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Front cover: Fer-de-lance (*Bothrops asper*) from Braulio Carrillo National Park, Costa Rica.
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Comparison of eDNA and visual surveys for rare and cryptic bromeliad-dwelling frogs

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Surveys of rare or cryptic species may miss individuals or populations that are actually present. Despite the increasing use of environmental DNA (eDNA) analysis to survey species in ponds, rivers, and lakes, very few studies have attempted to use eDNA for the detection of species using very small water bodies such as those accumulated within plants. Our aim was to investigate the feasibility of an eDNA sampling method for detecting *Crossodactylodes itambe*, an endemic bromeliad-dwelling frog from a remote location in Brazil. We collected water samples from 19 bromeliads for which we had observational data from direct visual surveys. We compared occupancy estimated from direct observations with the results from quantitative real-time PCR based eDNA assays. For observational surveys, we used a single season occupancy model. We applied a novel Bayesian occupancy model to estimate occupancy from eDNA samples, as well as false positives and false negatives at different stages of the workflow. eDNA from bromeliad tanks provided reliable estimates, with very low error levels and improved detection when compared to detectability from direct observation. Estimated occupancies using eDNA and visual survey methods were similar. The method is feasible for species restricted to small water bodies and exposed to direct UV radiation, and particularly useful to survey remote locations and confirm species presence. eDNA analysis provides a viable alternative to destructive sampling of bromeliads or direct observation methods that require logistically challenging repeated observations. Therefore, eDNA methods may be widely applicable to sampling programmes of other amphibians that live in plants.

Keywords: bromeliad, eDNA detection, false-positive, amphibian, occupancy, phytotelm

INTRODUCTION

Species surveys using direct observations suffer from the problem of individuals or populations being missed (MacKenzie et al., 2002). Such imperfect detection is caused by a wide variety of factors, including time of survey and temperature (Sewell et al., 2010), observer experience (Grant et al., 2005; Fitzpatrick et al., 2009) or simply individuals being obscured from view. When making observations, the presence of a surveyor can alter the behaviour of the target organism, reducing the likelihood of it being observed (Barata et al., 2017, 2018a). To account for imperfect detection, repeated visits are required to control for variation in detectability (MacKenzie et al., 2002). However, detection does not necessarily require direct observation. An increasing number of indirect survey methods are emerging such as environmental DNA (eDNA) analysis. Indeed, eDNA surveys can outperform direct observation surveys (Lopes et al., 2017; Burns et al., 2020), but this varies according to the ecological characteristics of the targeted organism (Takahara et al., 2019).

Surveys targeting eDNA involve the collection of environmental samples from a location which usually comprises water, soil or sediments (Turner et al., 2015; Buxton et al., 2018; Spitzen-van der Sluijs et al., 2020; Valentin et al., 2020). These samples are then processed following forensic protocols for the extraction of DNA that has been released by organisms into the environment. Where possible, the DNA that is extracted and amplified from these environmental samples is then identified to species level by comparing their sequences to a reference DNA library, thereby allowing for inferences concerning species presence in that habitat (Jane et al., 2015). Detection of species using eDNA methods has become commonplace in environments where detectability of a target species may be relatively low, such as ponds (Harper et al., 2018), lakes, rivers, and streams (Sales et al., 2019; Bedwell et al., 2020). However, very few studies have attempted to use eDNA methods for the detection of species using very small bodies of water such as aggregations of water collected within plants (also known as a phytotelm), e.g. within the tanks of bromeliads. Only two previous studies have used eDNA

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methods in the survey of bromeliad tanks (Brozio et al., 2017; Torresdal et al., 2017), both of which targeted the Critically Endangered Trinidad golden treefrog (*Phytotriades auratus*), an elusive species that requires destructive sampling (i.e., bromeliad destruction).

Crossodactylodes itambe is a small frog species endemic to the summit of one mountain, the Itambe summit, Minas Gerais state, in south-eastern Brazil (18°23'S 43°20'W; datum WGS84). The species exclusively lives within ground bromeliads (*Vriesea medusa*), which are found on high elevation rocky outcrops (Barata et al., 2013). *Crossodactylodes itambe* is restricted to bromeliads at 1800 m above sea level or higher with a total range of less than 0.5 km^2, although the plants can be found at lower altitudes (Santos et al., 2017). Species occupancy increases at higher elevation (Barata et al., 2017) and abundance of individuals is related to bromeliad structure, such as plant size and the volume of water retained by the central tank (Barata et al., 2018b). The restricted range and habitat requirements for the species make it highly vulnerable to extinction from climate change, wildfires or disease, and therefore a priority for conservation monitoring.

Due to the remote location, nocturnal activity of the species (Barata et al., 2018a) and a detection probability of 0.40-0.65 (Barata et al., 2017), visual surveys are labour intensive and costly. Power analysis conducted by Barata et al. (2017) suggests that when using visual encounters, an observer would be required to make at least three to four visits and 143 bromeliads would need to be surveyed to have an 80 % chance of detecting a change of 30 % in the population. Furthermore, when searching for new populations of rare and cryptic species, only a subset of the potentially highly suitable areas can be surveyed because of logistical and financial constraints (I.M. Barata, unpublished data). Consequently, it would be highly beneficial to develop a passive survey method with an equivalent or greater detectability from a single site visit than visual encounters. A passive method would provide substantial savings in terms of logistical and survey efforts, without compromising the ability to detect population changes within this highly vulnerable population or finding new populations at surrounding locations.

Here we develop species-specific PCR primers for *C. itambe* and test the practicalities of collecting eDNA samples from ground bromeliads in a remote location. Bromeliads in this location grow in an outcrop among shrub and herbs and are exposed to direct UV radiation, which can potentially increase DNA degradation rates (Strickler et al., 2015). We compare the known occupancy estimated from observational surveys (from multiple visits in 2014-17) with the results from quantitative real-time PCR (qPCR) based eDNA assays. Our main goal was to test the feasibility of a passive sampling method in detecting *C. itambe* in an exposed montane area with the challenges of high UV radiation, with a view to applying the method more widely to other amphibian species that are restricted to bromeliads.

METHODS

Site selection

A tank bromeliad is a type of phytotelm that accumulates rainwater at the base of each leaf axil and in the central tank, thereby providing a microhabitat for other species (Lehtinen, 2004). We selected 19 bromeliads for eDNA sample collection, 11 of which were known to have been occupied by *C. itambe* based on previous studies and detection histories (Barata et al., 2017). The remaining eight bromeliads had no *C. itambe* recorded within them over the previous four years and were therefore considered to be unoccupied by frogs. All samples from bromeliads were from within the known range of the frog, on the Itambe summit, Minas Gerais.

Additionally, two samples were collected from flowing water in the vicinity of the study site as field negative samples. These were collected to ensure no contamination occurred in the field, as this is a potential risk when sampling in remote locations. Whilst we acknowledge that filtering sterile water on site as a field negative is a more standard approach, it was decided not to increase the volume and weight of materials needing to be carried due to the logistics of accessing the remote location. As flowing water is not used by the target species, it was highly unlikely to contain target DNA. If these results returned negative, we could be confident there was no in-field contamination.

Visual encounter surveys

Observational surveys were conducted across four years, from 2014 to 2017, during the rainy season (between October to March). Bromeliads were tagged with individual numbers that allowed repeated visits in multiple years. For each year, visual encounter surveys consisted of three to six consecutive visits to the same site to create a detection history of presence (1) and absence (0). Visual surveys were conducted at night by a team of two people, with only the most experienced observer taking notes on species presence/absence to avoid observational bias (Barata et al., 2017). Bromeliad selection for eDNA samples was based on previously known detection histories between 2014 and 2016 and an additional survey in 2017 during four consecutive nights. Surveys and detection histories from previous years provided estimates of species occupancy and detection (Barata et al., 2017). The surveys in 2017 confirmed the same species presence/absence pattern observed in previous studies and allowed the collection of water samples from selected bromeliads.

Environmental DNA sample collection

Samples were collected using syringe filtration and 0.22 µm (PVDF membrane type, gamma irradiated) MilliporeTM SterivexTM filter capsules. To prevent contamination, we prepared individual sterile sample collection kits to be used at each bromeliad before conducting fieldwork. Each kit contained two pairs of gloves, a 0.22 µm filter capsule, a 60 mL luer-lock syringe, a 30 mL container filled with absolute ethanol, a 10 ml syringe with 1/2" needle, two luer-lock caps, a 50 mL centrifuge tube and a small zip-lock bag (Fig. 1A). In the field, water was drawn from either the centre or lateral leaves of the bromeliad using the sterile 60 mL luer-lock syringe (Fig. 1B); the filter capsule was attached to this syringe which was used to push the water across the filter membrane (Fig. 1C). This procedure was repeated twice to filter a total of 120 mL of water. Once the whole water sample was filtered or the capsule had become blocked (which was the case for one sample), 10 mL of absolute ethanol was added to each filter as a preservative using the 10 mL syringe and ½" needle. Each capsule was sealed with luer-lock caps, re-sealed in an individual 50 mL centrifuge tube and stored in a sample bag to prevent contamination while in storage and transport (Fig. 1D). We numbered each sample (filters, containers and bags) with the reference number of the bromeliad from individual tags.

Figure 1. Environmental DNA sample collection demonstration: A) sample collection kit; B) water collection from lateral leaves of the bromeliaed using a sterile 60 mL syringe; C) pushing the water across the 0.22 µm filter capsule; and D) individual capsule sealed in a tube and stored in a sample bag to prevent contamination.

eDNA extraction

DNA extraction was undertaken in a dedicated lab, within a UV hood. All equipment and work stations were sterilised using a combination of 10 % bleach solution and/or UV light in advance of use. Standard laboratory protective equipment was worn at all times. DNA extraction followed a modified Qiagen® DNeasy® blood and tissue kit protocol, adapted from Spens et al. (2016). The ethanol preservative was removed from the MilliporeTM SterivexTM filter capsule by attaching a sterile syringe and passing air across the capsule, collecting the liquid in a 2 mL micro-centrifuge tube. 50 uL of 3 M sodium acetate solution per millilitre of ethanol recovered was added, these samples were then incubated at -80 ˚C for 10 minutes to aid in DNA precipitation before being centrifuged at 14000 RPM for 15 minutes to collect any precipitate as a pellet on the side of the tube with supernatant discarded. 180 µL of ATL buffer and 20 µL of proteinase K from the extraction kit was added to each micro-centrifuge tube, which was then vortexed for 15 seconds to suspend the pellet and mix. Samples were incubated on a rotating block at 56 ˚C overnight. AL buffer and ice-cold absolute ethanol was then added to each micro-centrifuge tube in a 1:1:1 ratio with the incubated contents of the tube.

720 µL ATL buffer and 80 µL of proteinase K from the extraction kit were added directly into each of the filter capsules, the caps replaced, and filter units sealed with Parafilm®, and incubated at 56 ˚C overnight on a rotating block. The liquid was removed from the filter using a sterile syringe and by passing air through the capsule, collecting the buffer in a fresh micro-centrifuge tubes. AL buffer from the extraction kit and ice-cold ethanol were then added in a 1:1:1 ratio (samples were split across two tubes per capsule to accommodate the volume). Product extracted from the ethanol preservation buffer and the filter capsule for each sample were then combined, passing both across the same mini-spin column from the extraction kit. Extraction continued as per extraction kit manufacturer's protocol eluting into 200 µL of warm AE buffer.

Primer development

Primers suitable for use with eDNA were developed to amplify a short region of the Cytochrome Oxidase 1 gene (COI) of *C. itambe*, based on the sequence identified by Santos et al. (2017); NCBI accession number KY362551. Primers were designed using the program Primer 3 (Koressaar & Remm, 2007; Untergasser et al., 2012; Koressaar et al., 2018). Conditions were set to identify primers between 18 and 23 base pairs in length to amplify a region between 75 and 100 base pairs long. We specified that no runs of greater than three base pairs should be included, with a GC content of between 40 and 50 %, and an optimum melt temperature of 60 °C. A set of primers was identified to amplify an 83 base pair sequence (Table 1). The primer sequences were tested in silico with a NCBI blast search to check for cross amplification with other species. No species were found to have a 85 % or greater match to either the forward or reverse primer. Additionally, whilst other

frogs (*Bokermannohyla nanuzae*) were occasionally seen using the bromeliads in the study area over this 4-year period (Barata I.M., personal observation), no other amphibian species using the bromeliads were recorded during the observational surveys (i.e., the night before eDNA samples were taken). Primers could not be tested in vitro due to the difficulties in obtaining and exporting tissue samples of the relevant species.

