. However, the female periodically returned to water. We observed no differences in proximity to the nest between day and night-time, which could be possibly related to different egg predators in the area foraging at different times of the day. Furthermore, the period when the nest was unattended could be associated with either feeding activities or avoidance of human presence. This research brings light on an important part of the reproductive behaviour of broad-snouted caimans in their natural environment, highlighting the importance of GPS-telemetry as a tool for monitoring reproductive behaviour of crocodylians, while contributing to understanding their biology under human-influenced settings.

Keywords: Atlantic Forest, crocodylian, movement, parental care, reproductive biology

Parental care is a common behaviour observed in animals and is characterised by parents investing effort in order to enhance offspring survival and growth, sometimes at the cost of their own survival or health (Smiseth et al., 2012). In oviparous species, adults have developed specific strategies for attending their nests and protecting the eggs. This often benefits maintenance of the whole population, for example, by allowing egg maturation and juvenile survival, contributing to a higher number of reproductive adults (Klug & Bonsall, 2010). Crocodylians are egg-laying organisms, distinguished from most reptiles by consistently exhibiting parental care (Grigg & Kirshner,

2015). They typically lay eggs in excavated holes in the ground or in mounds of vegetation, with females often guarding their nests during incubation period (Thorbjarnarson, 1996). Their main behaviours while attending the nest include repeatedly approaching the nest, positioning themselves near or above it, directly attacking intruders and, in some cases, retreating to the water for self-protection (Kushlan & Kushlan, 1980). Females may also repair their nests after disturbance by predators (Charruau & Hénaut, 2012; Platt et al., 2024).

Several factors affect nest attendance in crocodylians, in addition to their natural behaviour. For instance, species that nest in holes excavated in the ground tend to be less territorial and less prone to defend the nest when compared to mound nesters, as the former possibly build communal nests more frequently (Murray et al., 2020). Another factor influencing parental care is sunlight. During daylight hours, females may also use the area over or near the nest for basking or resting (Grigg & Kirshner, 2015). Nest attendance during night-time has also been widely documented, and generally attributed to milder temperatures, protection of eggs against nocturnal predators, or increased activity after daytime basking (Balaguera-Reina et al., 2015; Campos et al., 2016; Combrink et al., 2017). The distance of water bodies also plays a role in female nest attendance, as they may return to the water for foraging more frequently when the nest is located near a water source, or experience starvation when nests are located farther from water bodies (Villamarín et al., 2011). Additionally, human disturbance including hunting, habitat loss or nest manipulation can negatively affect female attendance (Kushlan & Mazzotti, 1989; Simoncini et al., 2016; Mazzotti et al., 2022).

The broad-snouted caiman *Caiman latirostris* (Daudin, 1801), is a widely distributed South American crocodylian species, ranging from Argentina to north-eastern Brazil. Most of its global population is distributed within Brazilian territory (Coutinho et al., 2013), threatened by urbanisation, hunting and pollution (Yves et al., 2018; Mascarenhas-Junior et al., 2021;

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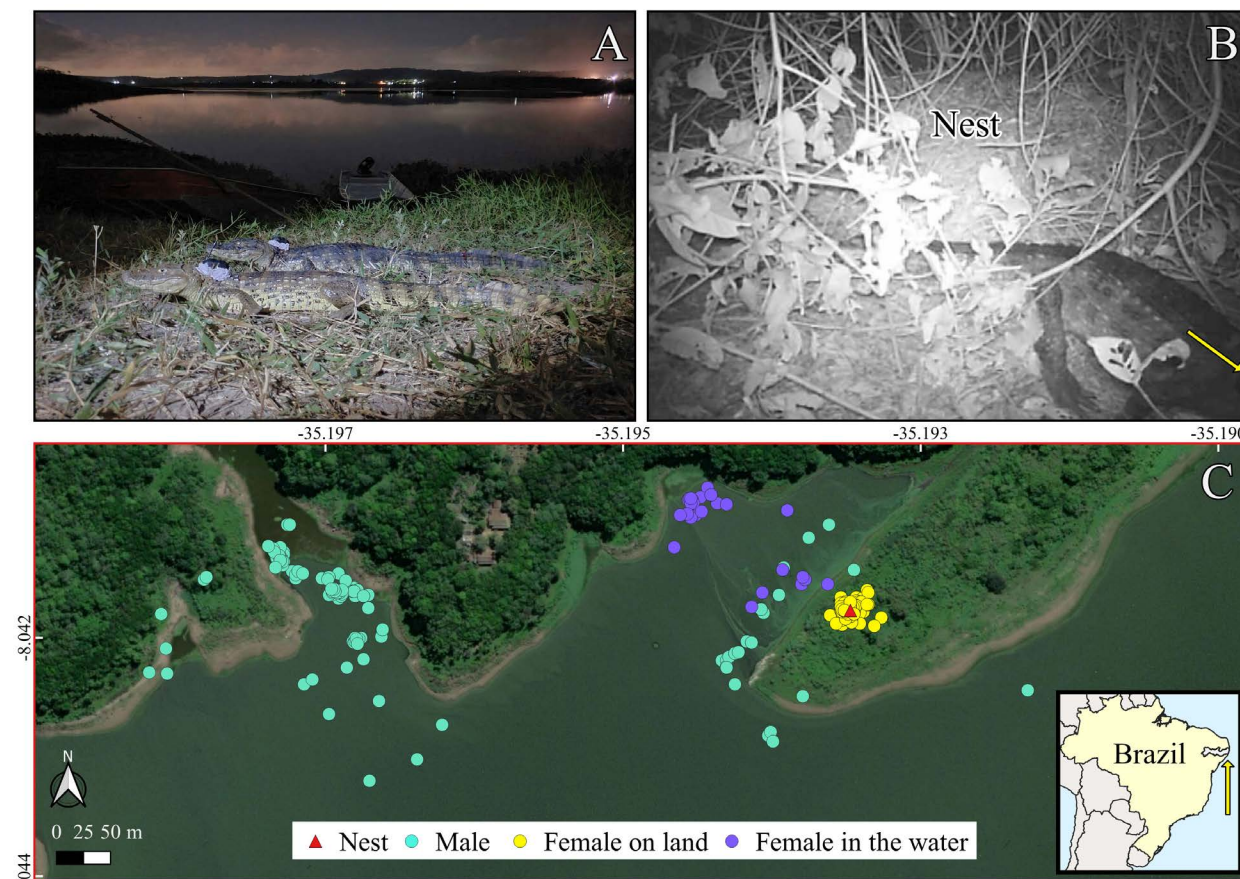


Figure 1. **A.** Deployment of GPS-telemetry transmitters on adult male (front) and female (behind) broad-snouted caimans *Caiman latirostris* in the Tapacurá Reservoir, São Lourenço da Mata City, Pernambuco, Brazil. **B.** Camera trap image with evidence of GPS-telemetry tracked female situated above a nest mound, with a yellow arrow indicating the transmitter attached to her neck. **C.** Geographic positions of GPS-telemetry tracked male (turquoise dots), female on land (yellow dots) and in the water (purple dots). Female's geographic position presented represents the period between the first access to the land (6 January 2022) until transmitter detachment (15 March 2022), and male's geographic position represents full monitoring (25 November 21 to 4 July 2022). Red triangle indicate nest geographic position.

Santos et al., 2021). Adults reach an average length of 2.5 metres (Siroski et al., 2020), and although their reproductive biology has been well studied in captivity (Verdade & Sarkis, 1998; Verdade, 2001; Bassetti et al., 2014), information on reproductive behaviour from Brazilian wild populations is relatively scarce. Studies on reproductive ecology of wild crocodylians traditionally utilise camera traps to investigate parental care, but information is limited to sensor and image capture range limit (Caravaggi et al., 2017). The employment of telemetry techniques could be an ally to improve data collection robustness (Campos, 2019; Mascarenhas-Junior et al., 2023). In this sense, we present novel information on nest attendance by a wild female broad-snouted caiman, revealed by GPS-telemetry data. This study is part of a systematic monitoring program aimed at unveiling critical aspects of broad-snouted caimans' reproductive ecology in the Atlantic Forest of Brazil (Barboza et al., 2021).

From July 2021 to July 2022, we started a telemetry-tracking program focused on adult caimans inhabiting the Tapacurá Reservoir (hereafter 'Tapacurá'), a lentic water body formed by damming of the Tapacurá River, in São Lourenço da Mata, State of Pernambuco,

Brazil (8.043856 °S, 35.195710 °W). The surrounding landscape comprises fragments of native Atlantic Forest and open fields where local communities practice agriculture and ranching. On 25 November 2021, we fitted GPS transmitters (Nortronic, Brazil) to an adult female caiman measuring 89 centimetres of snout-vent length (SVL) (24.5 kg) and an adult male caiman measuring 101 centimetres of SVL (> 40.0 kg) (Fig. 1A). Transmitters weighed less than 2% of individuals body mass, to avoid interference in their natural behaviours (Mascarenhas-Junior et al., 2023). The attachment procedure followed the protocol proposed by Brien et al. (2008). We programmed the transmitters to collect and transmit geographic co-ordinates by a flexible antenna at every six-hour interval, and both caimans were subsequently released at the same location where they were originally captured, 24 hours after attachment procedures. Preliminary tests indicated that the transmitter's error range was within a radius of five metres.

During the monitoring period, we observed a spatial and temporal overlap in the geographic position data for both individuals on 4 January 2022, the only spatial overlap between these two individuals during

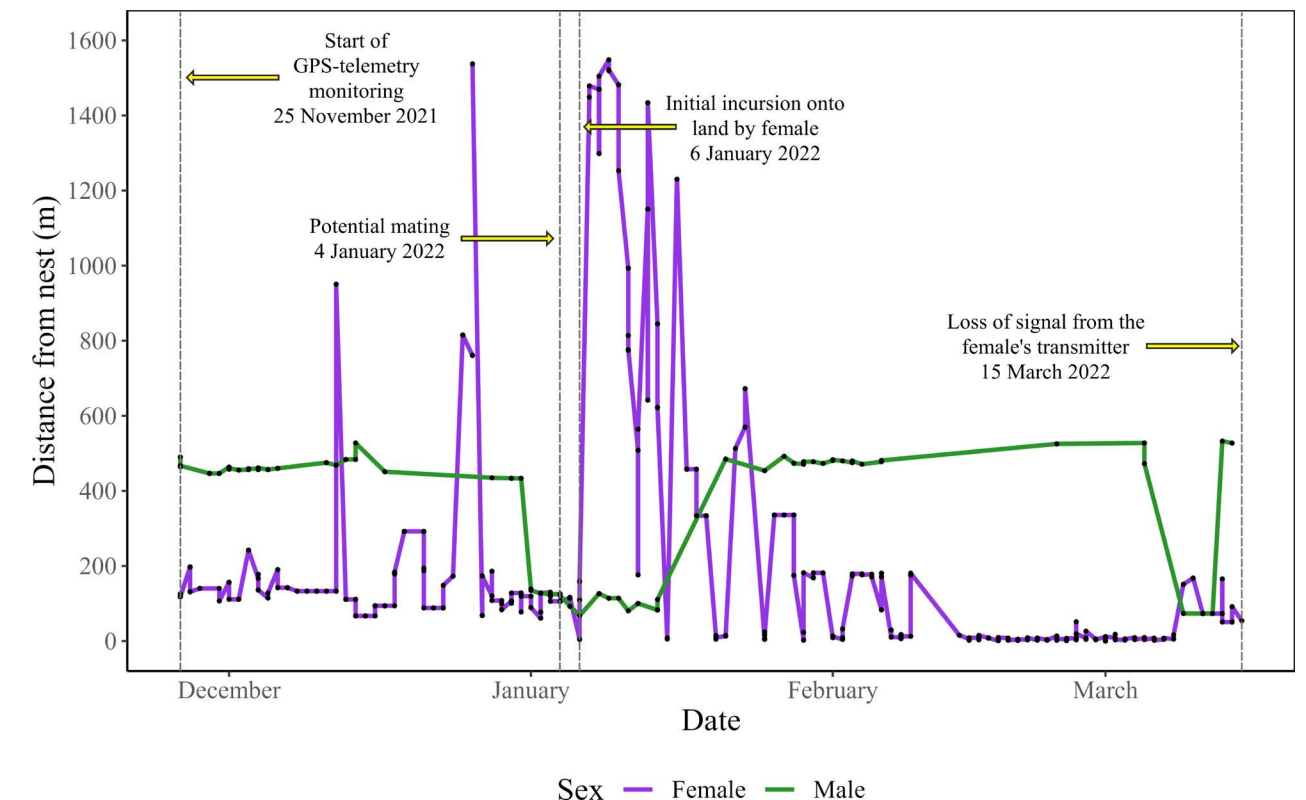


Figure 2. Daily distance from the nest of adult male (green line) and female (purple line) broad-snouted caimans *Caiman latirostris* based on GPS-telemetry monitoring between 25 November 2021 and 15 March 2022 in the Tapacurá Reservoir, São Lourenço da Mata City, Pernambuco, Brazil. Important events are highlighted by dark grey dashed lines: Potential mating between male and female (4 January 2022); Female's initial visit to the nesting site on land (January 6, 2022); Termination of female monitoring due to transmitter signal loss (15 March 2022).

monitoring, suggesting a potential mating. From 6 January 2022, GPS position data indicated that the female caiman started making incursions onto land. These were made in a small stretch of Tapacurá, in an area covered by grass and shrub vegetation. On 8 March 2022 we visited the same co-ordinates and found the female in the shrub vegetation, 4.7 m distant from a newly constructed nest. The nest was built under an emerging tree, protected from direct solar radiation, composed by a combination of branches, leaf litter and ground material, with 2.75 m of height and 56 metres distance from the water. We opened the nest and verified that the opaque band filled about 70% of egg's size, leading us to suspect that oviposition occurred about 30 days earlier (lungman et al., 2008). In the same day (8 March 2022), we installed a camera trap two metres from the nest, and directed at it. We used photos obtained from the next weeks to confirm nest attendance by the female (Fig. 1B).

We GPS-tracked the female's movements over a span of 110 days, obtaining 207 geographic positions from 25 November 2021 to 15 March 2022. Over the 42 days preceding the initiation of female nesting activities on the land, from 25 November 2021 to 5 January 2022, we obtained 81 geographic positions. Notably, we documented all these records (100%) within the water. The average $\pm$ SD distance of the female from the chosen nest construction site was 203 ± 272 metres, ranging from 61 to 1,629 metres (Fig. 2). In the remaining 68

days, from 6 January 2022 to 15 March 2022, after the female starting to access the land, the average distance of female from the nest was 36 ± 57 metres, ranging from 0 metres to 182 metres (Fig. 2). During this time, we obtained a total of 126 valid geographic position records. Among these, 75.8% were located on land, in proximity with the nest (Fig. 1C), averaging 8 ± 5 metres (Fig. 2). The remaining 24.2% records occurred in the water (Fig. 1C), averaging 128 ± 53 metres, with 182 metres as maximum distance (Fig. 2). We confirmed by a Wilcoxon test that the female remained closer to the nest upon initiating access to the land compared to the period before starting to visit the nesting site ($W = 1,547$, $p < 0.001$). We were also able to track the male for 221 days until 4 July 2022, when we lost transmitter signal. The male remained in the water during all of the monitoring period (100% of geographic positions recorded) but continued patrolling the breeding site even after mating (Fig. 1C). Prior to mating, the male remained 414 ± 124 metres distant from the constructed nest, which slightly increased to 432 ± 168 metres after mating (Wilcoxon test: $W = 3,386$, $p < 0.001$; Fig. 2).