Table 1. Primer sequences generated using Primer 3 for detection of *C. itambe* from eDNA.

Primer name	Length	Melting temperature	GC%	Sequence
CICO1-F	20	59.78	50	tacttgcttctgctggcgta
CICO1-R	20	59.59	55	ggcatgggctaagt-taccag

qPCR

qPCR was conducted in a separate room to DNA extraction. All equipment and work surfaces were sterilised using either a 10 % bleach solution and/or UV light in advance of use and appropriate personal protective equipment was worn. Plate set-up was conducted in a UV hood dedicated to low concentration DNA work. qPCR was performed using a SYBR Green assay, with eight replicates per sample. The qPCR amplification procedure consisted of 10 µL of Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix, 2 µL of each primer at a concentration of 10 µM /µL, and 4 µL of template DNA, in a final reaction volume of 20 µL. qPCR conditions consisted of two activation steps at 50 ˚C for 2 minutes followed by 95 ˚C for 2 minutes, then 40 cycles of 95 ˚C for 15 seconds, 59 ˚C for 15 seconds and 72 ˚C for 1 minute. A melt curve was then performed ramping up from 55 ˚C to 95 ˚C in 0.5 ˚C increments. Three negative control samples of ddH_2O were included in each qPCR run, to check for contamination. A replicate was classed as positive when an exponential growth phase in relative florescence was identified and the melt curve indicated a temperature of between 82 ˚C and 82.5 ˚C, indicating the fluorescence was not caused by primer dimer.

Following qPCR analysis, each sample was checked for inhibition by adding a known quantity of non-target DNA to each sample. The assay consisted of 10 µL of Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix, 4 µL of eDNA sample, 2 µL of a tissue extract from great crested newts (*Triturus cristatus*), 2 µL of forward and reverse primers for our targeted species (*C. itambe*), as well as the forward and reverse primers for great crested newts (TCCBL and TCCBR; Thomsen et al., 2012) at a concentration of 10 µM /µL, in a final reaction volume of 20 µL. The qPCR and melt curve conditions replicated those stated above, with two negative control samples included in each qPCR run. Samples failing to amplify our target species' DNA or amplifying more than 1 cycle later than control samples were considered to contain PCR inhibitors.

Data analysis for species detection

We used a single-season occupancy model (MacKenzie et al., 2002) to estimate detection and occupancy rates from the observational data collected in the field. We used previously published detection histories (Barata et al., 2017) and included only bromeliads for which we had eDNA samples. Although occupancy models can accommodate covariates that explain both parameters (MacKenzie et al., 2002), we opted to run a null model (i.e., with no covariates). As our aim was to obtain overall estimates for comparisons without exploring covariates; given the small size of our dataset, we wanted to avoid overparameterisation of the model. We controlled for variation in detection in our data by using observations from a single experienced observer (Barata et al., 2017). We used a free online tool to analyse qPCR based eDNA data to generate occupancy and detectability information (https://blogs.kent.ac.uk/edna). This tool is based on Griffin et al. (2020) and uses a Bayesian framework to identify: the probability of species occupancy; stage 1 (the sample collection phase) true and false positive rates; and stage 2 (the laboratory phase) true and false positive rates. Stage 1 true positive rate (θ11) is the probability that a water sample collected from an occupied site includes DNA of the target species, with stage 1 false negative rate being the complement of this. Stage 1 false positive rate (θ10) is the probability that a water sample collected from an unoccupied site includes DNA of the target species. Stage 2 true positive rate (p11) is that an individual PCR replicate of a sample containing target DNA is positive, with stage 2 false negative being the complement of this. Stage 2 false positive rate (p10) is the probability that an individual PCR replicate of a sample that does not include DNA of the target species returns amplification. These differ from standard observational occupancy models as eDNA sampling is a two-phase process with potential for error to be introduced at both sample collection and sample analysis phases. Conversely, direct observation has a single phase where the species is either observed or not observed.

RESULTS

No target DNA was amplified from field or laboratory negative control samples by 40 qPCR cycles, and no sample demonstrated characteristics that indicated PCR inhibitors were present. All 11 samples collected from bromeliads with known species presence amplified target DNA, of which 10 showed amplification in all eight qPCR replicates, with the remaining sample showing amplification in seven of the eight qPCR replicates (Table 2). Additionally, one sample from a bromeliad with no known occupancy showed amplification in a single qPCR replicate (bromeliad 8125; Table 2). One sample from a previously unoccupied bromeliad had amplification in five of the eight replicates (bromeliad 7007; Table 2). However, this filter was damaged and leaked during transportation, possibly leading to contamination and a false positive. Therefore, we excluded this result from any further data analysis. These two eDNA positive but

Table 2. Water samples collected in the field for 19 bromeliads and two field negatives (FN) with sample number, characteristics of bromeliads (elevation given in metres above sea level, size given as bromeliad height in centimetres), and results from direct observations detection history (0 = absence and 1 = presence) and environmental DNA (eDNA) analysis (P = positive and N = Negative).

Sample number	Bromeliad		Direct observation				eDNA	eDNA positive replicates	Signs of inhibition
	Elevation	Size	2014	2015	2016	2017			
8131	2063.4	63	0	1	1	1	P	7/8	N
6940	2047.6	52	1	1	1	1	P	8/8	N
1149	2013.7	61	0	0	1	1	P	8/8	N
6950	1987.1	44	1	1	1	1	P	8/8	N
6983	2029.6	40	1	1	0	1	P	8/8	N
7015	1934.3	78	0	0	1	1	P	8/8	N
2222	1885.9	56	1	1	1	1	P	8/8	N
6963	1874.8	48	0	1	1	1	P	8/8	N
6994	1873.8	44	1	1	1	1	P	8/8	N
2802	1911.5	-	-	-	-	1	P	8/8	N
8161	1769.4	53	0	0	1	1	P	8/8	N
7007	2039.9	52	0	0	0	0	P	5/8	N
8125	1713.1	69	0	0	0	0	P	1/8	N
2168	2024.8	43	0	1	0	0	N	0/8	N
6929	1927.7	52	0	0	0	0	N	0/8	N
7014	1920.2	65	0	0	0	0	N	0/8	N
8070	1771.8	56	0	0	0	0	N	0/8	N
8164	1841.7	62	0	0	0	0	N	0/8	N
6981	1716.6	62	0	0	0	0	N	0/8	N
FN 1	1597.1	-	-	-	-	0	N	0/8	N
FN 2	1558.9	-	-	-	-	0	N	0/8	N

Table 3. Parameter estimates from occupancy models derived from direct observational surveys and eDNA samples with multiple qPCR replicates (for observational surveys using occupancy models, CI = confidence interval; for eDNA samples using Bayesian framework, CI = credible intervals).

Parameter	Estimate	CI		Description
		Upper	Lower	
Observational surveys				
Ψ	0.61	0.38	0.80	Occupancy estimated from observational survey data
p	0.77	0.62	0.88	Detection probability for visual night encounters
eDNA sample				
Ψ	0.61	0.38	0.81	Occupancy estimated from eDNA samples
θ10	0.04	0.00	0.20	Stage 1 (sample collection) false positive rate
θ11	0.97	0.85	1.00	Stage 1 true positive rate
1-θ11	0.03			Stage 1 false negative rate (given by 1-θ11)
p10	0.02	0.00	0.07	Stage 2 (laboratory analysis) false positive rate
p11	0.98	0.94	1.00	Stage 2 true positive rate
1-p11	0.02			Stage 2 false negative rate (given by 1-p11)

observationally negative bromeliads were both found within the existing known range of the species. Overall, we had a naïve occupancy rate of 66.7 % using the eDNA method (12 positives out of 18 samples), compared to 61.1 % for direct observational surveys (11 positives out of 18 surveyed sites).

From direct observational survey data, the occupancy rate was estimated to be 0.61 and detectability was 0.77 (Table 3). From eDNA analysis, a bromeliad occupancy rate of 0.61 was estimated. A false positive rate at sample collection (θ10) of 0.04 was found, with a true positive rate at sample collection (θ11) of 0.97, equivalent to a 3 % false negative rate (Table 3). This compared to the false positive rate at the laboratory analysis stage (p10) of 0.02 and true positive rate at the laboratory analysis stage (p11) of 0.98, equivalent to a 2 % false negative rate (Table 3). The conditional probability of detection analysis given by the number of amplified qPCR replicates (Fig. 2) shows the probability that an occupied site is wrongly classified as unoccupied in relation to the

number of samples which amplify. Our results suggest a high probability of false positive detection for any sample amplifying with fewer than three positive qPCR replicates. Additionally, when five or more of the eight replicates amplify, we can be confident that the site is indeed occupied. We also observed that there is little gain in occupancy estimate with this additional effort (Fig. 2).

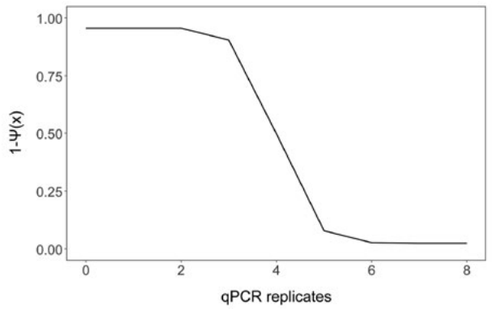

Figure 2. Posterior conditional probabilities of species absence (1- Ψ (x)) given by the number of amplifying qPCR replicates.

DISCUSSION

We found that eDNA from bromeliad tanks, including sample collection (stage 1) and laboratory analysis (stage 2), is highly reliable, with very low levels of error for both false negatives and false positives (false negative: stage 1 = 3 %; stage 2 = 2 %; false positive: stage 1 = 4 %; stage 2 = 2 %). Higher error rates were observed for commercial eDNA surveys for great crested newts in ponds within the UK, from both sample collection (stage 1: false positive rate = 15 %; false negative rate = 27 %) and laboratory analysis (stage 2: false positive = 5 %; false negative = 19 %) (Griffin et al., 2020). Phytotelm-breeding species are often elusive and difficult to detect using visual surveys, and occasionally require destructive sampling methods such as removal of plants (Brozio et al., 2017; Torresdal et al., 2017). In these cases, eDNA is a reliable non-invasive method that detects species presence with very low error rates (i.e., low false positive/negative rates). With a single set of eDNA samples we were able to accurately detect species presence in every bromeliad confirmed to be occupied through repeated direct observations, as well as in one bromeliad where species occupancy had not been identified. However, this additional detection had only a single positive qPCR replicate which is highly likely a case of false positive detection, as indicated by the conditional probabilities analysis.

We have demonstrated the feasibility of collecting eDNA samples in a remote setting and transporting them to a laboratory for analysis. Despite one sample being damaged in transit and the potential false positive result described above, it was possible to maintain a contamination-free environment during sample collection and transport as demonstrated by the absence of amplification in the samples of water collected from streams close to the study site. It is also evident from the high proportion of qPCR replicates amplifying in the confirmed positive samples that for a species which spends a large part of its life cycle within the phytotelm (such as a bromeliad), sampling only a small volume of water is not a limiting factor for the recovery of target DNA. Whilst we present the results from a relatively small sample size, we successfully demonstrate the feasibility of extracting and amplifying DNA from water samples as little as 120 ml. The conditional probability of species detection analysis showed that above five qPCR positive replicates, there is limited gain in the estimate of occupancy. Therefore, the number of qPCR replicates may be excessive and a reduction in laboratory replication may be possible without reducing the occupancy estimate; however further analysis would need to be undertaken to confirm this observation which would require a larger data set.

A major advantage of the use of eDNA methods in remote locations is that it can reliably confirm the presence of species in bromeliads, even when bromeliads have high UV exposure, such as at the mountaintop described here. In this scenario, eDNA surveys would have wide-ranging benefits compared to multiple observational visits. Firstly, it is challenging and costly to access remote areas, requiring an experienced team of observers within an expedition that lasts a number of days. Secondly, our described method reduces bias caused by variation in observer experience (Barata et al., 2017) and is sensitive to different life stages (e.g., eggs and larvae; Zinger et al., 2020) that can be missed during visual surveys. Thirdly, eDNA is a non-invasive method that can reduce environmental impact associated with direct observations (Brozio et al., 2017; Torresdal et al., 2017). Most importantly, because high prevalence of chytrid fungus can be found in bromeliads occupied by frogs (Ruano-Fajardo et al., 2016), reduced visits could also decrease the potential risks associated with the spread of wildlife pathogens by the survey team, while eDNA samples can also be reanalysed for the pathogen (Schmidt et al., 2013).