Our data revealed that 39.3% of the land-based geographic positions occurred during daylight hours, while 60.7% occurred during night-time. Geographic positions in the water were almost evenly distributed between daytime (45.6% of records) and night-time (54.4%). There was no significant difference in distance from the nest during daytime (44 ± 65 metres) and

night-time (30 ± 51 metres) (Wilcoxon’s test: W = 3,926, p = 0.912). On five distinct days, the female alternated between water and land in different periods of the day. During the monitoring, she went onto land eight times, typically staying for a duration of 5 ± 8 days, with a maximum consecutive stretch of 23 days, between 14 February 2022 and 8 March 2022. In the water, the female spent an average of 2 ± 2 days, with a maximum of six consecutive days, observed from 8 March 2022 to 15 March 2022. The transmitter ceased operation on 15 March 2022 due to detachment from female’s neck. After detachment, the female continued nest attendance in the land, but eggs failed to hatch due to predation by black-and-white tegu *Salvator merianae*, confirmed by camera trap data. The last record of the female on the nest was on 27 March 2022, 80 days after she initiated accessing the land. Raw data obtained from telemetry is available in the Supplementary Material.

The female altered her movement patterns upon accessing the land for the first time. Prior to starting movements towards the nesting site, she maintained farther from the land, remaining exclusively in the water. Female crocodylians typically relocate to deeper waters for mating but retract to smaller ponds or shallow areas after copulation (Joanen & McNease, 1989). Our tracked female first accessed the nesting site two days after the potential mating. We suspect that copulation occurred during the period of spatial overlap between the tracked individuals, as no additional records have been documented since the beginning of study (25 November 2021). Nevertheless, it remains plausible that the female was fertilised before this encounter. The duration between copulation and the initiation of nesting activities can vary, occurring either immediately or after a few days, as observed in wild American alligators (Lance, 1989), or even preceding mating, as documented in captive Philippine crocodiles *Crocodylus mindorensis* (Schneider et al., 2014). However, the interval between copulation and oviposition may extend over a more prolonged period. Studies on wild Nile crocodiles and spectacled caimans suggest that egg laying typically occurs a month after mating (Kofron, 1990; Thorbjarnarson, 1994). We presumed our telemetry monitoring occurred during the phases of nest construction and attendance, extending until approximately halfway through the incubation period. This assumption was based on the understanding that the incubation period for broad-snouted caimans spans about two months (Simoncini et al., 2019; Barboza et al., 2021), and the egg’s embryologic stage based on opaque band suggested one month of oviposition. Hence, our tracked female caiman probably laid her eggs in either the first or second week of February.

Since nesting, and throughout monitoring, the female exhibited a marked preference for terrestrial areas near the nesting site. This behaviour is commonly observed among crocodylians, when breeders typically stay close to their nests for defence (Murray et al., 2020). Proximity to the nest may reduce predation attempts on the eggs, as egg predators refrain from

foraging when females are near (Hunt & Ogden, 1991; Eversole & Henke, 2022). In Tapacurá, several species have been identified as potential threats to caiman eggs and hatchlings, such as *S. merianae* and the South American coati *Nasua nasua*, with records of females actively repelling these animals from their nesting areas (Barboza et al., 2021). The presence of female broad-snouted caimans near their nests has been associated with lower predation rates and offspring survival (Simoncini et al., 2016), similar to observations made on black caimans *Melanosuchus niger* in the Brazilian Amazon (Torralvo et al., 2017). However, outcomes of nest attendance by female crocodylians are variable, and the presence of females did not enhance eggs survival in other crocodylian species and areas (Vergne & Mathevon, 2008; Platt et al., 2021), suggesting they may be species- or site-specific. Moreover, during nest attendance, females have limited opportunities to feed, potentially reducing their body condition and overall nutritional conditions (Barão-Nóbrega et al., 2018). Consequently, some individuals occasionally return to the water to forage (Barão-Nóbrega et al., 2016). While we were unable to recapture the tracked female to confirm the body score, we suspect that regular access to water likely contributed to the maintaining of nutritional condition during nest attendance, as observed in other adult females captured in Tapacurá (unpublished data). Alternatively, another hypothesis for females moving into the water might be associated with human disturbances, particularly in wary populations (Ron et al., 1998). Crocodylians likely learn from previous negative experiences, such as hunting attempts and fishing, activities that are common in Tapacurá (Mascarenhas-Junior et al., 2020). In such situations, females may leave their nests unattended, as widely recorded in our target population (Barboza et al., 2021; Rodrigues et al., 2021).

Nest attendance occurred both during daylight and at night. Studies on American alligators *Alligator mississippiensis*, American crocodiles *Crocodylus acutus* and smooth-fronted caimans *Paleosuchus trigonatus* suggest that females may visit their nests at night for protecting eggs against nocturnal predators (Charruau & Hénaut, 2012; Balaguera-Reina et al., 2015; Campos et al., 2016; Merchant et al., 2018). However, primary predators of caiman’s eggs in Tapacurá present diurnal foraging habits (Emmons & Helgen, 2016; Srbek-Araujo et al., 2020), indicating that daytime nest attendance could also serve as a protective measure. In contexts with multiple predators with varying daily foraging behaviours, crocodylians may adjust their diel visits to the nest (González-Desales et al., 2023). Furthermore, female caimans may also visit their nests at night because human disturbance is lower during this period (Charruau & Hénaut, 2012). The presence of females near the nest during daylight hours may also be associated with basking or resting, a behaviour observed in other crocodylian species during the nesting care, such as the Nile crocodile *Crocodylus niloticus* and the spectacled caiman (Cott, 1971; Marioni et al., 2007). Crocodylians

often return to the top of the nest during the night to increase protection and remain near the nest in nearby vegetation-covered areas for protection from intense sunlight during daytime (Combrink et al., 2016), which aligns with our findings.

GPS-telemetry revealed that the breeder female spent most of the monitoring time near the nest, especially after oviposition. We suspect the infrequent access to the water was likely for feeding or to avoid human disturbance. This is the first description of movements of a broad-snouted caiman nesting female, providing valuable insights into the species’ reproductive biology. We assumed that our tracked individual was an experienced breeder, considering that caimans typically attain sexual maturity with a SVL of 75 cm in Tapacurá (Barboza et al., 2021). Further studies considering different sizes of reproductive females should clarify the generality of the movement patterns described here and allow a better understanding of broad-snouted caiman’s reproductive ecology. The utilisation of integrated methods, such as camera trap and telemetry, must be imperative to investigate nesting behaviour in broad-snouted caimans, and effectively addresses gaps that are frequently overlooked by traditional research methods.

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Author contributions

Research conceptualisation: PBMJ. Data collection: All authors. Data analysis: PBMJ. Data interpretation: PBMJ, RSLB, PIS, JC. Writing: All authors. Formatting: PBMJ, MC, GL, CFR.

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Small-scale geographic factors promote a high diversity of amphibians and reptiles in Colombia

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Analysing spatial biodiversity patterns in amphibians and reptiles is key to understanding the main drivers of community ecological structure, biogeography, and guiding conservation strategies. Our goal is to map patterns of taxonomic change and unravel the geographic and environmental factors driving species turnover among the biomes found in the Sogamoso river basin (SRB), which tributates to the inter-Andean middle Magdalena valley in Colombia, a hyperdiverse region at high-risk from human activities. The study area encompasses lowland forest, submontane, montane and high-elevation Andean habitats, with distinct biomes defined by elevation and physiographic characteristics. To do this, we compared species richness among different biomes and describe their variation along the altitudinal gradient. We collected data from literature and revision of specimens deposited in herpetological collections. Species distribution models were used to estimate geographic ranges and species composition was analysed among biomes using a presence/absence matrix. Generalised Dissimilarity Modelling (GDM) was employed to explore spatial turnover patterns of species composition. We observed that species richness of both amphibians and reptiles was the highest in the tropical humid forest biome and the lowest occurred mainly in the Sub-Andean semiarid vegetation. The spatial pattern of species turnover was the same for amphibians and reptiles and revealed an extreme similarity with the previous delimitation of the five biomes, which are supported by a combination of biotic, abiotic and anthropogenic factors. This study provides valuable baseline information for the conservation of amphibians and reptiles in the SRB and highlights the importance of considering geographic and environmental factors in understanding species distribution and turnover in the region.

Keywords: Sogamoso River, herpetofauna, richness patterns, turnover species, ecological structure

INTRODUCTION

Globally, the decline of many amphibian and reptile populations has been associated with important factors such as land-use change (Powers & Jetz, 2019; Cordier et al., 2021), introduced invasive species (Kraus, 2015), disease (Scheele et al., 2019), and global climate change (Hof et al., 2011; Mi et al., 2023). In Colombia, habitat loss and degradation are among the principal factors (Armenteras et al., 2014; Clerici et al., 2020). It is estimated that around 70% of Andean and 30% of lowland forests in Colombia have been lost (Etter et al., 2006), especially in the Magdalena river basin (Etter & van Wyngaarden, 2000). This basin is considered one of the most important regions of the country, due to its high economic productivity and population concentration. Furthermore, biologically and biogeographically, it represents an important reservoir of biodiversity, currently defined as the Province of Magdalena that belongs to the Pacific domain, sharing a composition of taxa with nearby provinces, such as Guajira, Chocó-Darién and Cauca (Hernández-Camacho & Sanchez, 1992; Kattan et al., 2004; Morrone, 2014).

One of the main tributaries of the Magdalena River is the Sogamoso River, which drains its waters on the western slope of the Cordillera Oriental and belongs to the great Magdalena-Cauca inter-Andean hydrographic area. The region is hydrographically delimited as a hydric subsystem with features of relief and homogeneous drainage, defined as the Sogamoso River Basin (SRB) (IDEAM, 2013). Located in north-eastern Colombia (Santander department), it is an important area for the extraction of oil, palm oil crops and cattle raising (Etter & van Wyngaarden, 2000; Etter et al., 2006), and it is the area of influence of one of the most important hydroelectric power plants in the country. This industrial development involves the establishment of new roads, petroleum wells, dams, and other infrastructure related to the economic activities that are developed in the region (Salgado et al., 2022).

In these terms, it is important to design conservation strategies that could reverse this trend in wild fauna populations, to prevent, mitigate, compensate or correct the negative effects of socio-economic activities on ecosystems (Schmeller et al., 2017). Hence, the starting point is to evaluate the state of biodiversity, generating

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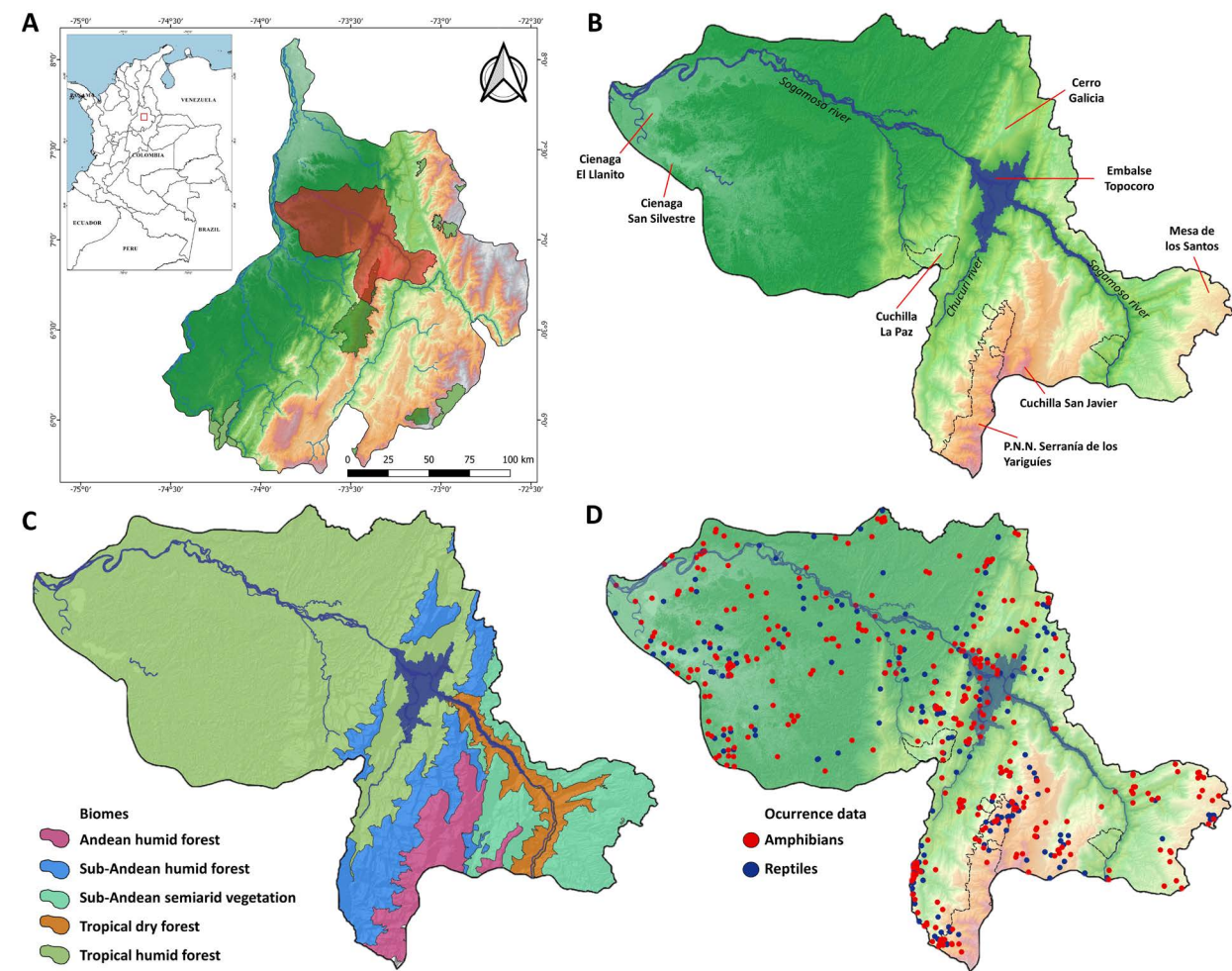


Figure 1. Graphical representation of the study area – **A.** The geographic location of the Sogamoso river basin within the department of Santander and Colombia, **B.** topography with major mountain massifs and rivers, **C.** major bioclimatic zones or biomes, and **D.** occurrences for amphibians and reptiles within SRB.

a solid baseline that allows the correct direction of conservation actions (Mace, 2004). In general, the conservation status and distribution of amphibians and reptiles in the SRB is generally unknown, and until recently, few studies have examined species composition and diversity in this river basin. Those studies correspond to technical reports and environmental management plans. However, those studies have not been carried out with a scientific approach and are not publicly available, because they are reports for the exclusive use of companies or Colombian environmental legislation.

Here, we provide the first comprehensive analysis of the species richness and diversity of amphibians and reptiles species in an important geographical area in the middle Magdalena valley of Colombia. We compare the species richness among the biomes and describe their variation in the altitudinal gradient. This study aims to identify and understand the geographic and environmental factors that influence the presence and distribution of species, and contribute to species turnover in the region. We expect that geographic distance and climate have strong explanatory power, mainly when considering the whole basin assessed for differences between species turnover, as they may affect herpetofauna independently of their

ability to disperse, niche partitioning, and interspecific competition along environmental gradients.

MATERIALS & METHODS

Study area

The Sogamoso river basin is located in the Santander department, north-eastern Colombia between 7° 18'39.60"–6° 38'16.80" N and 73° 1'12.0"–73° 55'26.4" W from middle Magdalena valley to the western slope of the Cordillera Oriental, and covers 3400 km² of lowland forest, submontane, montane and high-elevation Andean habitats between 60 m and 3200 m elevation, which represents 0.3% of the national territory (Fig. 1). The temperature ranges between 11.7 and 29 °C and the annual rainfall is between 950 and 3150 mm (CAS & CDMB, 2016). For this work, we recognise five well-contrasting biomes or terrestrial ecosystems (tropical forest, tropical dry forest, Sub-Andean humid forest, Sub-Andean semiarid vegetation and high-Andean humid forest). These biomes are defined by their elevation range, and physiographic and physiognomic characteristics (Hernández-Camacho & Sanchez, 1992; Latorre et al., 2014) (Fig. 1) as described below.

The tropical humid forest biome covers an area of 231,617 ha (67% of SRB) and ranges in elevation from 60 to 850 m. It is a lowland ecosystem that currently consists mostly of agribusiness systems (extensive livestock, African oil palm crops and rubber crops, among others) and large infrastructure projects (oil extraction, a hydroelectric power plant and heavy transport roads). Additionally, within this area, we find large areas of wetlands, forests and tropical marshes (Supplementary Material Fig. 1). The annual average temperature is 28.5 °C and rainfall is 3150 mm (Latorre et al., 2014; CAS & CDMB, 2016).

The sub-Andean semiarid vegetation biome covers an area of 38,010 ha (11% of SRB) and ranges in elevation from 1200 to 1800 m. It is a tropical dry forest formed by the rain shadow on the leeward side of the 'Serranía de los Yariguíes'. The vegetation of this biome consists of trees of low and medium height, thorny thickets, dry grasslands, and some areas transformed into tobacco, pineapple, vegetables, and goat grazing areas (Supplementary Material Fig. 2). The annual average temperature is 18.5 °C and rainfall is 1700 mm (Latorre et al., 2014; CAS & CDMB 2016).

The sub-Andean humid forest biome covers an area of 38,160 ha (11.1% of SRB) and ranges in elevation from 850 to 2000 m. It is a mountain ecosystem located between the north-western zone of the Natural National Park Serranía de los Yariguíes (P.N.N. Serranía de los Yariguíes), and the upper area of the Serranía de la Paz, forming the Chucurí river basin. The vegetation cover currently present in this biome is small-scale agroforestry systems of cocoa, coffee plantations, livestock, and relicts of conserved forests in the highlands of the Serranía de la Paz and low marginal areas of the P.N.N. 'Serranía de los Yariguíes' (Supplementary Material Fig. 3). The annual average temperature is 25.5 °C and rainfall is 2600 mm (Latorre et al., 2014; CAS & CDMB, 2016).

The tropical dry forest biome covers an area of 15,750 ha (4.6% of SRB) and ranges in elevation from 220 to 1200 m. In this ecosystem, lands with steep slopes predominate, with low-organic matter soils, high erosion rates with high leaching processes, and where thorny and cardonal scrublands are established. This vegetation is typical of the Chicamocha and Suárez river basins that together form the SRB. At present, some areas withstand pressure from grazing goats (Supplementary Material Fig. 4). The annual average temperature is 24.6 °C and rainfall is 950 mm (Latorre et al., 2014; CAS & CDMB, 2016).

The Andean humid forest biome covers an area of 19890 ha (5.8% of SRB) and ranges in elevation from 2000 to 3200 m. In this work, this biome corresponds mainly to cloud forests; however, in this area, we also include small sub-paramo and paramo zones that are above 3000 meters, which cover less than 0.1% of the SRB area. Currently, the main land use of this ecosystem is the protected area of the P.N.N. Serranía de los Yariguíes (Supplementary Material Fig. 5). The annual average temperature is 18.5 °C and rainfall is 2100 mm (Latorre et al., 2014; CAS & CDMB, 2016).

Data collection

The records presented here come mainly from literature, global databases (Gbif) and specimens collected in the study area and deposited in the herpetological collections of Universidad Industrial de Santander (MHN-UIS), Instituto de Ciencias Naturales (ICN), Museo de Herpetología Universidad de Antioquia (MHUA), and in the biological collections of the Instituto de Investigaciones de Recursos Biológicos Alexander von Humboldt (IAvH). Most of the specimens reviewed come from the collections made during the different phases of construction of the Sogamoso hydroelectric power project led by the company ISAGEN S.A. All procedures were conducted under collecting permit #4120-E1-29166, issued by ANLA (Autoridad Nacional de Licencias Ambientales) on 16 June 2014.

For the revision and identification of the specimens, the original descriptions of the species, taxonomic revisions, reference material and type series available in the collections visited were used. Amphibian nomenclature follows Frost (2024), and reptile nomenclature follows Uetz et al. (2024). We included in the list only the species having voucher specimens or photographs that allow unambiguous identification of taxa. All records were tabulated and organised in the standard template Darwin Core (version 2015-06-05), which contains the elements required for the documentation of biological records of amphibians and reptiles that were developed by Biodiversity Information Standards (TDWG, 2011). Additionally, the threat categories for each species were consulted in the IUCN Red List of Threatened Species (IUCN, 2023).

Species Richness

Because the distribution ranges of SRB's amphibians and reptiles have not been comprehensively mapped and distribution records are scattered, we used species distribution models (SDMs) to obtain an estimate of the geographic ranges. Species distribution models were limited to species that had, at minimum, three unique occurrence points at the spatial resolution (0.91 km²). The models were generated in MaxEnt v3.3.3e (Phillips et al., 2004) using the following parameters: random test percentage = 25, regularisation multiplier = 1, the maximum number of background points = 10000, replicates = 10, replicated run type = cross-validate, threshold = minimum training presence. Species distributions were modelled for the current climate using the 19 standard bioclimatic variables of WorldClim 2.1 (Fick & Hijmans, 2017). Non-climatic variables (geology, aspect, elevation, solar radiation and slope) were included. Before SDM analysis, using SDMtoolbox (Brown, 2014) we checked and removed highly correlated variables. All layers were projected to Colombian projection in ArcMap at a resolution of 0.91 km². Continuous SDMs were converted to binary models using the minimum training presence threshold to represent the potential habitat of each species based on its fundamental niche (Guisan & Zimmermann, 2000). We stacked SDM predictions separately for reptiles,

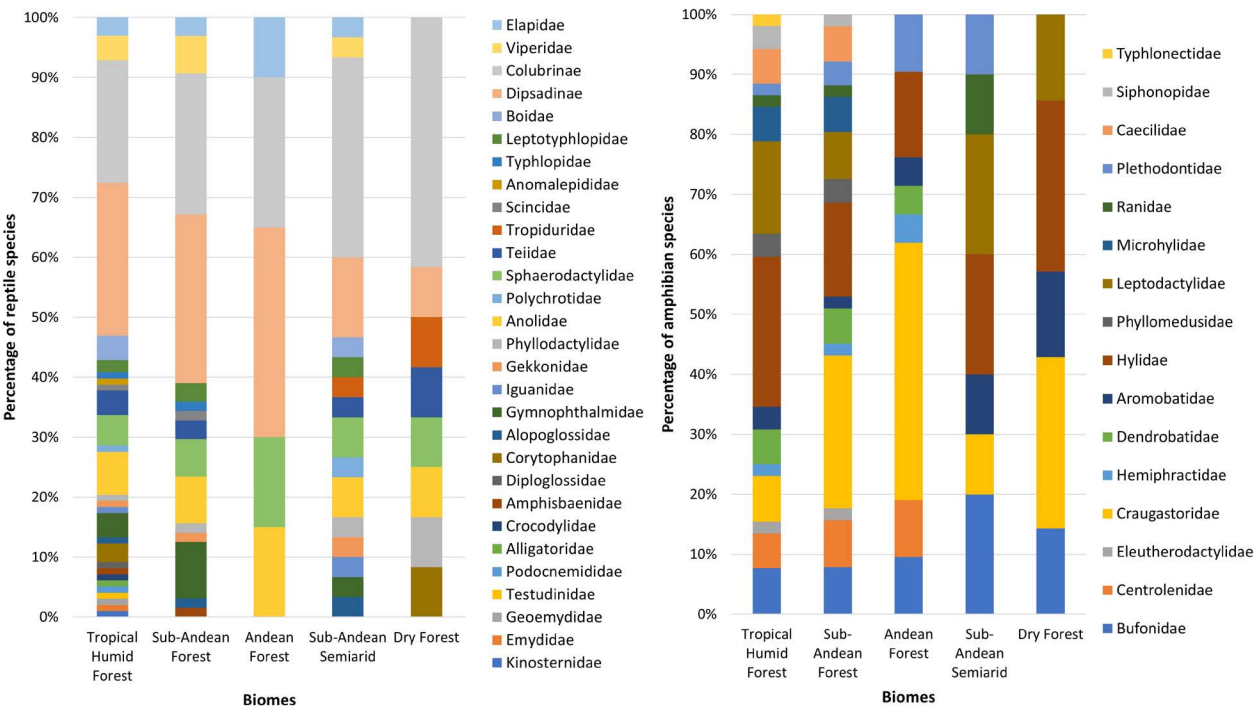


Figure 2. Bar graphics showing the reptile (left) and amphibian (right) families represented in each of the five major biomes of Sogamoso river basin. The percentage shows the representation of the species of each family within each biome with respect to the total number of species of the same biome.

amphibians, and both together using SDMtoolbox v1 (Brown, 2014) to estimate a threshold-based richness map. This methodology is useful as a baseline for analysing the abiotic deterministic processes that may shape and affect community assembly (Pouteau et al., 2019), and to define a reference pool against which null models or hierarchical analyses can be performed (Carstensen et al., 2013) for hypothesis testing modelling of biodiversity dynamics (Grenié et al., 2020).

Species richness among biomes and generalised dissimilarity modelling

Because the species richness of biological communities changes as the environment changes, we used species as an indicator of the state of the environment and evaluated the variation of species richness across biomes with distinctive physiognomic and physiographic characteristics. To do this, we first extracted the species richness of each grid from the threshold-based richness map to obtain a presence/absence matrix of species for each grid cell. We assigned each matrix grid cell to one of the biome categories. Then, with the species list for each grid cell and biome, was defined each biome's characteristic species as those that have the maximum proportion of their range in that biome. This approach is useful to assess a directional taxonomic overlap among biomes (see Echeverría-Londoño et al., 2018 for further details and use of this methodology).

Additionally, we calculate the Sørensen similarity index (Magurran, 2004) to estimate the similarity in species composition among biomes and extracted the maximum, minimum and mean elevations predicted for each species from the adjusted SDMs for calculating the

number of species estimated by the models to occur at intervals of 200 m above sea level. This analysis was performed in R v 4.2.3 (R Core Team, 2023). On the other hand, we explore the spatial turnover patterns of species composition using Generalised Dissimilarity Modelling (GDM, Ferrier et al., 2007), this analysis is a nonlinear matrix regression technique between pairs of locations as a function of environmental dissimilarity and geographical distance. GDM accommodates (i) variation in the rate of compositional turnover at different positions along a given gradient, and (ii) the curvilinear relationship between compositional dissimilarity and increasing environmental/geographical distance between sites (Ferrier et al., 2007). To fit GDMs, we used the site-by-species matrix obtained from previous analysis and the same environmental predictors used for the species distribution models (SDMs). This analysis was performed in R v 3.2.2 (R Core Team, 2023) with the package “gdm” (Manion et al., 2017).

RESULTS

General results

Currently, there are 2953 amphibian and reptile specimens registered that were collected between the 1940s and 2020 in the SRB and deposited in the herpetological collections visited during this work. With this review, we generated a list of 197 species, 76 of which are amphibians (16 families, 43 genera) and 121 reptiles (28 families, 83 genera; Supplementary Material Table 1, Fig. 2). Of these species, 49 are endemic to Colombia (35 amphibians, 14 reptiles), and 19 endemics to the Santander department (17 amphibians, 2 reptiles), indicating high levels of

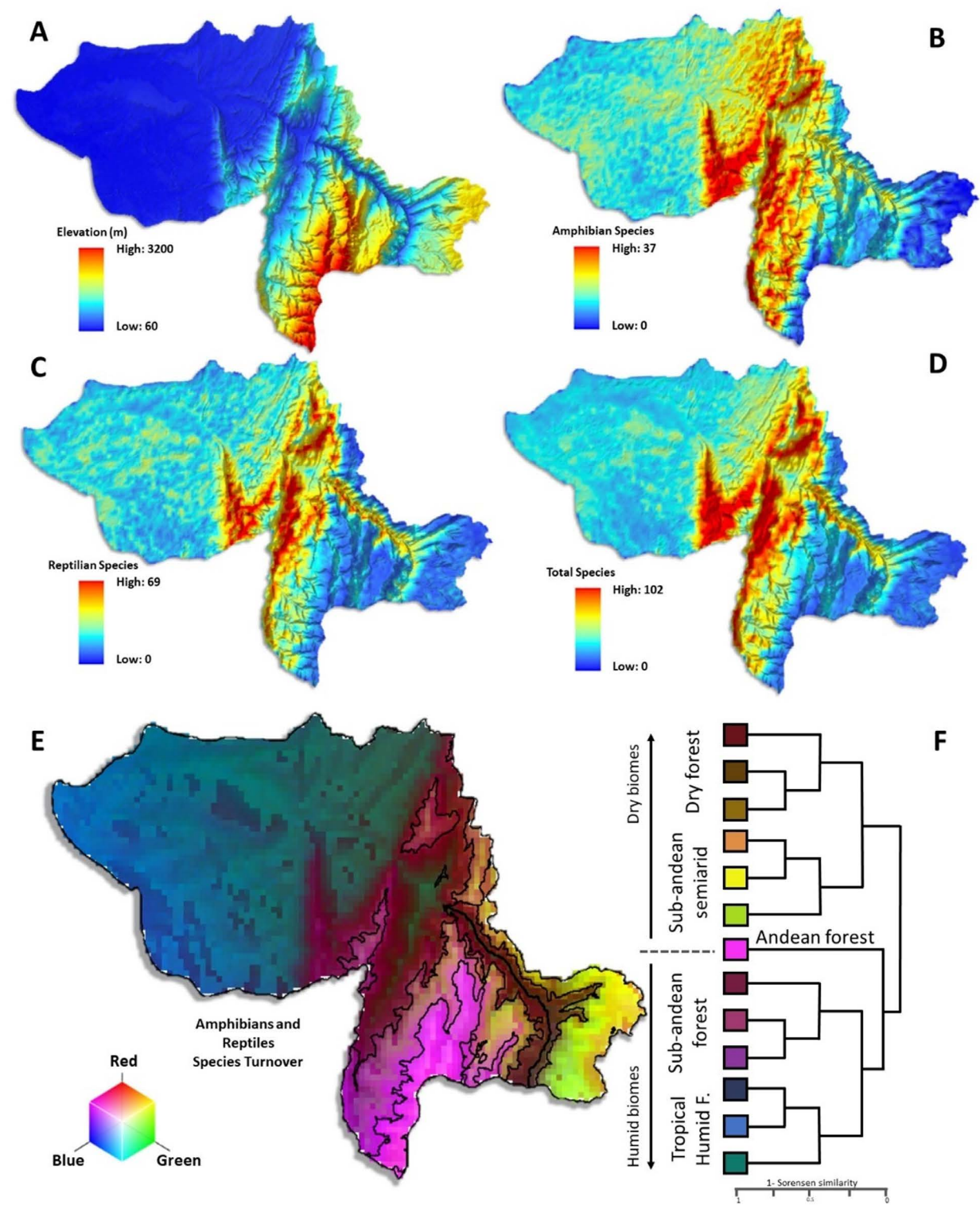


Figure 3. Biodiversity measures for reptiles and amphibians in the SRB – **A.** Elevation gradient, **B.** species richness of amphibians, **C.** species richness of reptiles, **D.** species richness of both, and **E.** visual representation of the turnover as measured by generality dissimilarity models (GDM), based on the distribution of 194 amphibian and reptile species from SRB. Similarity in colour represents similarity in species composition. The dendrogram depicts the relationships of each of the five biomes, where sister groups comprise communities of the highest similarity (1- Sørensen similarity) in species composition among biomes.

proportional endemism. Additionally, *Niceforonia aderca* (Lynch, 2003a), *Pristimantis ixalus* (Lynch, 2003b), *Bolitoglossa yariguiensis* Meza-Joya et al., 2017, and *Gonatodes chucuri* Meneses-Pelayo and Ramírez, 2020, are restricted to small areas in the SRB. We also included species that we have identified as putatively

new in molecular and morphological analyses, which are currently being described. This species richness represents 13% of the Colombian herpetofauna. The families with the highest proportions of richness are Hylidae (21.9%, 16 species), Craugastoridae (17.8%, 13 species), and Leptodactylidae (10.9%, 8 species)

for amphibians. For reptiles, richness is the highest in Colubridae (46.2%, 56 species), followed by Anolidae (8.2%, 10 species), Gymnophthalmidae (6.6%, 8 species), and Sphaerodactylidae (4.9%, 6 species). However, this representativeness of the families changes throughout different biomes of the SRB. In amphibians, 100% of the families (16) were registered in the tropical humid forest showing a gradual decrease across the biomes until the dry forest where only 5 families occur (31%). For reptiles, the humid tropical forest again supports 100% of the families, followed by the semiarid Sub-Andean biome with 16 families (57%) and the Sub-Andean forest with 15 (53.5%), in the dry forest biome 8 families (28.5%), and 5 families (17,8%) in the Andean forest (Fig. 2).