Despite the very high detection rates from eDNA samples, estimated occupancies using eDNA and direct observations were similar. This has implications for the use of eDNA methods for occupancy monitoring since eDNA analysis has laboratory and consumable costs above those incurred in direct observational surveys. For monitoring purposes, estimates of species occupancy can be available from a detection history, which would require multiple visits, and/or eDNA samples with laboratory replication. For our target species, an increase in detection does not improve statistical power and four visits are required to detect a change of 30 % in the population using direct observation (Barata et al., 2017). In our case study, the mean costs per bromeliad sampled by non-invasive observational surveys (four nights with a team of two people, 21 bromeliads = £21.29 per bromeliad) is lower than the costs per sample for a single set of eDNA analysis (a one-day visit by one person, two days of lab work by one person, and laboratory supplies, 21 bromeliads = £54.92 per bromeliad). Nonetheless, in cases where destructive sampling is required, the higher financial costs of eDNA methods could be outweighed by the costs to biodiversity conservation for habitat integrity. Therefore, a case-by-case cost-benefit analysis is recommended to ascertain whether the cost of running a full set of eDNA surveys offsets the cost of the observational survey visits.

A number of factors may influence the persistence of eDNA within the bromeliad phytotelm. Firstly, the ratio of target species biomass to available water volume in the phytotelm may influence the concentration of DNA in the water and therefore the sample. The ratio of the biomass of the species per unit volume of the water within the phytotelm will be high compared to other eDNA studies investigating species in more typical ponds, lakes and streams. Also, the volume of water retained in a bromeliad varies with the shape and size of a plant, reaching up to 2 L of water per plant in dry environments (Cogliatti-Carvalho et al., 2010). Considering *C. itambe* measures up to 18 mm in length with a mass of about 2 g, this would mean a biomass to water ratio of 1:1000 for one individual per plant. This is a much higher ratio than that encountered in eDNA surveys for larger amphibians in ponds or lakes (e.g. 10 g great crested newts in a 600 m^2 pond area (Jehle et al., 2011; Oldham et al., 2000), where a conservative biomass to water ratio would be 1:1,000,000 for 50 individuals, in a 500,000 L pond). This high ratio may contribute to the high amplification rate in positive samples, with most positive samples showing either seven or eight positive qPCR replicates.

Secondly, the degree to which the species is dependent on the phytotelm can influence DNA release into the water. If the species resides permanently in the bromeliad, more DNA may be released into the water than if it was a temporary visitor. Equally, the concentration of DNA within a sample may influence the chance of detecting the target DNA. Experimental studies could explore the relationship between persistence of eDNA in a phytotelm after introducing and removing individuals from water tanks to define an optimum time to detection. Finally, for pond breeding amphibians, surveys using eDNA methods can also account for a wide range of covariates associated with both the pond and the target species (Barnes et al., 2014), such as substrate type (Buxton et al., 2018) and seasonality (Buxton et al., 2017). For bullfrog (*Lithobates catesbeianus*) tadpoles in a controlled environment, degradation rates were lowest under low UV-B radiation and cold temperatures (Strickler et al., 2015). In a phytotelm, these external factors are likely to include seasonality, temperature and UV radiation exposure, particularly at high altitudes where UV rates are higher than at sea level.

We conclude that there are numerous advantages in the use of eDNA to survey cryptic species in remote locations, and to identify species presence with high detectability and low error rates. Factors influencing persistence of eDNA in small water bodies need to be further investigated to fully understand the challenges and limitations of applying eDNA methods within a phytotelm environment. The method has potential to uncover the extent of the distribution of many elusive phytotelm-breeding species with reduced expedition costs and environmental impacts. eDNA surveys are considered a promising method for amphibian monitoring regardless of species rarity (Burns et al., 2020) or population density (Lopes et al., 2017). However, the use of eDNA method for monitoring occupancy of phytotelm-dependent species will likely improve as the method becomes more cost-effective and we have a better understanding of the factors affecting detection probability in such environments. Our conclusions are applicable to other phytotelm-dependent species, but the feasibility of the method could vary with species' life history and the volume of water available for analysis.

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Reconstructions of the past distribution of *Testudo graeca* mitochondrial lineages in the Middle East and Transcaucasia support multiple refugia since the Last Glacial Maximum

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A cycle of glacial and interglacial periods in the Quaternary caused species' ranges to expand and contract in response to climatic and environmental changes. During interglacial periods, many species expanded their distribution ranges from refugia into higher elevations and latitudes. In the present work, we projected the responses of the five lineages of *Testudo graeca* in the Middle East and Transcaucasia as the climate shifted from the Last Glacial Maximum (LGM, Mid – Holocene), to the present. Under the past LGM and Mid-Holocene bioclimatic conditions, models predicted relatively more suitable habitats for some of the lineages. The most significant bioclimatic variables in predicting the present and past potential distribution of clades are the precipitation of the warmest quarter for *T. g. armeniaca* (95.8 %), precipitation seasonality for *T. g. buxtoni* (85.0 %), minimum temperature of the coldest month for *T. g. ibera* (75.4 %), precipitation of the coldest quarter for *T. g. terrestris* (34.1 %), and the mean temperature of the driest quarter for *T. g. zarudyni* (88.8 %). Since the LGM, we hypothesise that the ranges of lineages have either expanded (*T. g. ibera*), contracted (*T. g. zarudnyi*) or remained stable (*T. g. terrestris*), and for other two taxa (*T. g. armeniaca* and *T. g. buxtoni*) the pattern remains unclear. Our analysis predicts multiple refugia for *Testudo* during the LGM and supports previous hypotheses about high lineage richness in Anatolia resulting from secondary contact.

Keywords: *Testudo graeca*, niche modeling, Last Glacial Maximum, Middle East, Transcaucasia

INTRODUCTION

The spur-thighed tortoise, *Testudo graeca* complex (sensu Parham et al., 2006), has a wide distribution (Fig. 1) across a highly diverse landscape (e.g. from semi deserts, grasslands, traditional agricultural areas, shrublands and mixed forest). Previous studies have shown that, across this broad range, Pleistocene climate fluctuations and ecological conditions created profound morphological differences, especially in the Asian (Middle Eastern and Caucasian) parts of the range (Türkozan et al., 2010, 2018). Molecular studies revealed discordance between morphological variation and six mitochondrial clades (Parham et al., 2006; Fritz et. al., 2007; Türkozan et al., 2010; Mikulíček et al., 2013; Türkozan et al., 2018). The matter is complicated by the fact that specimens assigned to some mitochondrial DNA (mt hereafter) clades are morphologically distinct within some areas and not others (e.g., the mt clade that corresponds to "*T. g. armeniaca*" includes individuals that are phenotypically highly specialised only in Anatolia) and also that some mt lineages (e.g. those corresponding to "*T. g. zarudnyi*" of eastern Iran) require additional phenotypic study. Following Türkozan et al. (2010, 2018), we do not subscribe to the mitochondrial subspecies of Fritz et al. (2007). Still, for comparability purposes, we do continue to refer to the mt clades that are associated with the different mt lineages by their subspecific epithet (e.g. *armeniaca*, *buxtoni*). Meanwhile, one of the few analyses of nuclear markers (Mikulíček et al., 2013) shows some concordance with the mtDNA clades, suggesting that the mt lineages represent actual evolutionary lineages. Despite these unresolved taxonomic issues, comparisons of genetic variation and the application of ecological niche modeling to mitochondrial lineages have helped develop hypotheses about the paleobiogeographic history of the *T. graeca* complex in different parts of its range (Anadón et al., 2015; Graciá et al., 2017; Javanbakht et al., 2017).

There is clear evidence that the western range of the *T. graeca* complex has been contracting and expanding through time, driven by climate with the modifying effect of lithology and topography (Anadón et al., 2007; Graciá et al., 2017). Anadón et al. (2015) found apparent niche differences among five mitochondrial lineages of *T. graeca* in Africa (sublineages of the graeca mt clade in our terminology), with rainfall playing a primary role in

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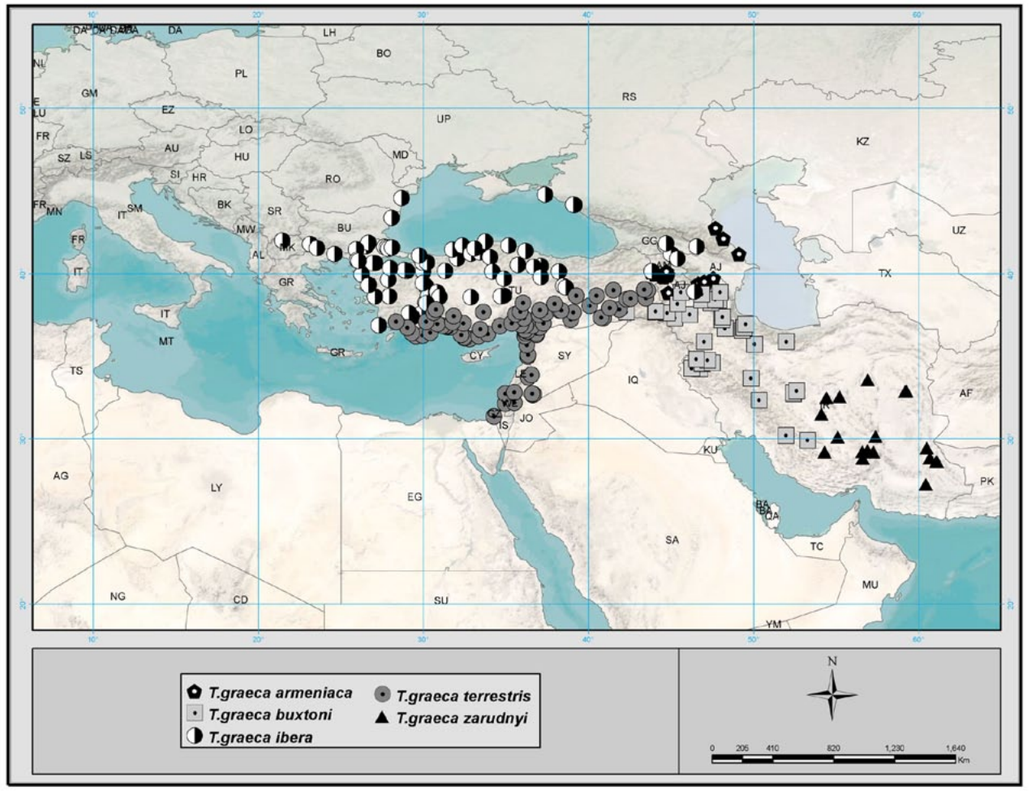

Figure 1. Occurrence records used for modeling *T. graeca* mt lineages in Maxent. Data were compiled from previously published papers (Parham et al., 2006; Fritz et al., 2007; Parham et al., 2012; Mashkaryan et al., 2013; Javanbakht et al., 2017; Türkozan et al., 2018)

shaping their distribution. In contrast, a comparison of the current distribution patterns and the reconstructed historical ranges of *T. graeca* hypothesised that the distribution ranges of the three mitochondrial lineages in Iran and Transcaucasia had not changed substantially since the Last Glacial Maximum (LGM; Javanbakht et al., 2017). In this work, we used distribution records of previous studies and our records to reassess past range dynamics in the eastern part of the range of the *T. graeca* complex (Middle East, Anatolia, and Transcaucasia). Our study differs from previous studies by including a large missing distribution of *T. graeca* from Anatolia.