Species richness and overlap between biomes

The mapping of the modelled distribution of 194 amphibians and reptiles species (with 3 or more occurrences; Supplementary Material Table 2) showed that the tropical humid forest near the foothills of the mountains and the Sub-Andean forest biome in the middle basin of the Sogamoso River holds the highest concentration (101.6 species per cell) of species richness of both amphibians and reptiles, and the lowest species richness occurring mainly in the sub-Andean semiarid vegetation with only 6 species per cell, the mean species richness per cell was 43.5 (±18.7 SD) in the SRB (Fig. 3D). In amphibians, the highest richness is found in the upper zones of the western flank of the Serranía de los Yariguíes with 37 species per cell and the lowest richness (3 species per cell) occurring in the dry forest and sub-Andean semiarid biomes, with the mean richness in 15.3 (±6.5 SD) (Fig. 3B). For reptiles, the highest richness occurred in the dry forest with 69 species per cell, and the lowest richness (5 species per cell) in sub-Andean semiarid biomes, with the mean richness in 28.2 (±12.8 SD) (Fig. 3C).

We found an important overlap in the total species composition across biomes, based on the intersection of modelled species distribution maps (Fig. 4). This taxonomic overlap is greater within all the biomes. Despite the high proportion of species characteristic of tropical humid forest (91%), this biome also shares large numbers of species with the sub-Andean forest biome. The high taxonomic overlap of arid biomes such as dry forest and sub-Andean semiarid suggests the potential for low functional distinctiveness (Fig. 4), with the dry biomes representing a poorer subset of the more species-rich, functionally diverse humid biomes (Fig. 3F).

Altitudinal distribution

We sampled the altitudinal species richness (SR) of amphibians and reptiles as the number of species predicted to occur in altitudinal sections of 200 m according to their modelled distributions (Fig. 5). With this, we analysed three important aspects of these data. First, we compared the variation in SR along the altitudinal gradient and found that the SR of amphibians increases slightly with increasing elevation, reaching a maximum between 400–800 m a.s.l., with the highest value at 600 m a.s.l. Above 600 m a.s.l., SR is negatively correlated

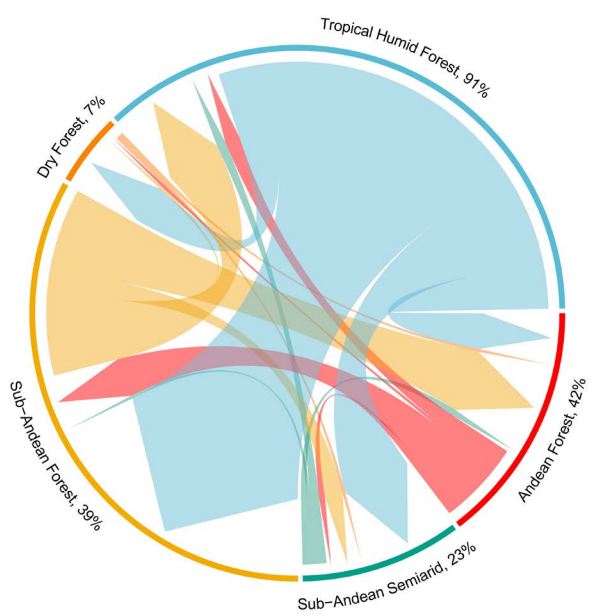


Figure 4. Overlap of species among biomes from Sogamoso river basin. Percentage values express the fraction of species occurring in a biome that has the greatest proportion of their geographic range in that biome, N = 196. The base of each branched arrow is positioned to show the biome that includes the greatest proportion of a species range, while the width represents the number of species shared with the biomes at the tips of the arrow.

with elevation, with a gradual reduction between each altitude interval. The altitudinal SR of reptiles shows the same trend in the lowland intervals, with the maximum SR value at 600 m a.s.l., and a moderately continuous decrease of SR with increasing elevation. In this case, the SR decreased dramatically above 1600 m, with amphibian SR being higher in the highland intervals above 2000 m a.s.l., relative to reptiles. The differences seen between amphibians and reptiles are statistically significant, with both minimum and maximum elevation per species, and altitudinal range, being on average higher in reptiles (t-test: P = 0.002, P < 0.001, P < 0.001).

Later, we graphically represent the distribution ranges of each species with vertical bars coloured according to their degree of threat or endemism. Also, these bars were placed on the biomes in which each species is distributed. We found that in amphibians the presence of endemic and threatened species increases with altitude but only in humid biomes; in dry biomes, we found only common species with wide altitude distribution ranges (Fig. 5A). In reptiles, the trend is similar, but the altitude distribution ranges of the species are wider than in amphibians (Fig. 5B). Finally, the altitudinal gradient showed a high turnover in species composition in mid-altitude intervals between 1400 to 1600 m a.s.l., both for amphibians and reptiles. These species clusters, along the altitudinal gradient, largely correspond to the different biomes present in the SRB.

Generalised Dissimilarity modelling

We applied Generalised Dissimilarity Modelling (GDM) to identify important areas for conservation

in the SRB, based on turnover patterns for reptiles and amphibians together. This method identifies changes in the communities that reflect high species turnover and can be interpreted as boundaries of biogeographic regions or biomes. The GDM model based on the data from amphibians and reptiles together captured 85.2% of deviance explained, and separately for each group 83.3% and 85.1%, respectively (Fig. 3E). In all cases, the distribution pattern of species turnover was generally the same. However, the level of importance of the predictors was moderately different between amphibians and reptiles. The top climatic predictors of species turnover (and percent of contribution to the model) for amphibians were, the maximum temperature of the warmest month (73.4%), elevation (72%), radiation of the warmest quarter (25.1%), annual mean radiation (23.4%), and precipitation of the coldest quarter (20.2%). For reptiles, the top predictors were the highest period moisture index (65.7%), the maximum temperature of the warmest month (41.2%), the mean moisture index of the warmest quarter (41.2%), the mean temperature of the wettest quarter (38.5%), and moisture index seasonality (35%). Given that the explained deviance is like other GDM models that are known for their robustness, but it is not close to 100%, indicating that the model does not capture all the variation present, the non-climatic species-specific idiosyncrasies were retained in input data, and thus, supported the use of GDM methods. The spatial pattern of species turnover reconstructed herein results largely mirrors the biomes previously delineated by Latorre et al. (2014), and the similarity relationships found with Sorensen's analysis show a strong separation between dry and humid biomes consistent with the turnover distribution pattern of species in the altitudinal gradient (Fig. 3F).

Conservation status

According to their IUCN status, in the SRB 129 species (64.7%) are of Least Concern (LC), 42 (21.4%) are listed as Not Evaluated (NE), 8 (4%) as Data Deficient (DD), 3 (1.5%) as Near Threatened, 8 (4%) as Vulnerable (VU), 4 (2%) as Endangered (EN) and 2 (1%) as Critically Endangered (CR). It was possible to show that species with significant degrees of threat or endemism tend to be more associated with highland biomes. In amphibians, 33 endemic species were found, of which 11 have a threat category between vulnerable, threatened and critically endangered. For reptiles, 20 endemic species were reported, and only two of them were within a threat category. This scenario may not represent the current reality in the region because for most species there are no population studies that provide evidence of the current status of their potential threats at a local scale.

DISCUSSION

The study of the spatial biodiversity patterns of Colombian amphibians and reptiles has been rarely addressed (Ortiz-Yusty et al., 2013, Herrea-Lopera et al., 2023; Saboyá & Urbina-Cardona, 2023). An important factor

to focus effective conservation strategies on territories with high rates of deforestation is the collection and analysis of basic information on the variation of richness and diversity of species from a spatial perspective, based on explicitly modelled distributions from a complete dataset of species as possible (Brown et al., 2016). In this work, we illustrate how herpetofauna communities are structured according to the main bioclimatic regions or biomes in the Sogamoso river basin and demonstrate an important effect of some geographic and environmental predictors in the variation of these patterns.

The first and most important result of this study is the compilation, depuration, and review of all records and specimens that have been collected within the SRB and deposited in the main scientific collections of the country and registered in global databases (Gbif). At this point, we list a total of 197 species between amphibians and reptiles, this being the largest currently reported herpetofauna for the Santander department (80% of the total, Meneses-Pelayo, 2024) and one of the most representative for the middle Magdalena valley and north-western Colombia. However, we are aware of several difficulties in this work. Such as the effects of taxonomic uncertainty, with some species included in this study belonging to species complexes that contain deep mitochondrial and nuclear lineages that might turn out to correspond to distinct species upon taxonomic revision. Some of these are currently in the process of description and are referenced in Supplementary Material Table 1 at the genus level. But, despite these, we are convinced that the results presented here reflect true biological patterns.

The spatial distribution of SR in amphibians and reptiles suggests a clear concentration of species around the Serranía de la Paz, foothills of the Serranía de los Yariquíes, between 400 and 800 m a.s.l (Fig. 3B, C & D). This accumulation of species richness (up to 102 species per cell of 0.91 km²) is found within the tropical humid forest biome and its transition to the sub-Andean humid forest. This altitudinal pattern could be partly explained by the species-area relationship since the tropical humid forest of the SRB has 56% more available area than the sub-Andean humid forest (Fig. 5A & B). Species-area relationships have been extensively studied in metapopulation dynamics and have demonstrated the importance of diversity analyses at a spatial scale (Rahbek, 1997; Lawton, 1999; Lomolino, 2000; McCain, 2007). Additionally, there is a strong contrast to dry biomes in which species richness is very low and is mostly represented by common and widely distributed species with some exceptions of endemic species found in tropical dry forests and the sub-Andean semiarid vegetation, such as *Bolitoglossa nicefori*, *Micrurus sangilensis*, *Stenocercus santander* and *Alopoglossus bicolor* (Fig. 3B & C). Therefore, the high degree of importance of the foothills of the tropical humid forest is evident, as it offers a bank of species with wide altitude distribution ranges that facilitate a large taxonomic overlap between the biomes (Figs. 3 & 5). This taxonomic overlap is complemented by the contribution of Andean and sub-Andean forests, which do not have a high species richness but are home

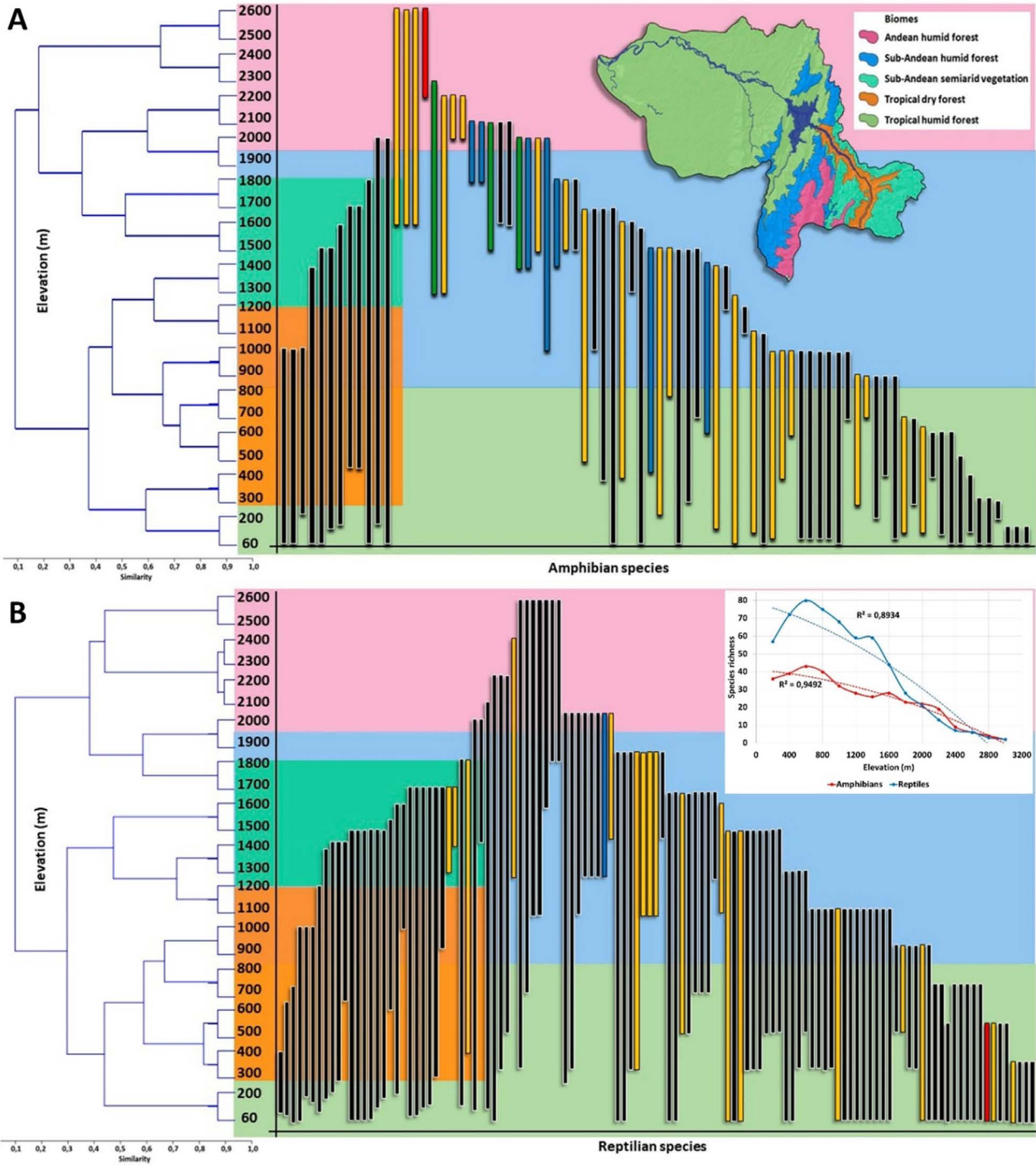


Figure 5. Distribution profiles of each species – **A.** amphibians and **B.** reptiles, based on the original records ordered according to their presence in each biome. The dendrogram represents the relationships of each of the five biomes, where the sister groups comprise communities of greater similarity (Jaccard similarity) in the composition of species between biomes. The colours of the bars represent endemic species (yellow bars), endemic and vulnerable species (green bars), endemic and threatened species (blue bars), and critically endangered species (red bars). The colours of the background stripes represent the spatial distribution of the biomes and coincide with their respective colour on the SRB map.

to 49% of endemic and threatened species, in only 17% of the total area of the SRB (Fig. 5A & B).

Moreover, whereas the patterns of species richness can give us a basis for directing conservation strategies to the areas, spatial analyses of species turnover, that integrate geographic and environmental information, could anticipate and mitigate the impacts of biodiversity threats. Since we can expect that areas with high

turnover (where the colours on the map contrast) and climatic stability, retain biodiversity in a scenario of climate warming or shifts in rainfall regimes (Sandel et al., 2011; Garcia-Rodríguez et al., 2021). In the SRB we found that areas of high species turnover identified by the GMD dissimilarity model agree remarkably well with the previously delimited biomes of Latorre et al. (2014) used in this study. However, we identified

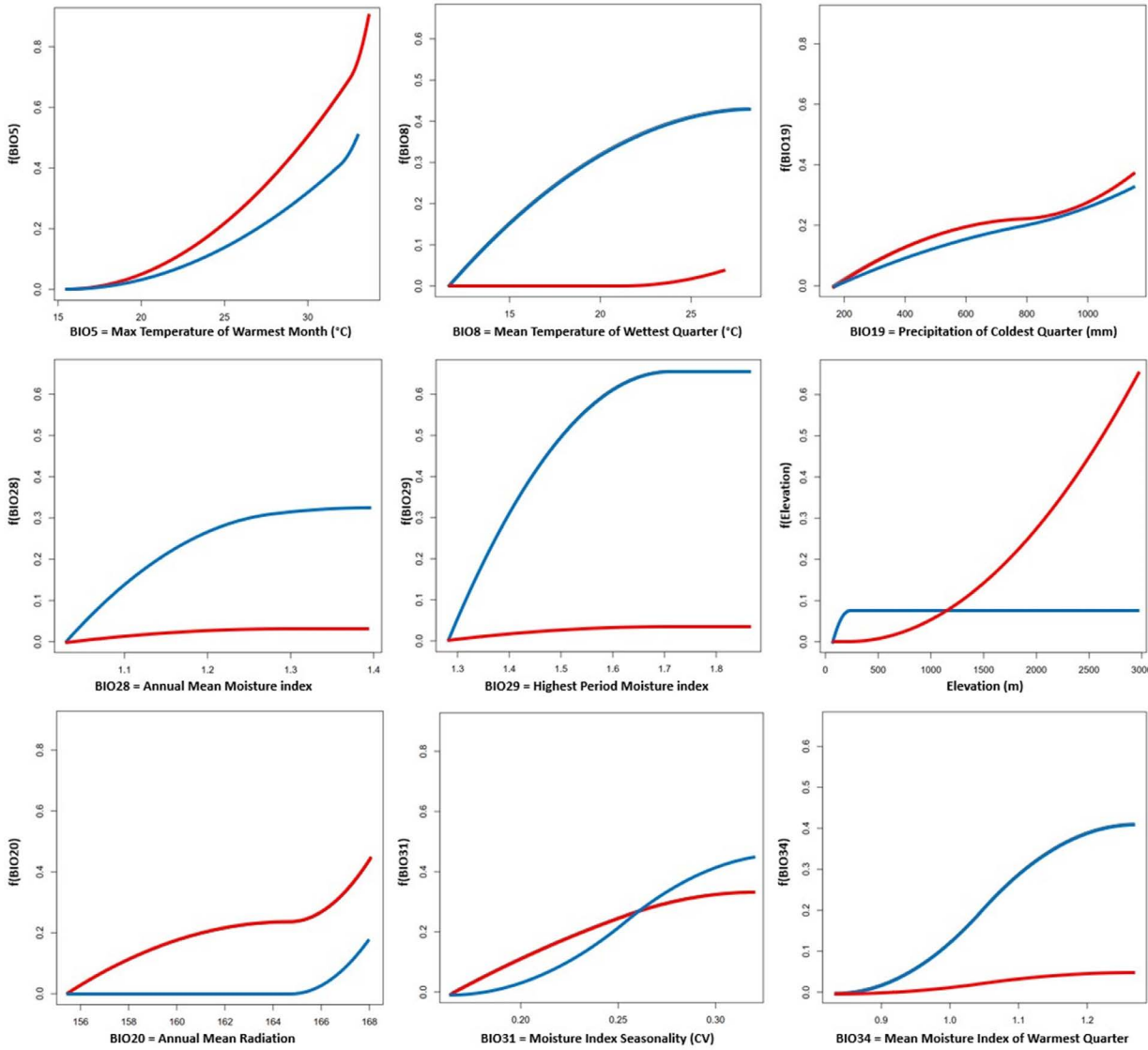


Figure 6. I-splines derived from a generalised dissimilarity model (GDM) for amphibians (red line) and reptiles (blue line). The maximum height of each spline indicates the total degree of community turnover predicted by that variable while holding the others constant. The shape of the spline corresponds to how the rate of turnover changes over the ecological gradient.

differences between amphibians and reptiles regarding the importance of the environment concerning species composition, which could show that small-scale habitat preferences and ecophysiological requirements can result in detectable patterns in the geographical distribution of different lineages. For example, in amphibians, abrupt changes in geography related to the elevation gradient facilitate the availability of many types of microhabitats. Each one of them, with differences in temperature and precipitation (Fig. 6), enhances the effects on the metabolism and demands additional physiological and behavioural adaptations (Wells, 2007; Hillman et al., 2009; Cunningham et al., 2016), potentially supporting a hypothesis of niche-filtering process (Carnicer et al., 2008). In contrast, in reptiles, the greater compositional turnover was explained by the temperature and moisture indexes (Fig. 6). These predictors have been widely accepted as important determinants of the distribution of reptile species (Huey, 1982), in which these variables

affect the hydoregulation and thermoregulatory behaviour (Rozen-Rechels et al., 2019). For example, competition for thermal and hydric resources can shape interactions between syntopic lizard species (Žagar et al., 2015) or be indirectly affected by dramatic thermal fluctuations that influence the adaptive capacity to cushion these fluctuations (Diele-Viegas & Rocha, 2018). These effects need to be monitored to plan effective long-term conservation strategies in the SRB, considering trends observed elsewhere in the world (Gibbons et al., 2000; Bickford et al., 2010; Dayananda et al., 2021). Patterns of amphibians and reptiles diversity in the SRB cannot be attributed to a single combination of explanation hypotheses, but rather to multiple biotic, abiotic and anthropogenic factors (Dainese & Poldini, 2012). The relation between species richness, turnover, and distribution of the threatened species between different biomes suggests that conservation efforts should focus on the whole gradient rather than just portions of

it. Currently, a large part of the sub-Andean humid forest and the entire Andean forest biome is protected within the Serranía de los Yariguíes National Park and civil society reserves. These areas are of vital importance for conservation because they are home to 49% of endemic and threatened species (Supplementary Material Table 1; Fig. 5A & B). However, in the tropical humid forest, where the highest values of species richness are concentrated, there are not enough areas designated for conservation, and where the greatest anthropogenic pressure is due to changes in the use of land destined for agriculture, livestock, and large infrastructure projects that put at risk the few relicts of forests that still exist. Consequently, it becomes imperative to implement more conservation actions, with a specific focus on creating and maintaining nature reserves in this area of the Sogamoso river basin and the broader middle Magdalena valley.

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Author contributions

Conceptualisation: EM-P, VHS-C, MPR-P.; Methodology: EM-P; Formal analysis: EM-P, Resources: VHS-C, M.P.R.-P.; Data curation: EM-P; Writing - original draft: EM-P; Writing - review & editing: EM-P, VHS-C, M.P.R.-P.; Supervision: VHS-C, M.P.R.-P.

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Scorpion mud turtle breeding in the Amazon: zootechnical and environmental activity for the species conservation

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Commercial captive turtle breeding is an opportunity for *Kinosternon scorpioides scorpioides* conservation, as predatory hunting, destruction and habitat degradation are the main threats to its natural populations. In this sense, the goal of this study is to provide a synthesis of knowledge about the species Embrapa-developed captive breeding system. Below, we have set up the themes in a way that makes sense in the captivity flow, discussing important information about the species in the environmental context and its management from the initial breeding stock formation up to reproductive aspects, highlighting animal behaviour and the environmental approach of the activity, to support commercial breeding from the species conservation perspective. The discussion and conclusions have shown that the species has a low invasive potential in Brazilian biomes; the captivity site needs not much space, facilities or water; captive breeding is a zootechnical and environmentally appropriate activity for the species' conservation because it is a way to avoid spending on programs that are made only for this purpose; and increasing knowledge of reproduction using molecular genetic tools is important.

Keywords: conservation, chelonian, *Kinosternon scorpioides*, jurará, turtle breeding

INTRODUCTION

In Brazil, *ex-situ* captive breeding of wildlife has been encouraged by public authorities to generate income and conserve species, within categories that include commercial, scientific, conservation and zoological purposes (Le Pendu et al., 2011). The Chelonians are the third-most commercial group in the 438 commercial captive breeding present in the country (Trajano & Carneiro, 2019). Although this commercial activity is centred on the species *Trachemys dorbigni* and *Podocnemis expansa* (Trajano & Carneiro, 2019; Dantas-Filho et al., 2020; Andrade et al., 2021), the semiaquatic turtle *Kinosternon s. scorpioides* (also known as scorpion mud turtle in English and jurará or muçua in Portuguese) has been standing out with progressive advances in the knowledge of its biology and parts of captivity management (Araújo et al., 2013a; 2013b; Costa et al., 2015; 2017; Ferreira et al., 2020a; 2020b; Andrade et al., 2021; Silva et al., 2021). This knowledge can promote the activity for commercial purposes, providing a great opportunity for species conservation in the Amazon (Le Pendu et al., 2011; Parry et al., 2014; Dantas-Filho et al., 2020) due to the threat of predatory hunting motivated by its meat (Alves et al., 2012; Cristo et al., 2017). There are many studies and most of them do not consist of comprehensive knowledge and experience

guides that holistically support new projects (Rocha & Molina, 1987; Rodrigues et al., 2017), with little emphasis on the environmental issues of the activity (Ferreira et al., 2020b) despite its importance (Boyd et al., 2020). The species has not yet been bred in captivity for commercial purposes and, therefore, research has only been conducted in zoos and scientific captive breeding for research purposes (such as Embrapa Eastern Amazon, which has been studying and conserving these species in its biological collection since 2004). However, animals cannot be commercialised within these two categories, thus favouring the illegal trade of *K. scorpioides* (Cristo et al., 2017), not contributing to the food safety of native Amazonian people. Currently, the species has two possibilities to be commercialised as an income resource, that is, for meat consumption (Smith, 1979; Baía Júnior et al., 2010; Marques, 2016; Cristo et al., 2017) or as a pet (Avendaño et al., 2002; Shiau et al., 2006; Berry & Iverson, 2011; Bedoya et al., 2018). Given the interest in understanding the species biology, this study aimed to provide a synthesis of knowledge about the Embrapa-developed *K. scorpioides* captive breeding system. To achieve this, we have organised the themes in a manner that enhances the workflow in captivity by discussing important information about the species, including its environmental requirements and

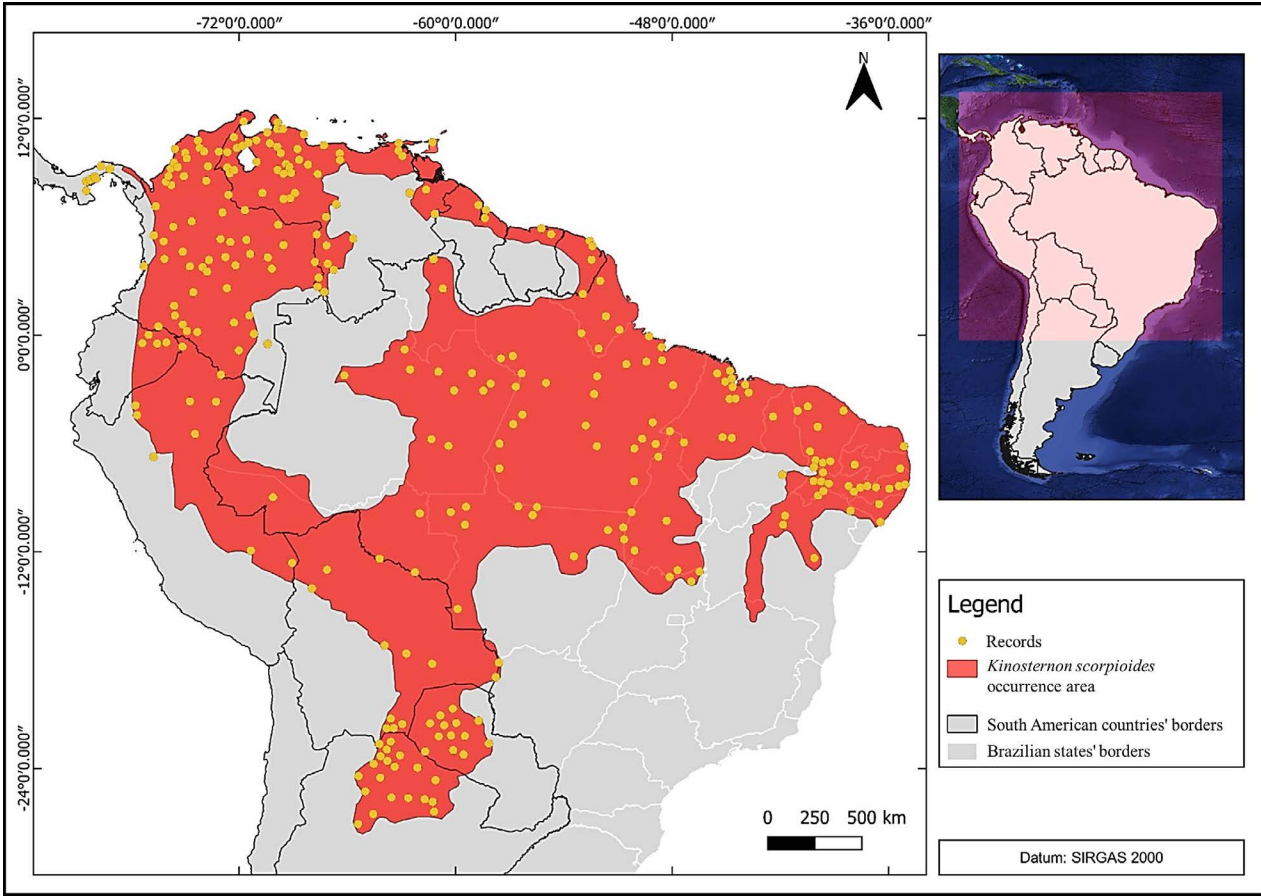


Figure 1. *Kinosternon s. scorpioides* geographical distribution: Argentina (Chaco, Formosa, Jujuy, Salta, Tucumán), Bolivia, Brazil (Acre, Alagoas, Amapá, Ama-zonas, Bahia, Ceará, Goiás, Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Pará, Paraíba, Pernambuco, Piauí, Rio Grande do Norte, Rondônia, Sergipe, Tocantins), Colombia (Amazonas, Antioquia, Arauca, Atlántico, Bolívar, Boyacá, Caldas, Caquetá, Casanare, Cauca, Cesar, Chocó, Córdoba, Cundinamarca, Guainía, Guaviare, Huila, La Guajira, Magdalena, Meta, Nariño, Norte de Santander, Putumayo, Quindío, Risaralda, San Andrés, Providencia y Santa Catalina, Santander, Sucre, Tolima, Valle del Cauca, Vaupés and Vichada), Ecuador, French Guiana, Guyana, Panama, Paraguay, Peru (Amazonas, Huánuco, Loreto, Madre de Dios, Ucayali), Suriname, Trinidad, Venezuela (Amazonas, Apure, Aragua, Bolívar, Cojedes, Falcón, Guárico, Lara, Monagas, Portuguesa, Sucre, Táchira, Trujillo, Yaracuy, Zulia). According to Berry & Iverson (2011), Dijk et al. (2014); Andrade (2019); Rhodin et al. (2021); Cassano & Alcade (2022) and Páez et al. (2022). Adapted from Rhodin et al. (2021).

management, from the formation of the initial breeding stock to reproductive aspects. Additionally, we highlighted their behaviour to support commercial captive breeding for species conservation.

Scientific breeding categories and authorisation aspects
The Embrapa scientific breeding for research purposes complies with one of the *ex-situ* categories of species captive breeding and use of allowed wildlife, according to Brazilian environmental legislation, especially by Resolution 489 of 26 October 2018, by the Environment National Council (CONAMA, in its original Portuguese acronym). The project is located on Marajó Island, city of Salvaterra, Pará, Brazil. Currently, the breeding stock numbers 599 animals, which supports all information and local studies with the species *K. scorpioides*.