MATERIAL & METHODS

We used the maximum entropy machine-learning algorithm Maxent version 3.4.1 (Phillips et al., 2006; Phillips & Dudík, 2008; Elith et al., 2011) to predict the geographically suitable habitats for *T. graeca* mt lineages in the present, LGM and Mid-Holocene. All GIS operations were conducted using ArcGIS 10.6 (www.arcgis.com) and SDMtoolbox 2.4 (Brown, 2014). Presence data for *T. graeca* lineages were compiled from previously published papers (Parham et al., 2006; Fritz et al., 2007; Parham et al., 2012; Mashkaryan et al., 2013; Javanbakht et al., 2017; Türkozan et al., 2018). However, we omitted

Table 1. Bioclimatic variables, bold variables are the subset used in ENMs

Bioclimatic Variable	Description
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

the record of *T. terrestris* from Sicily in our analyses since this population has been introduced (Fritz et al., 2007). A total of 247 occurrence records were used for modeling across the distribution range of each mt clade (85 *ibera*; 41 *buxtoni*; 81 *terrestris*; 22 *armeniaca*; 18 *zarudnyi*). The genetic assignment of each sample was confirmed by Turkozan et al. (2018). Data consisting of 19 bioclimatic variables for present (1950-2000), LGM (~ 22000 years ago) and Mid-Holocene (~6000 years ago) were downloaded from WorldClim database (Hijmans et al., 2005; www.worldclim.org) at a resolution of 2.5 arc minutes (approx. 4.5 km at the equator). The 19 bioclimatic variables derived from monthly temperature and precipitation values (for detailed information, see www.worldclim.org/bioclim). To identify and remove highly correlated variables, the Remove Highly Correlated Variables option in SDMtoolbox version 2.4 was used (Brown, 2014), and a maximum 0.8 correlation coefficient was allowed (Feldman et al., 2017). We used the subset of bioclimatic variables in ENMs (Table 1). We used the permutation importance to evaluate the relative contributions of bioclimatic variables.

To predict the suitable distribution habitats of lineages during the LGM and Mid-Holocene, CCSM4, MIROC-ESM, and MPI-ESM-P general circulation models (GCM) (see WorldClim database for further information) were used. The logistic values of three different GCM simulations were averaged to summarise predictions for the past. All bioclimatic variables were masked to cover the range of distribution of the species (as in Soberón & Peterson, 2005) for ENM.

As described before (Merow et al., 2013), MaxEnt contrasts presence data against background data where presence and absence are not measured. The issues related to MaxEnt background selection during model application are described before (Brown et al., 2016; Elith et al., 2011; Merow et al., 2013). To reduce the issues related to background selection, Minimum Convex Polygons (MCP) with presence data were created for background selection for each *T. graeca* lineage (by Background Selection via Bias File option in SDMtoolbox). The home range of *T. graeca* is 3 to 10 hectares (Cobo & Andreu, 1998; Attum et al., 2011; Anadón et al., 2012) and so a 1 km buffer distance was selected for MCPs.

Identifying the optimum model parameters for model performance of MaxEnt (Elith et al., 2011; Merow et al., 2013) was conducted by spatial jackknifing (k-fold cross-validation, k = 3) in SDMtoolbox. Multiple models with different feature class (FC) combinations (1=linear; 2=linear, quadratic; 3=hinge; 4=linear, quadratic, and hinge; 5=linear, quadratic, hinge, product, threshold) and regularisation multipliers (RM) (from 0.5 to 5 with 0.5 increments) were tested. The "minimum training presence" threshold was used during analysis. The best model criteria were the Omission error rate, then AUC in Spatial Jackknifing. After optimising Maxent models for each lineage, final models with optimised parameters were created. To quantify the niche overlap of *T. graeca* mt lineages, ENMTools 1.4.4 (Warren et al., 2010) software was used. The output ascii files of ENMs were used for niche overlap analysis. Niche overlap in ENMTools is calculated via Schoener's D (D); ranging from 0 (no similarity) to 1 (complete overlap) (Warren et al., 2008). An identity test was also performed, which is an indicator that the ENMs of the two species are more different from expected by chance. Identity test results show the degree of niche overlap when samples are from the same distribution. Comparisons of the D values of identity tests with the D values of Niche overlap analysis of actual data demonstrate whether populations are different. The accuracy of the ENM's was performed by Area Under the Curve (AUC) of the Receiver Operating characteristic Curve (ROC) (Fielding & Bell, 1997). An AUC > 0.5 indicates that the model performs better than a random prediction (Gassó et al., 2012). AUC is a reliable assessment method because it is not affected by choice of threshold (Fois et al., 2018; Yi et al., 2016).

RESULTS

The best model parameters determined by Spatial Jackknifing for each lineage were as follows; *armeniaca* FC=linear – RM=3.5, *buxtoni* FC=linear - RM=5, *ibera* FC=linear – RM=4, *terrestris* FC=linear, quadratic – RM=5, *zarudnyi* FC=linear – RM=5. According to Area Under the Curve (AUC) of the Receiver Operating characteristic Curve (ROC) values of ENMs, all models performed better than a random prediction (*armeniaca*: 0.83, *buxtoni*: 0.65, *ibera*: 0.73, *terrestris*: 73, *zarudnyi*: 0.75). The most significant bioclimatic variables in limiting the potential distribution of clades are Precipitation of Warmest Quarter (BIO 18) for *armeniaca* (95.8 %), Precipitation Seasonality (Coefficient of Variation) (BIO 15) for *buxtoni* (85 %), Min Temperature of Coldest Month (BIO 6) for *ibera* (75.4 %), Precipitation of Coldest Quarter (BIO 19) for *terrestris* (34.1 %) and Mean Temperature of Driest Quarter (BIO9) for *zarudyni* (88.8 %). The predictions for the reconstructed past (mid-Holocene and LGM) and present bioclimatic conditions (Fig. 2) suggested that the *armeniaca* clade survived in a potential refugium in the Caucasus and Central Anatolia and expanded its range from this refugium. Its ultimate possible range included north-eastern Anatolia and some parts of the Black Sea region during the mid-Holocene before the range retracted its distribution to Caucasus again as at present. The model also suggests additional suitable areas in central and northeastern Anatolia where these areas are currently occupied by *ibera* clade. In contrast, *buxtoni* may have survived the LGM in the Zagros, Caucasus, Elburz Mountains, Anatolia, and coastal parts of Greece and Bulgaria, but after the LGM, suitable areas included almost all of Anatolia, Greece, part of Macedonia and Albania during the mid-Holocene and contracted its range to south-eastern Anatolia and the Zagros mountains. The model suggests additional suitable areas in north-eastern Turkey and central Anatolia, European Turkey, the coastal belt of Greece, and some parts of Macedonia and Montenegro. Exceptionally, the potentially suitable areas for *ibera* during the LGM include the coastal belt of central and eastern Black Sea coast of Anatolia, some parts of the Levant, Northern Cyprus, Aegean Islands including Crete, and Greece. After the LGM, during the mid-Holocene,

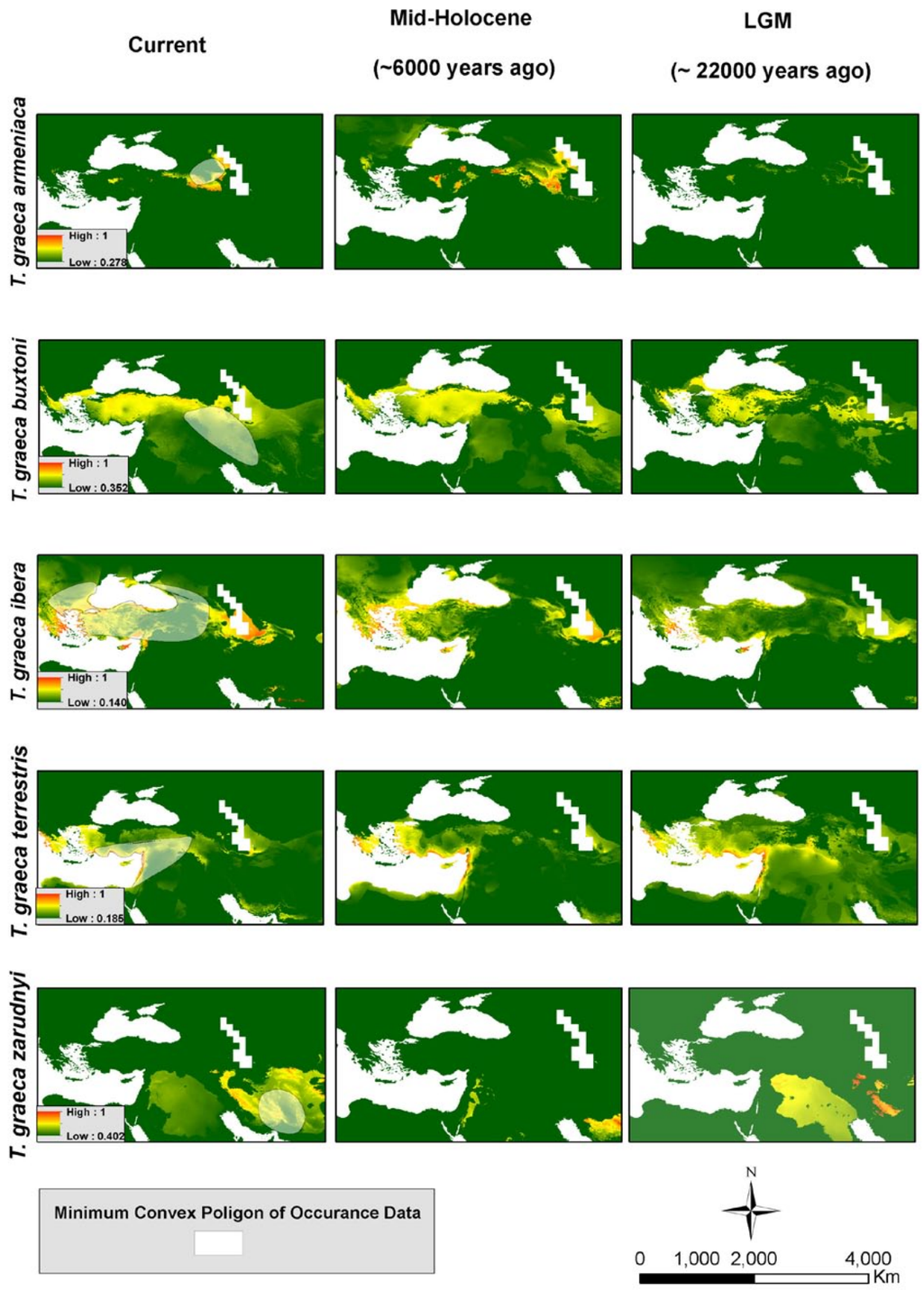

Table 2. Predicted habitat suitability maps of *T. graeca* lineages for Current, Mid- Holocene, and LGM

Table 2. Permutation contribution percentage of biological variables to the distribution of *T. graeca* mt lineages.

	Permutation Contribution (%)				
Variable	*ibera*	*terrestris*	*buxtoni*	*armeniaca*	*zarudyni*
BIO2	-	18.5	-	-	-
BIO3	0.4	-	-	-	-
BIO4	-	-	-	4.2	-
BIO5	-	-	-	-	11.2
BIO6	75.4	-	-	-	-
BIO8	9.5	2.7	-	-	-
BIO9	-	29.6	15.0	-	88.8
BIO15	1.7	-	85.0	-	-
BIO18	-	12.2	-	95.8	-
BIO19	13.0	34.1	-	-	-

Table 3. Schoener's D values of niche overlap scores.

Lineages	*armeniaca*	*ibera*	*terrestris*	*zarudnyi*	*buxtoni*
armeniaca	1.0000	0.2529	0.0882	0.0580	0.1618
ibera	x	1.0000	0.3138	0.0874	0.3115
terrestris	x	x	1.0000	0.0515	0.3473
zarudnyi	x	x	x	1.0000	0.2712
buxtoni	x	x	x	x	1.0000

Table 4. Minimum and maximum values of Schoener's D scores of identity tests.

Lineages	*armeniaca*	*ibera*	*terrestris*	*zarudnyi*	*buxtoni*
armeniaca	1.0000	0.5666 – 0.7842	0.5460 – 0.7870	0.4669 – 0.7457	0.5460 – 0.7870
ibera	x	1.0000	0.6639 – 0.8397	0.4309 – 0.7748	0.6531 – 0.8341
terrestris	x	x	1.0000	0.3839 – 0.7661	06120 – 0.8220
zarudnyi	x	x	x	1.0000	0.5379 – 0.7908
buxtoni	x	x	x	x	1.0000

suitable areas included the Caucasus, Aegean coasts of Anatolia, southern Cyprus, and the Balkans. The current distribution of *ibera* includes most of the suitable mid-Holocene range except the north-eastern Black Sea coast and Cyprus where no Testudo currently occur. The model suggests additional appropriate areas along the coastal belt of eastern Mediterranean and Levant where *terrestris* occurs presently. The models indicate that the *terrestris* clade may have survived along the Mediterranean coastal belt of Anatolia, including the Levant region and island of Cyprus, western coast of Greece, and Albania. The hypothesised suitable areas remained stable during the mid-Holocene and then retracted. The model suggests additional appropriate areas on the west coast of Anatolia, the west coast of Greece and Albania, Caucasus, and the island of Cyprus. The *zarudyni* clade may have survived in the Zagros Mountains and north-west Syria. The hypothesised suitable range contracted to the southern Zagros Mountains and the Dasht-e Lut during the mid-Holocene and remained in that region until recently. The model suggests additional appropriate areas at Kopet Mountains and Afghanistan where they do not occur.