All research with animals has been conducted under controlled conditions and following the national animal welfare criteria, and the specific projects have been approved by the internal technical committee under the Embrapa internal Seg Codes 22.1306023.00.00/01-2016 and 21159.002097/2022-85.

The species and its environmental requirements
The scorpion mud turtle is a native species of South American wildlife that is not listed in critical categories of threatened species of the International Union for Conservation of Nature (IUCN, 2023). Currently, the species has three subspecies (*K. s. scorpioides*, *K. s. cruentatum* and *K. s. albogulare*), and *K. s. scorpioides* has a wide distribution mainly in South America, part of Central America, and the Caribbean island of Trinidad (Fig. 1), ranging from eastern Panama to northern Argentina, excluding Chile (Rhodin et al., 2021). Cassano & Alcaldé (2022), sampled 207 units from three regions in Argentina and confirmed the absence of records of the subspecies in the humid portion of the Chaco region. Contrarily, Páez et al. (2022) expanded the occurrence record mainly towards the western region of Colombia in 14 new locations where the subspecies had not been previously documented. Moreover, Andrade (2019) expanded the knowledge of its occurrence in the north-east region of Brazil, filling the distribution gap between the States of Maranhão and Ceará and reporting the first record in the State of Piauí.

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The species is distributed in almost all Brazilian continental biomes (Rhodin et al., 2021), habiting in a wide variety of environmental conditions. This fact is an advantage for the policies of environmental agencies that restrict their authorising acts on the condition to places where this animal naturally occurs (according to IBAMA Normative Instruction 07 of 30 April 2015). This management is essential to prevent species invasion (Boyd et al., 2020), leading to achieving aquaculture sustainability.

The Brazilian Chico Mendes Institute for Biodiversity Conservation (ICMbio) classified the species conservation status as 'Least Concern (LC)', considering that there was no evidence of threats that could affect the species and risk the populations. Thus, *K. scorpioides* received less attention for the species population protection in management programs despite its strong hunting pressure for consumption and illegal trade in several places (Marques et al., 2008; Berry & Iverson, 2011; Frugoli et al., 2015; Auliya et al., 2016; Balestra, 2016; Cristo et al., 2017). It represents an advantage for captive breeding due to the absence of restrictions imposed on officially threatened species categories. However, it also means that the animal is underrated in the country, as reported by Lemos-Espinal & Smith (2020).

In addition to the already-mentioned benefits, *K. scorpioides* is a good species model because it can be maintained in captivity due to its small size, semiaquatic lifestyle and low environmental requirements (Berry & Iverson, 2011). These traits allow for low area requirements for facilities and water demand, important limiting factors that constitute a challenge in aquaculture to reduce environmental impact (Boyd et al., 2020).

Initial breeding stock recruitment

In 2004, a total of 175 individuals (43 males and 132 females) were recruited from Marajó island to make up the initial breeding stock at Embrapa, according to IBAMA authorisation. Animals in wildlife captive breeding projects must be obtained in three different ways: from other ready and authorised captivity, deposits of environmental agencies or collected from nature (Fig. 2). In the latter case, following the requirements of Chapter IV in CONAMA Resolution 489 of 26 October 2018 is necessary. Being careful with the degrading collection practices exposed by Cristo et al. (2017), such as fire implementation and extraction in the reproductive period, is required because they represent environmental crimes. Once the initial breeding stock is decided, the next step is to separate and manage the herd in different categories.

Animal categorisation

The first research records in Embrapa have already mentioned the separation of the breeding stock into zootechnical categories for easier handling (Marques et al., 2008). It is essential to avoid cannibalism and competition, which occur at all life stages but mainly when there is a clear difference in size. In this situation, older turtles attack hatched ones, biting the thinnest

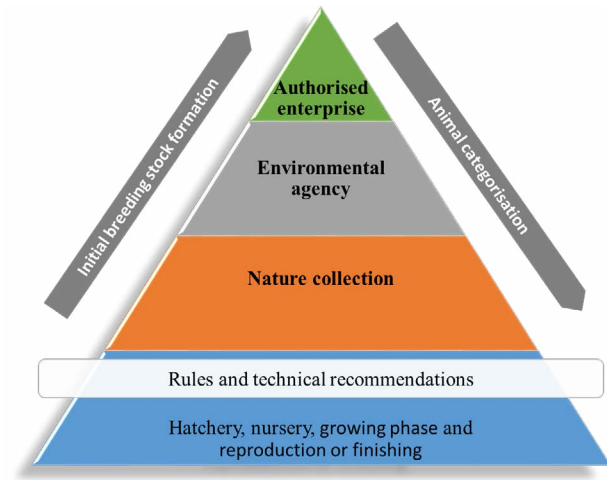


Figure 2. A scheme showing the different ways to obtain the initial breeding stock and the necessity for categorisation within the enterprise.

and softest parts such as the folds of the paws, eyes and the base of the neck and tail. Guimarães et al. (2016) confirmed these observations and demonstrated that soft tissue injuries in females and males account for between 60% and 81% of lesions in captivity. For this reason, four categories are determined by density, reproductive stage, and feeding management:

i) Hatchery: It is the egg incubation place until hatching takes place. The area should be closed, and the ceiling lined to avoid ant attacks and egg predation by rodents, birds and other reptiles.

ii) Nursery: It comprises all newly hatched individuals kept until 150 g of weight. Costa et al. (2017) examined 1,835 pups up to 12 months old and demonstrated that the average weight of this category is 36.54 g. In many cases, redividing them into two other nursery categories to keep individuals weighing 60 g and the largest up to 100 g, respectively, is necessary. This phase requires total care because the individuals are more susceptible to predation and ant attacks, especially the genus *Dinoponera*. In the latter case, the bite of a medium-sized ant can immobilise small individuals of scorpion mud turtle, allowing other ants to intervene and, in a matter of minutes, cause their death.

iii) Growing phase: Category in which animals weigh between 150 and 250 g. The analysis of 921 animals around 24 months old displayed an average weight of 199.20 g in this category (Costa et al., 2017). This phase allows to designate animals for the pet trade if there are legal permits for this purpose.

iv) Reproduction or finishing: Category in which animals weighing more than 250 g are kept, corresponding to two-year-old animals. Adult animals need to be separated in a commercial enterprise according to the purpose, whether reproduction or pet trade.

Breeding densities

There is no specific legal rule determining individual density for *Kinosternon s. scorpioides*. However, the literature shows flexibility in this aspect (Table 1),

Table 1. Stocking density of *Kinosternon s. scorpioides* in captivity, classified by breeding phase, as reported in the literature.

	Specimen density/m ²		
	Nursery	Growing phase	Finishing
Castro (2006)	-	-	7.65
Silva (2015)	-	-	3.27
Costa et al. (2017)	73.5	2.3	12.64
Ferreira (2017)	7.14	2.61	1.92

which can be explained by the gregarious behaviour and rusticity of the animal, supported by pulmonary respiration, which allows several chelonians to survive without breathing for hours if necessary (Girling, 2013). Up to 100 animals per m² with 10 cm of water depth are allowed in the breeding site until they reach 50 g, remaining at the bottom of the tank for a long period.

Sanitary problems and prophylaxis

As mentioned above, cannibalism and predator attacks are the most common causes of health problems. However, prophylaxis is fundamental and legally indispensable in breeding animals, requiring a trained responsible person. Disease prevention is the most crucial, economical and long-lasting procedure, as it is as important as trying to mitigate disease propagation in the breeding stock (Flosi et al., 2001). Footbaths, environmental disinfection and water quality monitoring should be considered. Guimarães et al. (2016) proposed that periodical clinical evaluations would be increased during the reproductive period to prevent mild lesions from progressing to systemic complications.

Additionally, individuals collected from the wild must be cleaned and quarantined because they usually contain helminths or ticks that disperse *Salmonella* (Pereira et al., 2018). However, Silva (2019) found that *Salmonella* bacteria is not very common in wildlife samples from Marajó Island.

Individual system identification

Animals in chelonian captive breeding systems can be individually identified with a transponder (microchip), an electronic radiofrequency marking consisting of a sterile microcircuit with 0.43" x 0.08" of dimensions and operating in a frequency range of 134.2 kHz (FDX-B).

These devices (Partners®) have been used in the Embrapa biological collection since the first days of life to almost 15 years without problems. Generally, it is implanted above the forelimb, below the carapace, away from vital organs. The anti-migratory capsule system, made of polypropylene, protects the fixation at the applied site.

CONAMA Resolution 487, dated 15 May 2018, has confirmed that these tools and aluminum band tags are the only two acceptable ways to mark reptiles in various captive breeding categories.



Figure 3. Overview of the reproductive infrastructure area, depicting the central tank. The white arrow indicates the shading mesh above the water area.

Area, facilities and water demand

The total area available at Embrapa for the captive facility is 200 m² divided into Nursery (9 m²), Growing phase (18 m²), Reproduction or finishing (50 m²), Hatcheries (9 m²) and Quarantine (8 m²).

There is a low structural requirement for species management compared to other Amazon chelonian breeding facilities (Dantas-Filho et al., 2020). The facility conditions are reflected in lower area demand, which was defined here as the facility portion required to be constructed. Given that Brazil faces deforestation threats (Azevêdo & Vieira, 2018), the development of productive activities in smaller spaces requires less suppressed vegetation for new enterprise establishment.

As required by Normative Instruction IBAMA 07 of 30 April 2015, the area is composed of 60% water and 40% sandy beach, with a little vegetation shade. Nevertheless, concerning the amount of water and depth of the tank, the species demands no more than 20 cm of depth. However, an interval between 50 and 70 cm by the regulation is recommended to avoid intense water temperature variations. For this reason, a shade is provided at Embrapa over the tank area (Fig. 3).

Rocha & Molina (1987), Molina (1992) and Rodrigues et al. (2017) also observed this low demand for water and facilities. This advantageous environmental condition is important because land and freshwater are limited resources in aquatic organism breeding programs (Boyd et al., 2020).

Kinosternon scorpioides has tolerance to survive in a variety of human-altered aquatic environments (Berry & Iverson, 2011; Cassano & Alcalde, 2022), supporting wide variations of ammonia, nitrite and nitrate, as well as slightly acidic pH, without physiological or respiratory harm. However, monitoring water quality indicators during breeding, ensures an environment suitable for growth, reproduction, health and survival, ensuring the species' welfare (Ferreira et al., 2020b).

Compared to other types of captive breeding, the amount of water required to produce one kilogram of scorpion mud turtle, with a semi-aquatic habit, is about

Table 2. The estimated water consumption in litres associated with one kilogram of mud turtle meat production, in comparison to other farming.

	Protein retention (%)	Edible meat per 100kg feed	Freshwater consumption L/Kg edible meat
Beef	15	4–10	15.400
Pork	18	17	6.000
Chicken	21	21	4.300
Mud turtle	20.44	26	4.871

Source: Adapted from Boyd et al. (2020)

Table 3. Summary of the environmental approach to mud turtle captive breeding

Considered Aspect	Environmental Advantages	Implications
Area	Possibility to avoid vegetation suppression	Non-conversion of green areas
		Project complexity reduction and increased environmental agency demand
Infrastructure	Possibility of breeding outside the countryside	Production outflow is accessible
		Inspection and regulatory access
	Low solid waste generation	Reduced labour demand Reduced building construction input use
Water demand	Possibility of using public or ground water	Exemption of unenforceability of granting water resources Reduction in the environmental agency demand
	Reduction of effluent generation	Non-contamination of natural water resources

13% more than the demand to produce the same amount of meat of terrestrial animals, which has less demand for water (Table 2). This estimate is based on the Embrapa captive breeding management, according to previous studies on carcass yield by Fernandes-Neto (2013) and feed conversion by Ferreira et al. (2020b). Therefore, measuring it according to a specific water footprint methodology is important.

This amount of water would be reduced with treatment and reuse, ensuring the recommended range for species tolerance. Table 3 shows the benefits of *K. scorpioides* captive breeding for the environment and its effects.

Food management

The animals have been fed commercial omnivorous fish feed since 2007. This food contains 42% protein

levels during the nursery and growing phases or 32% protein levels during the reproduction phase. Generally, individuals fed 1% of their live weight once a day, around midday when the photoperiod accelerates the metabolism of chelonians (Souza, 2004; DeNardo, 2006; Girling, 2013; Guimarães et al., 2017). This pattern has also been found in the giant Amazonian turtle captive breeding population *Podocnemis expansa* (Dantas-Filho et al., 2020). Ferreira et al. (2020a) demonstrated that scorpion mud turtles expressed better growth when receiving 0.6% of their live weight three times a week and 0.5% five times a week in the dry and rainy periods, respectively. An optimal food composition requires a high protein content (20.44% ± 8.02), low lipid composition (1.86% ± 0.61) and high moisture content (77.29% ± 2.12) (Ferreira, 2017).

The animals remain in the hatchery for varying periods during birth, from 24 hours up to 7 days, deprived of food until complete absorption of the residual yolk (Guimarães et al., 2017). After this period, each individual is implanted with a microchip and placed at the nursery site.

Feed management in the growing phase is conducted according to the production goals. The feeding system can be intensified if the production is focused on meat, causing turtles to obtain a minimum of 14% of their weight (Costa et al., 2015). In contrast, feed with a 5% average weight is recommended when the objective is for the improvement to guarantee the mean weight of the species (Castro, 2006). Fruits and vegetables are alternative feed resources that can be used (Souza, 2004; Anjos et al., 2014; Silva et al., 2021; Cassano & Alcalde, 2022). The authors tested the feed predilection among 13 Amazonian and non-Amazonian fruits in the reproduction and finishing phases and the animals preferred peach palm (*Bactris gasipaes* Kunth), followed by melon (*Cucumis melo* L.) and mango (*Mangifera indica* L.). However, some fruits are highly nutritious whereas others are not and can be toxic to some reptiles (DeNardo, 2006).

Moreover, the diet can be supplemented with calcium and phosphorus, which facilitates the optimal development of morphological structures in the growth (2.015 ± 0.115 Ca:P) and finishing (1.92 ± 0.26 Ca:P) phases (Fernandes-Neto, 2013). Egg laying performance can be improved during the reproduction phase in terms of frequency and shell thickness by incorporating diets containing proteins of animal origin with 70% commercial fish feed (22% crude protein) associated with 30% of a paste mix of bovine offal, fish and shrimp three times a week (Araújo et al., 2013a).

Furthermore, previous studies have exposed that the supplementation with 4.2% calcium in the reproductive phase increases the quality and quantity of eggs (Fernandes-Neto, 2018). However, the author emphasised the importance of avoiding excess when supplying nutrients, as it can cause problems for individuals and the environment. Boyd et al. (2020) attempted to show that increasing the amount of feed does not lead to more production because most of the feed consumed is not turned into protein. Instead, it goes into the water as waste, which reduces the environmental quality.

Table 4. General information about *K. s. scorpioides* raised under controlled conditions in the Embrapa's captivity, Marajó, Pará, Brazil

Reproductive variables	Information	Measurement
Ideal male/female ratio	1:10	Specimen
Mating starts	December (end)	Days
Reproductive period	January–June	Months
Mating peak	March–June	Months
Mating amount/period	1–3	Unit
Average nesting time	0.03–3.35	Hours
Laying period	April–October	Months
Laying peak	June–August	Months
Laying amount	1–2	Unit
Embryonic development	26	Stages
Egg incubation period	129.31	Days
Eggs number/female	4–5	Unit
Egg viability	20.5	Percentage
Average eggs measure (L x W)*	34.5 C x 18 L	Millimetre
Average egg weight	1.60–13.40	Grams
Birth period	April–October	Months
Births Peak	June–July	Months
Embryonic specie differentiation	18	Stage number

Source: Compiled information from Costa et. al. (2017) and Braga et al. (2021). * (L x W) = length x width in cm.