Schoener's D Values for niche overlap scores between lineages are given in Table 3. Niche overlap scores appear to be compatible with the known distribution of species. The highest overlap values (D>03) were observed between the pairs of *ibera – terrestris*, *ibera – buxtoni*, and *terrestris – buxtoni* clades. The lowest overlap score was 0.08 between *armeniaca* and *terrestris* clades. Clearly, *zarudnyi* occupy a differentiated niche, among others (Table 3).

Minimum and maximum Schoener's D values of 100 replicates of identity test for *T. graeca* mt lineages are given in Table 4. The results indicate that ENMs of the species are more different than expected by chance because, for any pair of species, the Schoener's D values are higher than niche overlap values (Schoener's D value of Niche overlap analysis for *ibera-terrestris* pair is 0.3138 and Schoener's D value of identity test for the same pair is between 0.6639 and 0.8397). The potential distributions of clades at present is generally in line with the known distribution ranges of the clades.

DISCUSSION

The ecological niches of mt clades were similar, with temperature extremes and precipitation related variables playing the most crucial role in determining suitable habitats (Table 2). Similar results were presented for the western range of *T. graeca* lineages in Africa (Anadón et al., 2015). In west Asia, Javanbakht et al., (2017) found that *armeniaca*, *buxtoni*, and *zarudyni* mt clades did not significantly expand their distribution after the LGM, with precipitation delimiting the distribution. However, it seems that the authors erroneously interpreted their results by not understanding that in principal component analyses, both negative and positive principal components loadings have the same power, and the negative value shows only the direction of the relationship. As shown in Javanbakht et al. (2017: Table 3), temperature related factors (maximum temperature of warmest month BIO 5 and mean temperature of the coldest quarter BIO 10) were more responsible for delimiting the distribution of the lineages than precipitation. Therefore, the combination of temperature extremes and precipitation appears to shape the distribution pattern of *T. graeca*. However, it is not the only factor shaping the distribution of the species but also the biotic factors, accessibility of the region, and evolutionary capacity of the population to adapt to new conditions (Soberón & Peterson, 2005). This explains why some clades are missing from suitable niches during the mid-Holocene and the present time. Extensive deserts in Iran with arid climate (Javanbakht et al., 2017) and the Anatolian Diagonal with a steep environmental gradient associated with temperature seasonality (Gür, 2016) seem to be shaping the distribution of *T. graeca* in the east and mainland Anatolia. The range of *T. graeca* covers areas with rainfall values of 800-1200 mm (north-western Africa; Anadón et al., 2015) to arid conditions in semideserts in Iran, which is a sign of ecological plasticity (Javanbakht et al., 2017). Such plasticity was recently represented for *T. armeniaca* populations (Arakelyan et al., 2018) with a low domed shell shape living in the burrows, and high domed tortoises living in the steppes. The climate was humid and cold during LGM in Anatolia, and forest vegetation covered 80-90 % of the land cover in north-western Anatolia and the Black Sea coast and 50 % of the Mediterranean coast. Similarly, eastern Anatolia and western Iran were cold and arid during the LGM (Şenkul & Dogan, 2013). During the Pleistocene, glacier development within Anatolia was limited to higher mountain peaks (Atalay, 1996) while the lowlands remained open, developing steppe communities (Michaux et al., 2004). This provided suitable habitats for temperate species to survive the LGM (Rokas et al., 2003; Fritz et al., 2009). During the Holocene, humidity and temperature increased, and present day climatic conditions in eastern Turkey and Lake Van region probably started 8200 years ago (Wick et al., 2003). It is therefore probable that low genetic distance among Testudo clades (Turkozan et al., 2018) may be the result of a very recent distribution pattern.

The distribution model of *T. graeca* clades in the present work are in line with the classical glacial range contraction and interglacial range expansion model (Stewart et al., 2010) except the *zarudyni* clade which contracted during the interglacial period. Our analysis supports multiple potential refugia during LGM, namely Caucasus, Anatolia, and Balkans. This is in line with the concept that temperate adapted taxa are confined to southern refugia (Stewart et al., 2010). These refugia are well defined for other species in the Balkans, Anatolia, and the Caucasus during glacials (Hewitt, 2000; Joger et. al., 2007; Wielstra et. al., 2013).

During the Quaternary ice ages, Anatolia is known to have served as a vital refugium for species that later expanded their distributions during interglacial periods to Europe and Caucasus (Hewitt, 2000; Gür, 2013; Korkmaz et al., 2014). Anatolia is the region where three of the world's 35 biodiversity hotspots meet and interact, namely the Caucasus, Irano-Anatolian, and Mediterranean hotspots (Mittermeier et al., 2004). The north-east and south-west orientation of the Anatolian Diagonal provided a continuous mountain chain connection between the Mediterranean and the Caspian Sea that is linking the southern Taurus diversity hotspot to the nearby Caucasus and Near East mountain system. This connection was used as a corridor for the expansion out of Anatolia during a favorable glacial period by other species such as the Anatolian mountain frogs (*Rana macrocnemis* group) and oriental green lizards (*Lacerta trilineata* group) (Veith et al., 2003; Ahmadzadeh et al., 2013). Similarly, high lineage diversity in Anatolia is clearly due to secondary contact after range expansion, and Anatolia is likely an essential corridor for this. On the other hand, the Anatolian diagonal can also be an environmental barrier with a steep ecological gradient associated with temperature seasonality (Gür, 2016).

The south-eastern Taurus Mountains are located on the junction between Turkey's Taurus Mountain and Iran's Zagros mountains, where *the buxtoni* mtDNA clade is limited by forest steppe. This clade comes into close contact with the *terrestris* mtDNA clade (Türkozan et al., 2018) in southern Anatolia. Together, the Taurus-Zagros range separates the Anatolian-Iranian plateau from the Mesopotamian Lowlands (Sarıkaya et al., 2011). These physical barriers, together with precipitation and temperature related factors, help to delimit the distribution of *Testudo graeca*.

The models predict range expansion for most clades, except *terrestris* and *zarudyni*, in the mid-Holocene, which is typical for other species of vertebrates in Anatolia and the European Peninsula (Taberlet et al., 1998; Hewitt, 2000; Schmitt, 2007). Based on our model, *T. graeca* retreated to glacial refugia during the LGM and expanded its range during the mid-Holocene. However, this is not the case for *zarudyni* clade, which occupied a broader distribution range during LGM and contracted during the mid-Holocene. Javanbakht et al. (2017) also showed a range contradiction in *zarudyni* clade. In conclusion, our study supports multiple refugia for *Testudo* during LGM and that Anatolia was an important corridor for the range expansion of *Testudo graeca*.

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The effects of two calcium supplementation regimens on growth and health traits of juvenile mountain chicken frogs (*Leptodactylus fallax*)

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The mountain chicken frog (*Leptodactylus fallax*) is among the 42 % of amphibians threatened with extinction and is dependent upon ex situ populations to recover in the wild. Amphibian captive husbandry is not fully understood and empirical data are required to optimise protocols for each species in captivity. Calcium metabolism and homeostasis are areas of importance in captive husbandry research and have been identified as a challenge in maintaining ex situ populations of *L. fallax*. We trialled two frequencies (twice and seven times weekly) of calcium supplementation via dusting of feeder insects in two groups of *L. fallax* juveniles and measured growth and health effects through morphometrics, radiography, ultrasonography and blood and faecal analysis over 167 days, followed by a further 230 days of monitoring on an intermediate diet informed by the initial dataset. We showed that supplementation treatment did not affect growth or health status as measured through blood analysis, radiography and ultrasonography. More frequent supplementation resulted in significantly more radiopaque endolymphatic sacs and broader skulls. Frogs fed more calcium excreted twice as much calcium in their faeces. The intermediate diet resulted in previously lower supplementation frogs approximating the higher supplementation frogs in morphometrics and calcium stores. Comparison with radiographic data from wild frogs showed that both treatments may still have had narrower skulls than wild animals, but mismatching age class may limit this comparison. Our data may be used to inform dietary supplementation of captive *L. fallax* as well as other amphibians.

Keywords: Amphibia, amphibian, anuran, diet, nutrition, zoo, calcium

INTRODUCTION

The international conservation response (Wren et al., 2015) to the global amphibian extinction crisis (Stuart et al., 2004; IUCN, 2020) involves the establishment of ex-situ populations for conservation, research and education. Unfortunately, data on optimal husbandry practices and natural history are often lacking (Michaels et al., 2014a; Tapley et al., 2015a). In a few species of amphibian, multiple investigation streams are converging to highlight these taxa as emerging conservation model species. The large, terrestrial and Critically Endangered mountain chicken frog (*Leptodactylus fallax*) is endemic to Montserrat and Dominica in the Eastern Caribbean (IUCN SSC, 2017) and is one such species. It has been the focus of well-rounded research and conservation activity in- and ex-situ (Adams et al., 2014; Tapley et al., 2014). Outputs include description and quantification of population trends (Hudson et al., 2016a), disease dynamics and mitigation (Hudson et al., 2016b; Hudson et al., 2019), translocation attempts in the field (Adams et al., 2014), local cultural associations (Nicholson et al., 2020), life history and reproductive data (Gibson et al., 2004), the development of field methods, captive husbandry protocols (Nicholson et al., 2017; Jameson et al., 2019) and the empirical quantification of some of the requirements of this species in captivity from captive populations (Tapley et al., 2015b; Jayson et al., 2018a; b). Importantly, these data streams have been derived from co-ordinated approaches between captive and field components, in line with the One Plan Approach of the IUCN Conservation Planning Specialist Group; however, there is much work to be done to optimise captive husbandry protocols.

The provision of adequate nutrition is a current challenge in amphibian husbandry (Dugas et al., 2013; Gagliardo et al., 2008; Antwis & Browne, 2009; King et al., 2011; Verschooren et al., 2011; Ogilvy et al., 2012; Michaels et al., 2014b; 2015). The physiological requirements of most amphibians for a suite of different nutrients are unknown (Jayson et al., 2018a) and only a narrow range of prey items are available to feed captive

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Table 1. Design, and thermal and photo parameters of enclosures used to house *L. fallax* in this study. Detail is given to heating and lighting arrays due to their impact on calcium metabolism.