The puppy and juvenile stages have a preference for feed of animal origin but they accept fruits and vegetables without compromising their weight gain (Anjos et al., 2014). However, Assad & Bursztyn (2000) pointed out to be careful with the carnivorous diet by assessing environmental quality and economic demand. In this case, waste increases turbidity due to emissions of effluents rich in organic matter and suspended solids (Valenti, 2002; Valenti et al., 2018). Also, it undermines conservation, as non-competitive prices do not grace the reduction of hunting pressure on chelonian due to their inability to replace illegal trade (Rebêlo & Pezzuti, 2000). Nutrition accounts for more than half of rearing costs, that is, the highest in the system, similar to other turtles (Andrade et al., 2021).

Reproductive management

Table 4 summarises the data about the reproduction of *K. scorpioides* in Embrapa. The control and reproductive planning, as well as neonatal care, are conducted through direct observation in Embrapa. The individuals are separated and placed in the reproduction tank during the reproductive management, maintaining the ratio of one male to ten females. This ratio avoids injuries caused by mating behaviours, such as female persecution, pre-coupling and copulation (Molina, 1992; Guimarães et al., 2016).

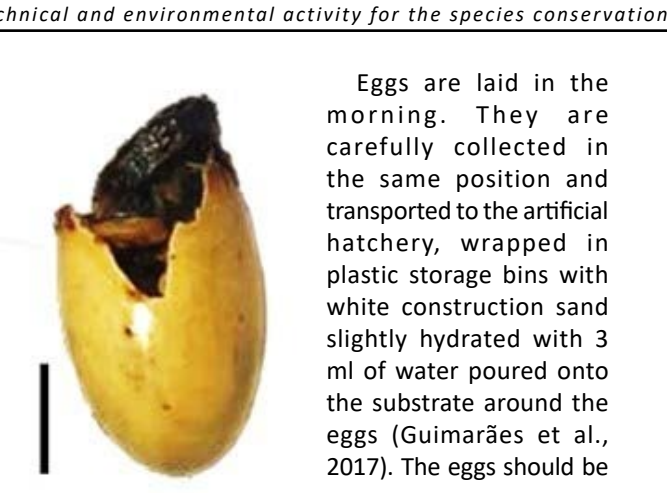


Figure 4. Photograph of failed hatching when the egg was covered with a layer of compacted sand larger than two centimetres. Scale bars: 2 cm.

completely buried with a layer of two centimetres of sand to avoid difficulties during hatching (Fig. 4). This artificial incubation is necessary to ensure successful breeding in *ex-situ* programs (Ferreira, 2017).

The average number of eggs laid by a female is four, the incubation period is approximately 122.98 ± 45.38 days, and the embryo develops in 26 stages, with speciation occurring at stage 18 (Costa et al., 2017; Braga et al., 2021). The literature shows similar records about laying values in captive breeding (Silva, 2011), even values up to six and seven eggs (Rocha & Molina, 1990; Castro, 2006). As previously stated, mineral and protein supplementation provided twice the average number of eggs in the Embrapa scientific breeding and more resistant eggs in other captive breeding, respectively (Araújo et al., 2013a; Fernandes-Neto, 2018).

Previous research has found low egg viability, with an average of 20.5% in the studied stock (Costa et al., 2017). This was attributed to primiparous females, egg predation, ant attacks, unfertilised eggs and embryonic death after hatching (Alves et al., 2014). The eggs have been opened at around the 122nd day of egg incubation to assist in hatching and prevent embryo death, thus minimising this low viability. Importantly, this low egg viability represents an 8-year average, in which the maximum reached 45.15%. Moreover, adjustments were performed in incubation management through the application of Good Incubation Practices and Standard Operating Procedures (SOPs), which showed a small improvement in the hatchability rate (Silva & Ferreira, 2017). In contrast, Leal (2018) studied four incubation protocols, changing the temperatures and humidity, and reached 54.2% egg viability with a handmade incubator. However, higher values have been reported in the literature, including 67.8% and 83.3% (Anunciação et al., 2011; Castro, 2006).

In general, males and females are born due to the average annual temperature (27.3 °C) of Salvaterra, located in the eastern region of Marajó (Lima et al., 2005). This is related to the pivotal (or threshold) temperature-dependent sex determination (TSD) of the species, in which both sexes develop in equal proportions around 27.5 °C (Ewert et al., 2004; Júnior-Ferreira, 2009).

Reproductive age is reached at 250 g of live weight, consistent with previous findings for animals maintained in a zoo in the capital of the State of Pará (Castro, 2006). In general, higher weight and female age result in larger and more numerous eggs, favouring individual selection and genetic improvement initiatives, although there is no molecular marker available for this purpose (Costa et al., 2017).

A relevant tool to be considered in the reproductive management of the species is assessing the genetic variability in the founder individuals to manage the genetic diversity of the captive-bred chelonian population, which is lost in each generation due to an inbreeding rate maintained below 2% (Williams & Osentoski, 2007). Silva et al. (2011) observed that the captive-bred population at Embrapa is not at risk of genetic erosion due to the high level of genetic variability, which aids in the genetic improvement and conservation of the species.

This information associated with advanced knowledge about the morphology of male and female reproductive systems (Machado-Júnior et al., 2006; Carvalho et al., 2010; Anunciação et al., 2011; Chaves et al., 2012), embryonic development (Braga et al., 2021) and mating behaviour (Costa et al., 2017) provides a satisfactory overview of the zootechnical and reproductive responses of the species. It is important because the number of births in captive breeding for commercial purposes is the most important indicator of the program's success (Trajano & Carneiro, 2019).

However, He et al. (2010) and Francisco & Silveira (2013) pointed out some instances in which reproduction does not occur. The authors attribute the absence of courtship behaviour in males and oviposition in females to inadequate facilities in captivity. This is manifested in reduced gonadal development, lack of reproductive hormones and/or insufficient nutrition to promote ovarian follicle maturation. The latter contributes to reproductive success outside the natural environment given individual ecological and physiological needs, such as nutrition, space, light, humidity, temperature, shelters, nests and social structure.

CONCLUSIONS

The scorpion mud turtle *K. scorpoides* is naturally found in almost all Brazilian biomes and is easy to contain and breed in a captivity environment, making it less likely to spread into other parts of the country so that the captive breeding activity can comply with the law.

The physical facilities require not much space and water compared to other Amazon species.

The knowledge about how the individuals are cared for, behave, breed, and affect the captive environment indicates their management as an alternative to turtle breeding in the Amazon. However, the economic aspects still need to be further examined.

The *Kinosternon s. scorpoides* captive breeding is a zootechnical and environmentally suitable activity for the conservation of the species because it helps to avoid spending on programs created exclusively for this purpose.

Enhancing our understanding of reproduction by employing molecular genetic tools is important to facilitate control and genetic improvement initiatives.

CONSERVATION APPROACH

Turtle captive breeding is considered a conservation strategy (He et al., 2010; Dantas-Filho et al., 2020; Andrade et al., 2021; Kim et al., 2022), as the *ex-situ* program mitigates the population decline generated by hunting, habitat destruction and degradation. In Brazil, the muçuã activity comprises large-scale capture using degrading methods without estimating natural population sizes. This activity is detrimental to population size because individuals remain under hunting pressure even if hunters know about biology and animal behaviour (Cristo et al., 2017). Therefore, there is a need for strategies that prioritise its conservation, associated with its legalised commercialisation (Marques et al., 2008; Berry & Iverson, 2011; Alves et al., 2012; Marques, 2016; Cristo et al., 2017; Bedoya et al., 2018).

Another preventative approach to captive breeding programs is to ensure the genetic identity and variability of the turtle population originating from certain areas (Williams & Osentoski, 2007). Recently, Kim et al. (2022) showed that turtles born in captivity can contribute to the conservation of globally threatened species, as they present migration behaviour similar to those born in the wild.

Officially, these animal captivity breeding practices are recognised, supported and mandated by several countries that have joined the Convention on Biological Diversity (CBD), of which Brazil is also a party. In this sense, Trajano & Carneiro (2019) stated that commercial breeding must be seen as an instrument for conserving Brazilian biodiversity in a unified manner, as international agreements and federal regulations have been pointing in this direction for some time.

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Author Contributions

Palmira Francisca Gonçalves Ferreira, Ednaldo da Silva Filho and José Ribamar Felipe Marques were involved in the conception, planning, methodology, development and design, as well as the article writing. The last two people oversaw the survey and were also in charge of the formal analysis. José Ribamar Felipe Marques, Larissa Coelho Marques and Relionan Pimentel Leal were involved in the provision of study materials, the curation of animals, estimations and the provision of all documents relevant to the data and information research. All authors discussed the results, commented, assisted in interpreting and worked on the manuscript.

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Differential longevity in free-living smooth snakes *Coronella austriaca*

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During a 31-year study (1992–2022) of reptiles inhabiting a 10 ha area of lowland heath, within a planted coniferous forest in southern England, UK, smooth snakes *Coronella austriaca* Laurenti, 1768, were individually marked with passive integrated transponder tags. New-born smooth snakes were also captured, measured and weighed to estimate size at birth. Growth curves were estimated for females and males which, combined with their estimated size at birth, provided an estimate of age at the time of death. The estimated mean age at death of females (7.8 years) was significantly lower than that of males (10.1 years), with females and males reaching a maximum age of 15 and 25 years respectively. Reproductive effort was considered as a possible explanation for the observed reduced longevity of females compared to males. Female reproductive success increased between 4 and 10 years of age and then declined in the largest breeding individuals, regardless of age. This finding suggested the possible presence of size/growth-related reproductive senescence. This is the first known study to provide reliable estimates of longevity in free-living smooth snakes. It demonstrated a significant difference between females and males in both mean and maximum ages attained, and the potential impact of breeding stress as an explanatory factor for increased female mortality and reproductive senescence.

Keywords: predicted age, body size, SVL, reproductive output, senescence

INTRODUCTION

The study of wildlife population dynamics relies on the ability to sex and age individuals, determine longevity, fecundity and age at death (Townsend et al., 2003). Due to the lack of long-term, individual-based and species-specific data (Colchero et al., 2018), this has largely been addressed through the use of mathematical modelling techniques. However, it is recognised that determining longevity in wildlife populations remains a problem that requires further reliable data on population parameters data to be collected from populations of tagged individuals that are frequently recaptured (Lind et al., 2005; Colchero et al., 2018; Cayuela et al., 2019). Birth and especially age at death are difficult parameters to determine in cryptic species such as amphibians and reptiles. The age of individual snakes is typically estimated using either growth curves for a given species (Plummer, 1985; Shine & Charnov, 1992; Stanford & King, 2004; Fornasiero et al., 2016; Dick & Mebert, 2017), or skeletochronology techniques to count annual growth rings in selected tissues, the latter of which may require either killing an individual animal or removing a bone/tissue sample from it (Waye & Gregory, 1998; Waye, 1999).

In snakes, an estimate of fecundity can sometimes be obtained by palpation to detect developing eggs or embryos (Reading, 2004). Determining the relationship between the number of eggs and/or embryos versus

snake size and estimated age, has often resulted in positive relationships (Luiselli et al., 1996; Sparkman et al., 2007; Bronikowski, 2008; Cayuela et al., 2020; Tully et al., 2020). However, this relationship was weak in *Thamnophis radix* (Stanford & King, 2004) and may also not occur in all geographic locations for a given species such as the smooth snake, *Coronella austriaca* (Dick & Mebert, 2017). Overall, a common factor is that incorrect age estimation can lead to significant error in all of these reported relationships.

Although it has been postulated that in both European and tropical reptiles there may be an increase in mortality (actuarial senescence) or a decrease in reproductive success (reproductive senescence) of females related to age (Dmi'el, 1967; Cayuela et al., 2020; Tully et al., 2020), female mortality due to other causes such as predation, resulting from a reduction in other physical attributes, such as a decline in response time to external stimuli, reduction in visual acuity and overall muscle strength decline due to actuarial senescence may occur before reproductive senescence is either apparent or detectable (Cayuela et al., 2019).

The current 31-year study of individually marked, and frequently recaptured smooth snakes at a heathland site in southern England, has provided a potential opportunity to address the problems associated with the reliable estimation of age and fecundity in a wild snake species and to test the hypothesis that reproductive senescence may

occur, and/or can be detectable in the oldest females. The objectives of this study were, therefore to 1) determine the age and longevity of wild female and male smooth snakes *C. austriaca* and 2) determine the relationships between body size and age, with fecundity in wild female smooth snakes.

MATERIALS & METHODS

Study site

The study site is an area of approximately 10 ha of lowland heath within a managed coniferous forest in southern England (50°44' N, 2°08' W). It comprises a mosaic of dry heathland, dominated by a fragmented area of heather (*Calluna vulgaris*) and gorse (*Ulex europaeus* and *Ulex minor*), wet heath, dominated by purple moor grass (*Molinia caerulea*) and cross-leaved heather (*Erica tetralix*). Bristle bent (*Agrostis curtisii*) is also present throughout the study area along with small (< 2 m high) regenerated conifers (*Pinus sylvestris*). Much of the ground surface, beneath the vegetation, is covered with moss. Small areas of open sandy soil were also present within the dry heath (Reading & Jofré, 2020).

Data collection and analysis

Between 1992 and 2022 (31 years) multiple surveys for smooth snakes within the study site were completed annually. With the exception of 2002, when only 3 surveys were completed, surveys were spaced approximately one week apart (mean = 21.27/year, SD = 2.406, n = 30; range: 13–28) between the beginning of May and the end of October (depending on weather conditions) using permanent hexagonal arrays of 37 artificial refuges (corrugated steel sheet: 76 cm x 65 cm; Reading, 1997). In each array, refuges were spaced 10 m apart. The total number of refuges used each year varied between 333 and 488 with 407 used annually between 1997 and 2022. During each survey the sex of all captured individuals was determined using the length and shape of the tail (Reading, 2004), their body mass (g) was determined using a Salter spring balance, snout-vent length (SVL) and tail measured (mm) using a 30 cm ruler. On initial capture, snakes were individually marked with a sterilised (70% isopropyl alcohol) Passive Integrated Transponder tag (PIT-tag: ID100) implanted sub-dermally 3–5 cm anterior to the cloaca, and towards the cloaca so that forward movement of the snake would not massage the PIT tag back to the implant needle puncture site before it had healed. The implant puncture site was also sterilised prior to PIT tag implantation. In addition, the lower abdomen of females was gently palpated to determine if they were gravid and if so, the number of embryos they contained. For fecundity analysis only palpation estimates were used if a gravid female was captured during the late summer (July and August) to reduce the likelihood of mistaking food in the gut for early developing embryos. New born individuals were not PIT-tagged as they were considered to be too small, but their head pattern was photographed for individual identification in case of future recapture. Refuge checks

during each survey also included a visual search along a 360 m transect walk between refuges in each array.

The determination of smooth snake age, at assumed death, was only estimated for individuals that were considered to be resident within the ten-hectare study site. The criteria used for residency depended on whether an individual snake had been captured within the study area for at least three consecutive years thereby eliminating potential transient individuals from the analysis. No dead individuals were found during the 31 years of the survey and therefore if an individual was not captured for at least two consecutive years it was assumed to have died.

Many of the snakes that met these two criteria were not first captured as very young juveniles so their year of birth had to be estimated using von Bertalanffy growth curves which were determined by a) an estimated mean SVL at birth and subsequent annual increases in SVL, for each sex, obtained from growth curves plotted for those individuals that had been first captured at a small size and then subsequently recaptured over many years. This growth curve (one for females and one for males) was then plotted with that for each snake that had been first captured as a subadult/adult, and its position along individual growth curves manipulated to enable the birth year, and subsequent age, for each snake to be estimated thereby enabling a determination of age at death.

A cubic regression analysis was used to determine the relationship between the number of offspring produced by gravid females in relation to either their SVL or estimated age. Data analysis was completed using Minitab software (Version 18.1) with p = 0.05 used as the threshold for statistical significance in all analyses.