Enclosure	Animals held	Study days occupied	Dimensions (LxWxH)/m	Furnishings	Heating and Lighting array	Thermo- and photo-gradients
1	Parents --- Study animals	N/A --- 200-398	2 x 2 x 3	Artificial planting, over-turned plastic dog-beds, clay-lined nest box. Water dishes provided. Bark chip substrate.	300 W Ultravitalux lamp (Osram, Germany) LightWave T5 lighting unit (Growth Technology, UK) with 2x 54W D3+ 12 % T5 HO lamps (Arcadia, UK) and 6 x 54W Prolite T5 HO lamps (Phillips, Germany).	Basking zone temperature: 29–31 ˚C Ambient temperature: 26–27 ˚C Cool zone temperature: 23–25 ˚C Nocturnal ambient temperature: 21–23 ˚C UVI: 0–3 correlated with heat Photoperiod: 12:12
2	Study animals	0-200	0.875 x 0.655 x 0.705	Leaflitter and cork flats. Water dish provided Peat-free compost substrate.	160 W mercury vapour lamp (Arcadia, UK) LightWave T5 lighting unit with 1 x 24 W 12% T5 HO D3+ 12 % T5 HO lamp (Arcadia, UK) and 3 x 24WProlite T5 lamps (Phillips, Germany)	Basking zone temperature: 29–34 ˚C Ambient temperature: 25–26 ˚C Cool zone temperature: 23 ˚C Nocturnal ambient temperature: 23 ˚C UVI: 0–3 correlated with heat Photoperiod 12:12

UVI = UV Index

amphibians (Michaels et al., 2014b). Calcium metabolism is a case in point as it relies on the appropriate replication of sunlight (specifically UVB radiation and heat) and the provision of dietary calcium, phosphorus and vitamin D3 sources in appropriate amounts and combinations (Antwis & Browne, 2009; Baines et al., 2016). This is further complicated by the tendency for commercially raised invertebrate species to be calcium-deficient in both absolute terms and relative to phosphorus content (Barker et al., 1998; Finke et al., 2002; Jayson et al., 2018a). In *L. fallax*, comparison of wild and captive diets (Jayson et al., 2018a) and empirical refinement of UVB provision (Tapley et al., 2015b) have demonstrated that supplementation of diets with calcium along with appropriate provision of UVB lighting is important to meet the needs of the species in captivity. However, empirical data regarding the optimal amount of supplementation are lacking, and the complexity of factors influencing calcium metabolism precludes simple replication of wild diets (see Michaels et al., 2014b; 2015). Moreover, husbandry professionals tend to be particularly focussed on avoiding calcium deficiency for captive amphibians due to the high frequency of nutritional secondary hyperparathyroidism (NSHP) commonly known as nutritional metabolic bone disease (NMBD) in anurans (Wright & Whitaker, 2001), and in *L. fallax* specifically (King et al., 2011; Tapley et al., 2015b). The potential for over-supplementation of calcium, however, has been less explored and is currently unknown. There have been increasing reports of choleliths (or, gall-stones) and cholecystitis in captive *L. fallax* in recent years; in some cases, cholecystitis was severe and likely associated with the cause of death in affected individuals (Jameson et al., 2019). The choleliths analysed to date have been composed of almost pure calcium carbonate ($CaCO_3$) and one hypothesis is that choleliths may form as a result of an excess of calcium in the standard captive diet used for this species (Jameson et al., 2019). It is therefore important to be cautious in attempts to supplement calcium in the diets of these frogs and to conduct empirical work to underpin species-specific captive husbandry (Michaels et al., 2014a). To this end, we present the results of trialling two different calcium supplementation regimens for captive *L. fallax*.

METHODS

Ethics statement

All aspects of this study were conducted within the routine husbandry and health monitoring practices for captive amphibians at ZSL London Zoo; the study was conducted between November 2016 and December 2017. All diets used met required calcium needs for vertebrates (see below). Animals were monitored continuously for clinical signs indicative of negative effects, but none were observed. The methods were reviewed by the Zoological Society of London and it was determined that they required neither internal ethical review nor a Home Office Licence under the Animals Scientific Procedures Act 1981; the project was approved and assigned the reference code ZDZ72.

Study animals and husbandry

The study used a split clutch design using 12 juvenile *L. fallax* captive bred at ZSL London Zoo as part of the Mountain Chicken Recovery Programme. These animals were the offspring of a three-year-old captive bred female and a four-year-old captive bred male. These parental animals were housed in Enclosure Type 1 (Table 1). Enclosure temperature and Ultraviolet Index (UVI) gradients (Table 1) were measured using a Ketotek laser thermometer gun (model KY600Y) and a Solartech 6.5 UVI meter.

The study animals hatched from a single clutch laid on 12th May 2016 and metamorphosed between 15th–22nd July 2016. The clutch was randomly split into two groups of six animals each on the 4th November 2016 (day 0). Visible Implant Elastomer tagging was used to identify each individual (Nauwelaerts et al., 2000), along with photographic IDs. From day 0 until day 200, animals were housed in two separate identical enclosures (Enclosure

Type 2, Table 1). On day 200, at approximately 1 year old, the animals were combined in a single enclosure with the same conditions as the parents (Enclosure Type 1, Table 1) although not co-housed with them.

Animals were fed a varied diet following Jayson et al. (2018a). All feeds for adults and pre-experimental juveniles were supplemented with a 1:1 by weight mix of Vetark Nutrobal® (VETARK Professional, Winchester, UK) and powdered calcium carbonate (product code P0302, Cambridge Commodities, Cambridge, UK), which was dusted onto prey items (Michaels et al., 2014b). Nutrobal® is a vitamin and mineral feeding supplement containing calcium carbonate and vitamin D_3, as well as other vitamins, in sufficient levels to mobilise the calcium provided. The 1:1 mixture contains 31.02 % Dry Matter (DM) calcium based on calculations from calcium content analysis of Nutrobal® and calcium carbonate powder (Supplementary Materials). Approximately 2–3 prey items per animal were added to the enclosures at each feed.

Experimental treatments

Two experimental treatments were implemented based on the variations in routine supplementation regimens reported anecdotally by amphibian keepers. The animals were randomly assigned to either a control group (HighCal) or an experimental (LowCal) treatment group. The LowCal group was regarded as the experimental group as it was the treatment that deviated from standard practice. Prior to the start of the study, all frogs were given the control diet. From day 0, HighCal frogs were offered supplemented food every day, whereas LowCal frogs received supplemented food twice a week while still receiving un-supplemented food the rest of the week. Sex was determined towards the end of the study when sexual dimorphism became apparent; the HighCal group comprised four males and two females, the LowCal group comprised two males and four females. The supplement was a powder comprising a 1:1 mix of calcium carbonate and Vetark Nutrobal®. Food items were bagged and coated in the supplement just prior to feeding to maximise supplement powder retention (Michaels et al., 2014b). The animals were fed daily between 0830-0900. The calcium content of the supplemented insect diet offered to HighCal frogs seven times per week and LowCal frogs twice weekly was approximately 3.94 % dry matter (DM) with a calcium:phosphorus ratio of approximately 6.86:1 at the point of feeding (Jayson et al., 2018a). The unsupplemented diet offered to LowCal frogs five times weekly constituted approximately 0.11 % DM calcium and had a calcium:phosphorus ratio of approximately 0.18:1 (Jayson et al., 2018a). Therefore, averaging across supplemented and non-supplemented days, LowCal frogs received a diet containing approximately 1.69 % DM calcium and a Ca:P ratio of approximately 2.03:1.

Data collection

Data collection began on day 0 when LowCal frogs began receiving the experimental treatment. As part of routine health checks carried out on all juvenile individuals of this species, frogs were captured and weighed using digital balances (Salter Housewares, UK) approximately every 14 days, starting on this date. Snout-vent lengths (SVLs) were measured using digital callipers (Transcat, USA) to the nearest millimetre every three months. After data collection on day 167, data were reviewed to inform management moving forwards.

Whole body dorsoventral radiographs (X-ray generator: Ultralight 300 (veterinary X-rays); Processor: Fujifilm FCR Prima II) and coelomic ultrasound images (Ultrasound Machine: Logiq E BT12 Console; Ultrasound probe: L8-18i-RS Wide-band high-frequency linear array with 6.7–18.0 MHz Imaging frequency) were obtained by veterinarians from all frogs on days 0, 87 and 167 as part of routine health checks for this species. Blood samples were collected on the two latter dates (on the initial date the animals were too small for samples to be collected). These procedures, which were conducted under isoflurane induced anaesthesia by veterinary staff, are used to detect the onset of choleliths in the captive population of this species. Blood samples were tested for plasma biochemistry and complete blood count, including plasma glutamate dehydrogenase (GLDH) (an inflammation marker), and plasma calcium and phosphorus levels.

After day 167, all animals were switched to a supplementation regimen intermediate to the experimental treatments as preliminary assessment of data suggested that either extreme may be suboptimal (see Discussion). The intermediate supplementation regimen involved supplementing live food four times weekly; the diet offered therefore had an average calcium content of approximately 2.30 % DM and an approximate Ca:P ratio of 4.0:1 (Jayson et al., 2018a). Final SVL and bodyweight measurements were recorded on day 396 and final radiographs on days 396–8.

Radiographs were analysed using ImageJ (Schneider et al., 2012) following the methods used by Michaels et al. (2015). SVL, femur length, femur width, skull length and skull width were measured. ImageJ was used to calculate the standardised mean greyscale value of the cranial and paravertebral endolymphatic sacs, which are used to store calcium in anuran amphibians (Stiffler, 1993). A polygon was drawn around the paravertebral calcium sacs and the mean greyscale value calculated using the inbuilt program function (following Michaels et al., 2015). To standardise for variation in greyscale value between radiographs, this value was divided by the mean greyscale value of a 52 x 52-pixel square sampled from the radiograph background to generate a standardised mean greyscale value for each frog. Intra-observer reliability of this method has already been established with the same observer by Michaels et al. (2015).

Dorsoventral radiographs from seven wild collected adult *L. fallax* were provided by JD King; these radiographs are referred to by King et al. (2011) and are derived from animals collected in Dominica in 2001 and maintained for c. 3 years in captivity in the USA before radiographs were taken. ImageJ was used to calculate skull length:width and femur length:width ratios from these radiographs.

Fresh faeces were collected daily from enclosures from day 180 to day 186 (inclusive) to ensure that

material from supplemented and non-supplemented feeds were represented; this was repeated for three consecutive weeks. Faecal samples were pooled for each week/treatment combination and frozen at –20˚C. They were then transported frozen to Manchester Metropolitan University where they were analysed for calcium and phosphorus content by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (see Michaels et al., 2014b; 2015).

Statistics

All statistics were conducted using SPSS 25.0 (IBM) for Windows 10. Non-parametric tests were used because of the small sample size. Mann-Whitney U tests were performed to compare SVL, bodyweight, skeletal measurements and greyscale between treatments at each timepoint. Ultrasound findings and blood results were compared qualitatively. A Friedman test for repeated measures was used to compare skull proportions within treatment groups between the final two radiographs. A Kruskall-Wallis test was used to compare skeletal proportions between wild, HighCal and LowCal frogs. A Mann-Whitney U test was used to compare mean plasma glutamate dehydrogenase (GLDH) activity for each frog between treatments. Mean results from faecal data were calculated for comparison between treatments, but N was too small for statistical analysis.

RESULTS

Summary morphometric and radiographic data are presented in Table 2 and Figure 2. P values for bodyweight data, where 13 tests were performed, were not corrected as no comparisons were significantly different with an alpha of 0.05. There were no significant differences between treatment groups for SVL, bodyweight, or femur length:width ratio. Frogs were not significantly different between treatments in terms of skull length:width ratio for the first two radiographs, but HighCal animals had a significantly lower ratio (U_5=3, $p < 0.05$) on the third radiograph, before diets were changed to the intermediate regimen. A significant change in skull proportions was observed in animals in the LowCal group in that they became relatively broader within-group between the penultimate and final radiographs (X^2_5 = 6, p=0.014). There was no significant change over the same timeframe in HighCal animals (X^2_5 = 0, $p > 0.999$) and after eight months on the intermediate diet at the final radiograph, no significant difference between treatments was apparent (Fig. 1B). Wild collected frogs had significantly proportionately broader heads than either captive experimental groups on day 167 (H_2=14.9684, p = 0.00056). Wild collected frogs had significantly proportionately thicker femora than either captive experimental groups (Table 2).

Frogs in the HighCal and LowCal groups did not have significantly different mean greyscale values of the paravertebral endolymphatic sacs at the start of the experiment (day 0), but for the following two radiographs on day 87 and day 167, frogs in the HighCal group had significantly higher greyscale values than frogs in the LowCal group (U_5 = 1, $p < 0.05$; U_5 = 0, $p < 0.05$, respectively). On days 396-398 (which were 229-231 days after changing to the intermediate diet), the statistically significant difference between groups seen at earlier timepoints was no longer apparent (Fig. 1A). Mean phosphorus content of faeces (collected days 180-86 inclusive) was similar between the treatment groups (2385 vs. 2241 mg/kg), but calcium content of faeces from frogs in the HighCal group was double that of frogs in the LowCal group (33,381 vs. 13,394 mg/kg).

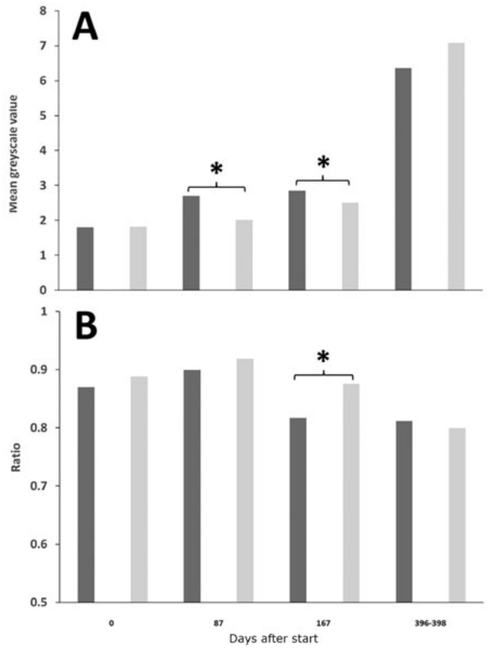

Figure 1. Medians of mean greyscale of the cranial and paravertebral endolymphatic sacs (bar chart A) and skull length:width ratio (bar chart B) of frogs in the HighCal group (dark grey bars) and LowCal group (light grey bars) measured from radiographs taken over the duration of the study. * indicates a statistically significant difference between the HighCal and LowCal groups.