RESULTS

For comparison with other studies of smooth snake growth, that measured total body length (TBL: SVL + tail length) rather than only SVL, the relationship between these two parameters, for females and males within the study area is shown in Fig. 1.

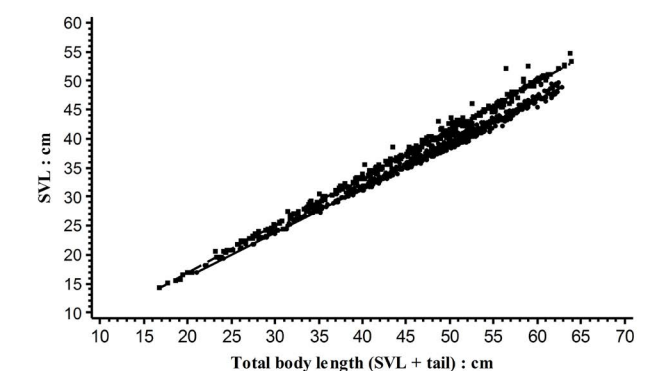


Figure 1. Relationship between SVL and total body length (TBL: SVL + tail length) in smooth snakes in southern England. ●—: mean male SVL = $0.9252 + 0.7635$ mean male TBL; $r^2 = 99.11\%$; $p < 0.001$; $df = 598$. ■---: mean female SVL = $0.1190 + 0.8287$ mean female TBL; $r^2 = 99.47\%$; $p < 0.001$; $df = 338$.

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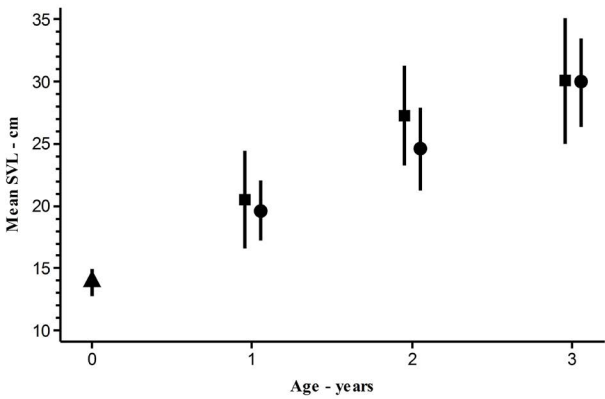


Figure 2. Mean SVL $\pm$ SD against age for female and male smooth snakes (females: ■; males: ●) during their first three full growth seasons plus SVL at birth (▲).

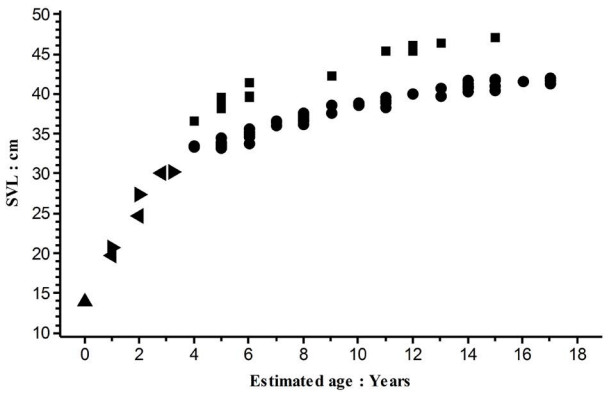


Figure 3. Growth curves for a female and a male smooth snake illustrating how age was estimated from recorded SVL measurements (female: ■; male: ●), mean predicted measurements (female: ▲; male: ►) and mean SVL at birth (▲).

Between 1992 (14 August) and 2022 (22 September) a total of 298 smooth snakes were captured and individually marked (♀: 127; ♂: 169; undetermined sex: 2). Of these, 102 (♀: 38; ♂: 64) met the criteria for residency (consecutive capture years: ♀: mean = 5.37 years; SD = 2.410; n = 38; range = 3–12 years; ♂: mean = 6.94 years; SD = 4.790; n = 64; range = 3–24 years) and their age at assumed death estimated after no captures for at least 2 years (♀: mean = 14.71 years; SD = 7.591; n = 38; range = 2–27 years; ♂: mean = 14.203 years; SD = 7.622; n = 64; range = 2–27 years).

The mean SVL of new born smooth snakes, born in late August/early September (13.84 cm; SD = 1.021; n = 26) was determined following captures under refuges where recently gravid females had been frequently found. Following birth, female and male smooth snakes grew at essentially the same rate during their subsequent first three years (Fig. 2). The predicted mean SVL of females and males against age, using the SVL values in Figure 2 to predict the birth year, are given in Figure 3. Female growth subsequently exceeded male growth from the fourth year onwards and also attained a lower maximum age (15 years) than males (25 years; Fig. 4). The oldest female was captured 33 times over 11 years (1993–2003) and the oldest male 167 times over 24 years

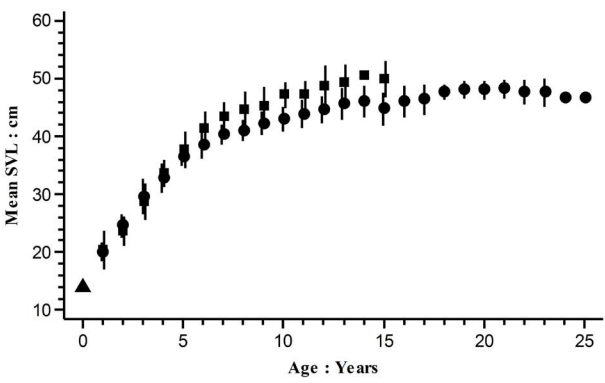


Figure 4. Female (■), male (●) and new born (▲) SVL $\pm$ SD against age.

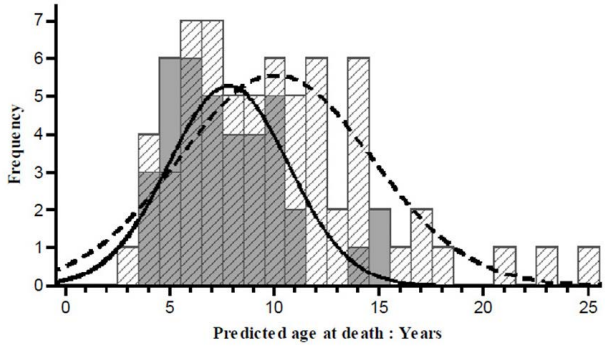


Figure 5. Histogram of female (dark columns) and male (striped columns) predicted age at death with a fitted normal distribution curve (females: —; n = 38; males: ---; n = 64).

(1997–2020). Overall, the mean age of assumed death for females (mean age = 7.82 years; SD = 2.87; n = 38; range = 4–15 years) was significantly lower ($t = 3.04$; $df = 99$; $p = 0.003$) than that attained by males (mean age = 10.06 years; SD = 4.58; n = 64; range = 3–25 years; Fig. 5).

One variable possibly correlated with increased female mortality compared to males, is higher reproductive effort. The number of young produced by females of different SVL (Fig. 6a), and estimated age (Fig. 6b) was plotted and analysed using a cubic regression analysis. This showed a significant positive relationship ($p = 0.021$) between the number of young produced by females from an SVL of about 40 cm (approximately when breeding starts), and an estimated age of 4 years, to an SVL of about 49 cm, at an estimated age of 10 years, after which there was an apparent decline in the number of young produced. The fecundity of the largest female (snake #162: ◆) increased with increasing SVL (45.7–49.2 cm) and then declined (SVL: 52.4/52.5 cm; age: 8–15 years). Female #103 (▲), which grew to a relatively large size (SVL: 50.8–51.5 cm) but at a younger age (7/8 years old) than female #162, also showed an apparent decline in fecundity. However, there was no significant relationship ($p = 0.057$) between the number of young produced and the age of females.

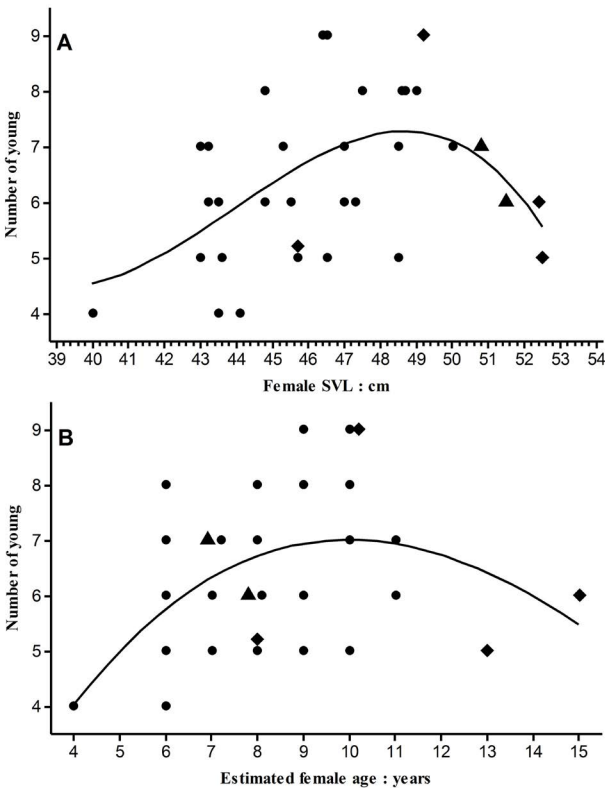


Figure 6. Plots of the number of young produced by female smooth snakes of different SVL – **A.** No. young = $543.5 - 37.59 \text{ SVL} + 0.8656 \text{ SVL}^2 - 0.006566 \text{ SVL}^3$; $r^2 = 27.30\%$; $p = 0.021$; $df = 33$ and estimated age. **B.** No. young = $2.201 + 2.061 \text{ Est. age} - 0.0354 \text{ Est. age}^2 + 0.002143 \text{ Est. age}^3$; $r^2 = 21.83\%$; $p = 0.057$; $df = 33$. ▲: female #103; ◆: female #162; ●: all other females.

DISCUSSION

Overall, the mean age at death of male smooth snakes was two years greater than that of females, the maximum age attained by males was ten years greater than that of females and, although fecundity generally increased with SVL, there was an indication that it started to decline in the largest females, possibly indicating evidence of size related reproductive senescence.

In this study, smooth snake size was determined by SVL, rather than total body length (SVL + tail), as used in some previously reported studies (Luiselli et al., 1996; Dick & Mebert, 2017; Dalessi et al., 2021; Struijk et al., 2023), as this excludes potential error in determining total body length resulting from tail damage/shortening, an occurrence that was occasionally found in this study.

The largest source of potential error was the determination of death for each individual snake, since dead individuals were never found. This was addressed using the capture data for individuals that had been caught many times over many years. For example, male #89 was first captured on 8 September 1997 and then recaptured for 24 years (mean: 6.87 captures/year; range: 1–13) so its absence in 2021 and 2022 indicates death, after its last capture on 23 July 2020. Similarly, female #87 was first captured on 24 June 1997 and then recaptured annually

for 9 years (mean 5.0 captures/year; range 2–10), so its absence in 2006, and all subsequent years up to 2022, suggests that it died after its last capture on 26 May 2005. The long periods of capture of an individual within the study site were also used to indicate residency within the site, thereby further reducing error in estimating age at death if a ‘transient’ individual simply moved away from the study site. These two examples, along with numerous others, were the basis for reducing error as much as possible in determining both residence and the occurrence of death, for both sexes.

The mean size (SVL = 40 cm) and age (4 years) taken to reach sexual maturity in female smooth snakes in this study differs from some other geographical locations where sexual maturity was reached in one female at two years of age (total body length = 50.9 cm; SVL = 42.2 cm) in the Netherlands (Struijk et al., 2023), but at a longer total body length (mean = 73.4 cm) and probably a greater age in Norway (Dalessi et al., 2021). In the eastern Italian Alps smooth snake females reproduced for the first time with a total body length of 43.5 cm and an estimated age of four years (Luiselli et al., 1996). Apart from the one reported exceptional female from the Netherlands (Struijk et al., 2023), most from that region are similar in size to the females from the UK and suggests that snakes from a similar latitude present with similar growth parameters compared to those from much further north (Norway) and that size at first reproduction in females declines as latitude declines (Norway: 59° N, 10° E; the Netherlands: 51° N, 5° E; UK: 50° N, 2° W; Italian Alps: 46° N, 13° E). Similarly, black rat snakes *Elaphe obsoleta* grew more slowly and matured at a greater age in Canada compared to those further south in the USA (Blouin-Demers et al., 2002) as did garter snakes *Thamnophis elegans* (Bronikowski, 2008).

Sexual size dimorphism in free-living snake populations is not consistent between species. In the smooth snake, females were larger than males in Germany (Dick & Mebert, 2017), Norway (Dalessi et al., 2021) and southern England (Reading, 2004) and also in the plains garter snake *Thamnophis radix* in the USA (Stanford & King, 2004). However, no difference was found between the sexes in the size of European whip snakes *Heiophis viridifavus* and *H. carbonarius* in France and Italy (Fornasiero et al., 2016).

The attained maximum age of females and males was also inconsistent between species. In meadow vipers *Vipera ursinii* in France, males have a higher adult mortality and a shorter life expectancy than females (Tully et al., 2020) possibly due to increased activity during the mating season and suggested avian predation. In smooth snakes the home range of males is approximately double that of females (Reading, 2012) and suggests that increased male activity does not necessarily result in increased mortality, as males lived to a greater age than females in the present study (females: mean age = 7.8 years; maximum age = 15 years; males: mean age = 10.1 years; maximum age = 25 years). Conversely, life expectancy in female Norwegian smooth snakes was possibly higher than in males (Dalessi et al., 2021). It is

tempting to suggest that reduced female survivorship may be the result of reproductive stress. For example, in a study of a captive colubrid snake *Spalerosophis cliffordi*, old females showed an inability to maintain body weight after successive breeding events suggesting a degree of reproductive senescence (Dmi’el, 1967). Similarly, the mortality rate of Italian smooth snakes was also highest in breeding females and those emaciated following parturition (Luiselli et al., 1996). Conversely, in a 16-year study of garter snakes *Thamnophis atratus* in California, females exhibited a higher survival rate than males, though the need for intensive sampling to gain reliable population parameter estimates was also acknowledged (Lind et al., 2005).

Perhaps the biggest problem that has been recognised with respect to determining senescence in wildlife populations is that mortality may occur before reproductive senescence occurs, or can be detected, (Cayuela et al., 2019). With respect to this study on smooth snakes a significant cubic regression analysis of offspring number against female SVL, indicated that fecundity may have declined in the biggest (SVL > 50 cm), but not oldest individuals. The capture data for females #103 and #162 indicate that reproductive senescence may occur at large size irrespective of age (female #103: SVL = 51.5 cm and 8 years old; female #162: SVL = 52.5 cm and 15 years old). The increase in female fecundity with increasing age/ SVL has been reported in many snake species (Luiselli et al., 1996; Stanford & King, 2004; Sparkman et al., 2007; Bronikowski, 2008), whilst in this study SVL appeared to take precedence over age with respect to correlating with reproductive decline. This being the case then it potentially supports the view that senescence may be caused by telomere shortening in relation to increased cell division during rapid growth (Bronikowski, 2008). This was found in garter snakes *T. elegans*, where fast growth resulted in early maturation, production of large annual litters, low annual survival and a reduced lifespan whilst slow growth resulted in late maturation, infrequent production of small litters, increased annual survival and an increased lifespan (Bronikowski, 2008). In the current study both fast and slow growing individual smooth snake females were found, with the fast growing individual (female #103) attaining a lower terminal age (8 years) than the slow growing individual (female #162; 15 years)(Bronikowski, 2008), despite both females being of a similar size.

The length of this individual-based study (31 years) is one of the longest on a wild snake species, and coupled with its high number of annual surveys, has provided a rare opportunity to investigate both sex-related longevity and female reproductive senescence in smooth snakes with potential relevance to other snake/reptile species for which long-term studies are not feasible.

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DATA ACCESSIBILITY

Data is available following an appropriate request to the author.

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