All ultrasound examinations showed grossly normal internal structures with no distinct choleliths, apart from in two animals within the HighCal group, where potential small (< 0.6 mm diameter) stones or echodense aggregations were detected on day 167, but which were not detectable in any of their previous or later ultrasound exams and were not visible on radiographs at any point.

Blood analyses (plasma biochemistry and complete blood counts) were performed from 1–4 times throughout the study, depending on the success of attempted blood draws at each health check event. All findings were unremarkable in comparison with available reference ranges for *L. fallax* (Species360,

Table 2. Median morphometric and radiographic data for *L. fallax* in the HighCal and LowCal treatment groups at the start (day 0) and end (days 396-8) of the study, and morphometrics of wild-collected conspecifics derived from radiographs.

Parameter	Day	HighCal median	LowCal median	Wild-collected
Bodyweight (g)	0	30	24.5	N/A
	396–8	39.5	33.5	
SVL (mm)	0	47.5	38.5	N/A
	396-8	56.5	46	
Femur length/width	0	22.799	23.815	15.60
	396–8	18.316	18.157	
Skull length/width	0	0.870	0.888	0.65
	87	0.900	0.919	
	167*	0.817	0.875	
	396–8	0.811	0.800	
Adjusted mean greyscale of endolymphatic sacs	0	1.808	1.820	N/A
	87*	2.705	2.010	
	167*	2.845	2.502	
	396-8	6.362	7.080	

SVL = snout-vent length. * denotes where High- and Low- Cal groups were significantly different from one another. Beginning and end data only are given for parameters where no significant differences were found between treatments throughout the study.

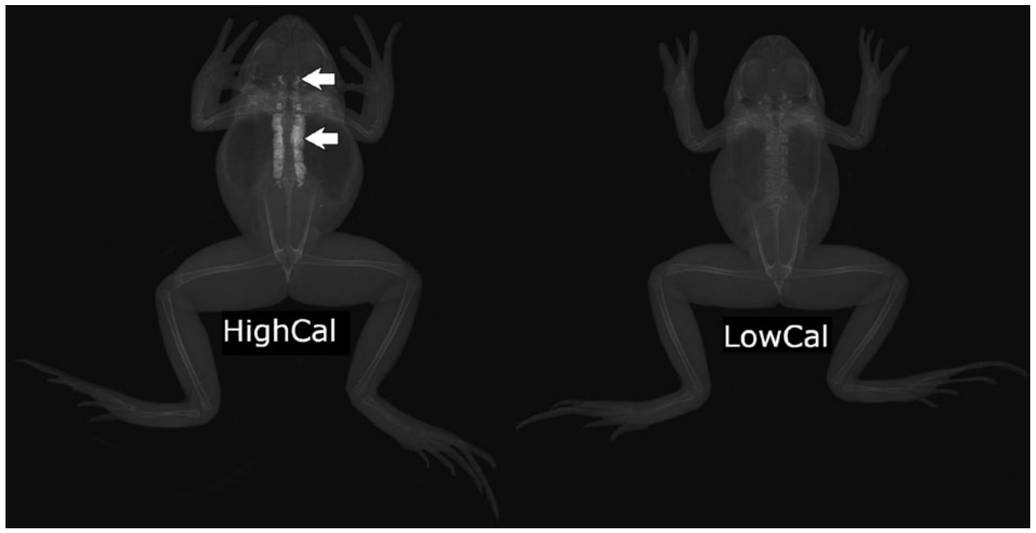

Figure 2. Representative dorsoventral radiographs of frogs from the HighCal and LowCal groups, taken on 20 April 2017. The more radiodense cranial and paravertebral endolymphatic sacs, indicating greater calcium stores, in the frog from the HighCal group are indicated with white arrows. Please note that this is a composite image for illustrative purposes and is not suitable for further analysis.

2020), except for a large variation in the range of GLDH results for which no healthy reference values in amphibians currently exist. Mean (±SD) GLDH across all measurements for all frogs in each treatment was 61.7 (±58.4) U/L for the HighCal group and 74.0 (±78.3) U/L for the LowCal group. A Mann-Whitney U test comparing the mean GLDH value for each frog across treatments showed that there was no significant difference in this parameter between groups, however (U_5=13, $p<0.05$). Compared to expected values in mammals and birds some results appear elevated, but these do not appear to be associated with any appreciable hepatic, gall bladder or other abnormalities in the frogs studied. The number of frogs presenting possible cholethiasis on ultrasound scan was too small for a statistical approach to testing for association between GLDH and signs of cholelithiasis; nevertheless, the frogs with the suspected choleliths did not have more elevated GLDH levels than the rest of the frogs.

DISCUSSION

We found that the frequency of dietary calcium supplementation of *L. fallax* content affected the radiopacity of endolymphatic sacs, the calcium content of faeces, and the proportions of the skulls of frogs, but not any other measures of growth or skeletal structure. Deficits were eliminated in LowCal animals after change to an intermediate supplementation regimen. Importantly, based on clinical examination, plasma calcium and phosphorus levels, and radiography, none of the frogs in this study was suffering from Nutritional Secondary Hyperparathyroidism (NSHP; often called nutritional metabolic bone disease). In particular, proportions of calcium and phosphorus in the blood, which ultimately become imbalanced in NSHP (Wright & Whitaker, 2001), were normal at around 2:1 in both groups, and no reduction of calcification or pathological deformities or fractures of bones, as reported in NSHP in this species by King et al. (2011) and Tapley et al. (2015b), were apparent. Moreover, both HighCal and LowCal diets met the approximate calcium requirements for vertebrates (Robbins, 1993; NRC, 1994; 2006; Oonincx & Dierenfeld, 2012).

HighCal *L. fallax* had relatively broader skulls (lower median skull length:width ratio) by day 167 in this study, which were not apparent earlier at 87 days. Michaels et al. (2015) found significant effects of high vs. low calcium supplementation on skull length:width ratio after 160 days in *Bombina orientalis* (Anura: Bombinatoridae). Michaels et al. (2015) only performed radiography at a single timepoint so comparing the timeline of skeletal changes to that observed in *L. fallax* is not possible. As in this study, Michaels et al. (2015) found no effect of supplementation on growth rates.

Proportionately broader heads may improve fitness as anuran feeding is gape limited and a wider head may facilitate consumption of a broader range of prey (Emerson et al., 1994). As *L. fallax* is a top vertebrate predator and its diet includes very large food items (Brooks, 1982; Rosa et al., 2012), this effect may be important for animals translocated to the field from captive breeding facilities. Moreover, this result supports the recommendation that skeletal health of frogs is assessed prior to translocation (Tapley et al., 2015b).

The more radiopaque paravertebral endolymphatic sacs in HighCal frogs indicate storage of at least some of the additional calcium provided. These stores of calcium carbonate may be mobilised to provide additional calcium in times of need, such as injury, metamorphosis or to provide a buffer in times of acidosis (Schlumberger & Burk, 1953; Pilkington & Simkiss, 1966; Stiffler, 1993; Warren & Jackson, 2005; Wongdee & Charoenphandhu, 2013) and potentially reproduction or periods of reduced feeding (e.g. breeding males). Increased calcium stores may therefore be interpreted as a benefit of more frequent supplementation. However, as the endolymphatic system in frogs is not fully understood and no data are available on either the status of these sacs in wild *L. fallax*, or their optimum physiological state, it is also possible that excessive radiopacity may also be possible in response to over-supplementation. Mean greyscale values for calcium stores in both groups of animals increased substantially between the penultimate and final radiographs (Fig. 1A). This is following nearly ten months of growth wherein the animals increased in size substantially, which is the likely cause of overall increased radiopacity.

The calcium content of faeces from HighCal frogs was double that of LowCal frogs. Given that unabsorbed dietary calcium from the gastrointestinal tract was likely the main source of faecal calcium (environmental contributions would be similar between enclosures, and urinary excretion is a near-negligible contribution in anurans; Stiffler, 1993), this suggests that the more frequent dietary supplementation regimen provided more calcium than could be absorbed by *L. fallax*. It is unclear whether this might be because of absolute physiological limitations of *L. fallax* in gastrointestinal calcium absorption, or if other aspects of calcium metabolism are not yet optimised for the species under captive conditions (Tapley et al., 2015b). There have been recent reports of choleliths and cholecystitis in captive *L. fallax* and choleliths analysed to date have been composed of almost pure calcium carbonate ($CaCO_3$) (Jameson et al., 2019). One hypothesis is that choleliths may form due to an excess of calcium in the diet (Jameson et al., 2019). Potential very small choleliths or aggregations of echogenic material in the gall bladder were observed in two individuals from the HighCal group on ultrasound scan on day 167, but these findings were not present on subsequent health checks and were not appreciable on radiographs. Given that supplemented calcium was provided in the form of calcium carbonate in this study, it is possible that excess calcium carbonate in the alimentary canal could have contributed to the presence of choleliths or echogenic material in the gall bladder. However, there is insufficient evidence at this stage to determine whether supplementation regimes influence the development of cholelithiasis in *L. fallax*.

The combined faecal and radiographic data support the management decision to move all frogs to an intermediate supplementation regimen (see Methods), which aimed to provide sufficient calcium to saturate calcium stores but avoid excessive supplementation that could not be absorbed by the gut. Our data suggest that this was successful; after several months on this regimen, differences between treatment groups in skull proportions and mean greyscale value were no longer apparent; the skull proportions of LowCal frogs approximated those of HighCal individuals, while the latter group did not change significantly. This is an important increase in knowledge from Michaels et al. (2015), as it indicates that, at least in this species and in young animals, skeletal and calcium store differences linked to captive diet may be corrected by adjustment of the diet. No further evidence of cholethiasis was seen in any animals after the switch to the intermediate supplementation regimen, but no causative link could be established. Kawamata (1990a; b) reported rapid $CaCO_3$ crystal formation in anuran endolymphatic sacs

after exposure to calcium supplementation, which is consistent with the response seen in this study to changes in supplementation regimen. This suggests that calcium supplementation should be a priority in captive diets and that even a short time with inadequate supplementation may result in depleted stores.

Data concerning the skeletal structure of wild *L. fallax* are lacking. Both HighCal and LowCal frogs had significantly narrower heads and thinner femora than wild-caught animals. Although these effects may be the result of age class (the wild-caught animals were adults), or levels of locomotive or foraging activity influencing bone structure, these data indicate that the wider skulls observed in HighCal frogs and all animals under intermediate supplementation were more similar to the skulls of wild animals than to those of LowCal frogs, further supporting the adoption of the intermediate supplementation regimen. The wild caught frogs had been in captivity for three years, so meaningful comparison of endolymphatic sacs was not possible. Comparison of age-matched captive and wild animals would be preferred to quantify skeletal differences between populations.

Although calcium carbonate is the primary constituent of the supplement used in this study, it also contained vitamin D_3, which facilitates uptake of calcium from the intestines (Antwis & Browne, 2009). Therefore, increased dietary vitamin D_3 provision, as well as higher total dietary calcium, may have contributed to some of the changes observed in the group provided with more frequent diet supplementation in this study. However, Michaels et al. (2015) found that UVB radiation had a more significant effect on serum vitamin D3 levels in anurans than dietary supplementation with a product containing vitamin D_3 and all frogs in this study had access to a similar gradient of UVB radiation. Sufficient blood samples were not recovered from frogs in the present study to measure serum vitamin D_3 levels using validated methods.

The data collected in this study derive from a relatively small sample size with only one enclosure for each treatment group. Statistically, our data may not be extrapolatable to other populations of *L. fallax* if the enclosure is considered the experimental unit. However, as is often the case with non-model and especially conservation dependent species, this limitation is difficult to overcome without creating additional confounding variables. Moreover, the small sample size prevented us from controlling statistically for sex. Sexes could only be determined towards the end of the study rather than when animals were allocated to groups, but both sexes were represented in both populations.

The findings from this study increase knowledge about calcium supplementation for *L. fallax* and highlight that empirical investigation into optimal captive diet preparation is important when developing captive husbandry protocols for amphibians. We recommend that more research into this field is required, particularly further wild data collection so that comparisons between wild and captive frogs can be made in order to better identify and quantify shortcomings and successes in captive diet formulation and husbandry.

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A new genus and species of rhinatrematid caecilian (Amphibia: Gymnophiona: Rhinatrematidae) from Ecuador

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A new genus and species of rhinatrematid caecilian, *Amazops amazops* gen. et sp. nov., is described based on a single specimen from Orellana, Ecuador collected in 1990. Among other features the new taxon differs from all other rhinatrematid caecilians in having less than four annular grooves interrupted in the region of the vent and in the squamosal contributing to the bony margin of the orbit. A consideration of its distinctive morphology suggests that it is plausible that the new taxon is the sister taxon of all other rhinatrematid caecilians. That the genus is known from a single specimen, and that this is the first new rhinatrematid species from the Andes described for more than 50 years, highlights the poor sampling (collecting) of rhinatrematid caecilians and limited knowledge of their diversity.

Keywords: Andes, biodiversity, computed tomography, morphology, South America, systematics, taxonomy

INTRODUCTION

The caecilian family Rhinatrematidae was established by Nussbaum (1977) to receive the neotropical caecilian species that Taylor (1968) had assigned to his family Ichthyophiidae, thereby restricting the latter to Asia. Rhinatrematids are the only caecilians with open skull roofs (zygokrotaphy) associated with primary jaw adductor muscles extending through the upper temporal fenestrae (Nussbaum, 1977), and that lack an elongate truncus arteriosus (Wilkinson, 1996). They are relatively short and stout bodied caecilians that retain short tails, have many annuli and annular scales, and, as far as is known, are oviparous with an aquatic larval stage. Phylogenetic analyses of both morphological and molecular data agree that the Rhinatrematidae represents one side of the basal divergence within the extant caecilians and is thus the sister group of all other living caecilians (e.g., Wilkinson & Nussbaum, 1996; San Mauro et al., 2014).

In 1990, two of us (RPR & JFJ) made a brief unscheduled stop on route from Quito to Loreto in the Amazonian versant of Ecuador. Beneath a rock or rocks on a dirt road, with a very muddy, soft, red substrate and much seeping water they found a small, unicoloured caecilian. Based on the position of the tentacle and length of the tail, among other features, this caecilian was readily identifiable as a rhinatrematid and tentatively identified as a species of *Epicrionops* Boulenger, 1883. However, based on their initial investigations its specific status was less clear. Four of the eight species of *Epicrionops* recognised at that time lacked a stripe but these were reported as having rather more or substantially fewer annular grooves than the new specimen, suggesting either that the ranges of annular grooves of the described species, many of which were known only from very small samples, were underestimated or that the new specimen represented an undescribed species. Subsequent investigation by the senior author and anatomical comparisons with type specimens of all other species of *Epicrionops* and of three of the five currently recognised species of the other rhinatrematid genus *Rhinatrema* Dumeril & Bibron, 1843, confirms the specimen belongs to a previously undescribed rhinatrematid species and reveals that its morphology is sufficiently distinctive to warrant the establishment of a new genus to receive it.

MATERIAL AND METHODS

Upon collection the specimen was immediately closed inside a plastic bag with moist ferns, kept cool and euthanised within 12 hours of capture by submerging in a solution of hydrous chlorobutanol (Chloretone), positioned and fixed in 10 % buffered formalin, and subsequently washed and transferred to 70 % ethanol for long term storage.

Cranial and caudal osteology and scales were visualised with high-resolution x-ray computed

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tomography (the results referred to here as CT scans) using a Metris X-Tek HMX ST 225 System with a molybdenum target set at 180kV and 200µA. Scan data were collected over 3142 projections (two frames per second) in 360˚, with reconstructed voxel size of 8 & 10µm respectively, and rendered as a three-dimensional volume using VGStudio MAX v2.1 (Volume Graphics, http://www.volumegraphics.com) which was used to generate images. Comparisons were made with information on cranial morphology including published (Nussbaum, 1977; Reiss, 1996; Gower et al., 2010) and unpublished observations, illustrations and CT scans of all other rhinatrematid species with the exceptions of the recently described *Rhinatrema gilbertogili* Maciel, Sampaio, Hoogmoed, and Schneider, 2018 and *Rhinatrema uaiuai* Maciel, Sampaio, Hoogmoed, and Schneider, 2018. CT scan data are available from the senior author upon reasonable request.

Total lengths and circumferences were measured to the nearest millimetre (mm) with a ruler, the latter by wrapping a piece of string around the body. Other measurements were made to the nearest 0.1 mm with dial callipers. Observations and direct counts of teeth were facilitated by the Nussbaum technique, i.e., using a directed stream of compressed air to temporarily dry and shrink the gingivae (Wilkinson et al., 2013). Tooth counts were also made from CT scans. In both cases, tooth counts include empty sockets and must be considered estimates rather than definitive. Sex was determined by direct examination of gonads via a midventral incision in the body wall. Number of vertebrae was determined by X-radiography, using a Matchlett Solus Schall, beryllium window, copper target tube exposing Kodak MX100 film for 25 seconds at settings of 25 kilovolts and 10 milliamps. Annular scales were sought by opening selected scale pockets by running a needle along the corresponding annular groove. Posterior scales were also visualised through CT scanning.

Following common usage we refer to an area around the vent that is differentiated in colour or structure from the adjacent skin as the disc and the fleshy margins of the upper and lower jaws that form the edges of the mouth as lips. Following Wilkinson et al. (2014; 2017) we use first and last to denote the anteriormost and posteriormost units of serial homologues, and front and back (and behind) to denote anterior or posterior (to) respectively. Where helpful, observations were made with the assistance of a dissecting microscope.

RESULTS

***Amazops* gen. nov.**
{urn:lsid:zoobank.org:act:D9F1C39D-4B47-46CF-AB9D-51896D4C619C}

Diagnosis. Rhinatrematid caecilians with the squamosal contributing to the margin of the orbit.

Content: A single species *Amazops amazops*, sp. nov., the type by monotypy and by designation.

Etymology: The name is a portmanteau word combining reference to the Amazonian provenance of the type and only known species and the distinctive topological relationships of its eye and orbit, particularly the contribution of the squamosal to the bony margin of the orbit, which is unknown in any other rhinatrematid. As mandated by the code, gender is masculine.

Remarks: Three other features of the only known specimen of this genus are distinctive, known in no other rhinatrematids and might be diagnostic for the genus: lack of contact between the quadrate and maxillopalatine, contact between the squamosal and frontal and the small number of annular grooves that are interrupted by the vent.

***Amazops amazops* sp. nov.**
{urn:lsid:zoobank.org:act:55BF6D3F-2B30-4EB2-BEFE-917532513286}
(Figs. 1-5)

Holotype. United States National Museum of Natural History, Smithsonian Institution (USNM) 320729, a female collected by Jeremy F. Jacobs and Robert P. Reynolds from Finca Virgen La Dolores, at km 57 sign on Hollin-Loreto Road (E20), Orellana, Ecuador, c. 0 degrees 43' 50" South and 77 degrees 30' 25" West, and c. 1000m above sea level, 16th August 1990. According to the map available at the time (titled "Republica Del Ecuador" and "Compilado Por El Instituto Geografico Militar," Escala 1:1'000.000, published by the Ministerio De Relaciones Exteriores in Quito, Ecuador, dated 9 November 1981), the type locality, which was in the Province of Napo (Orellana having been established in 1998), is in the vicinity of the "Cordillera Galeras" and featured hillsides through cutover forest with abundant epiphytes. The "Hollin-Loreto road" was not on the map but was provided by the driver, Ramiro Donoso. This is shown as E20 on modern maps.

Diagnosis. As for the genus.

Identification. Based on external morphology alone, the new species is readily distinguished from all other rhinatrematids by having very few (less than four) annular grooves interrupted by the vent. Further, the combination of low number of annular grooves (< 275) and its uniform colour distinguishes it from all other rhinatrematids except *E. colombianus* (Rendahl & Vestergren, 1938), the only known specimen of which has even fewer annular grooves (< 225).

Description of holotype. Good condition, an c. 20 mm midventral longitudinal incision c. 30 mm anterior to tail tip, some open scale pockets, mouth preserved open. Total length 173 mm. Body subcylindrical, mostly somewhat dorsoventrally compressed (at midbody: width 7.3 mm, depth 4.5 mm, circumference 20 mm), less so posteriorly, not compressed (width 4.0 mm, depth 4.0 mm) at level of vent, fairly uniform, narrowing slightly anteriorly and more notably posteriorly. Tail moderately long, 7.6 mm, slightly laterally compressed, 3.0 mm wide and 3.5 mm deep c. half way between vent and tail tip, tapering more strongly in dorsal than in lateral view, tip much more broadly blunt in lateral than in dorsal view, dorsal and ventral margins symmetrical in lateral view, ventral surface not flat.

Head 7.0 mm from snout tip to corner of mouth, 9.0 mm from snout tip to first nuchal groove behind corner

Figure 1. USNM 320729, holotype of *Amazops amazops* sp. nov. with head end (top), whole body (middle) and tail end (bottom). Scale bar gradations in mm. Photo by Harry Taylor (Natural History Museum, London).

of mouth, in dorsal view slightly more V- than U-shaped, dorsoventrally compressed, as wide (6.1 mm) and deep (4.9 mm) as adjacent nuchal region posteriorly, narrowing gently anteriorly up to about a third of the way between eyes and nares in front of the eyes. In ventral view, lower jaws virtually as wide as head, tip more bluntly rounded than snout anteriorly, mouth marginally subterminal (anterior of mouth to snout tip 0.7 mm). In lateral view, head tapers very gently anterior to eye level, sharply from level of nares, edges of mouth (lips) fairly straight, slightly downturned at corner of mouth, corner of mouth slightly further from top than from bottom of head, lower jaws robust, almost as deep as upper jaws at eye level.

Circular eyes small (diameter 0.4 mm), central grey lens and darker periphery clearly visible through skin, elevated above adjacent skin, equidistant from top of head and lip in lateral view, inset by almost one diameter from outline of head in dorsal view. Tentacular apertures

Figure 2. USNM 320729, holotype of *Amazops amazops* sp. nov. in life. Photo by William W. Lamar.

small (0.6 mm) almost horizontal arc-like slits, curving ventrally at their ends, extending anteriorly from the middle (i.e., at three o'clock) of the anterior edge of eye, their margins slightly elevated, the tips of the paired tentacular ducts of each side (leading from the tentacle to the vomeronasal organ) visible through the skin adjacent to the anterior end of the tentacular apertures. Nares small (0.3 mm), subcircular on left, more tear shaped on right, deeper and broader anteriorly, visible dorsally inset about one and a half diameters from outline of head; in lateral view, slightly closer to tip than to top or bottom of snout; slightly closer to lips (0.7 mm) than are eyes (0.8 mm), visible in anterior but not ventral views. Distance between nares (1.5 mm) half the distance from naris to eye (3.0 mm).

Teeth pointing posteriorly (at angles of 30˚ to 45˚ from the jaws), not strongly recurved, bicuspid, anterior and posterior teeth of each series smaller than those in between, none hypertrophied, outer mandibular ("dentary") teeth (36) generally a little larger than opposing premaxillary-maxillary teeth (42) and vomeropalatine teeth (40), inner mandibular ("splenial") teeth (24) a little smaller. Curvature of premaxillary-maxillary tooth series following that of upper lip, vomeropalatine tooth series straighter except anteriorly, extending slightly (about three teeth on each side) beyond the last premaxillary-maxillary teeth, distance between upper series narrowing posteriorly, maximal laterally (about the level of half way between the eye and the naris). Inner mandibular tooth series straightening a little anteromedially, much shorter than the outer mandibular series, about one quarter of the length of the outer mandibular tooth series (about six teeth on each side) posterior to the last inner mandibular teeth.

Based on the CT scan there are nine premaxillary teeth (five right, four left), 33 maxillary teeth (17 right, 16 left) for a total of 42 premaxillary-maxillary teeth, 13 vomerine (six right, seven left) and 26 palatinal teeth (12 right, 14 left, two on each side posterior to the last maxillary teeth) for a total of 39 vomeropalatine teeth, 37 outer mandibular teeth (19 right, 18 left) and 22 symmetrically disposed inner mandibular teeth, with seven (right) and eight (left) outer mandibular teeth behind the level of the last inner mandibular teeth.

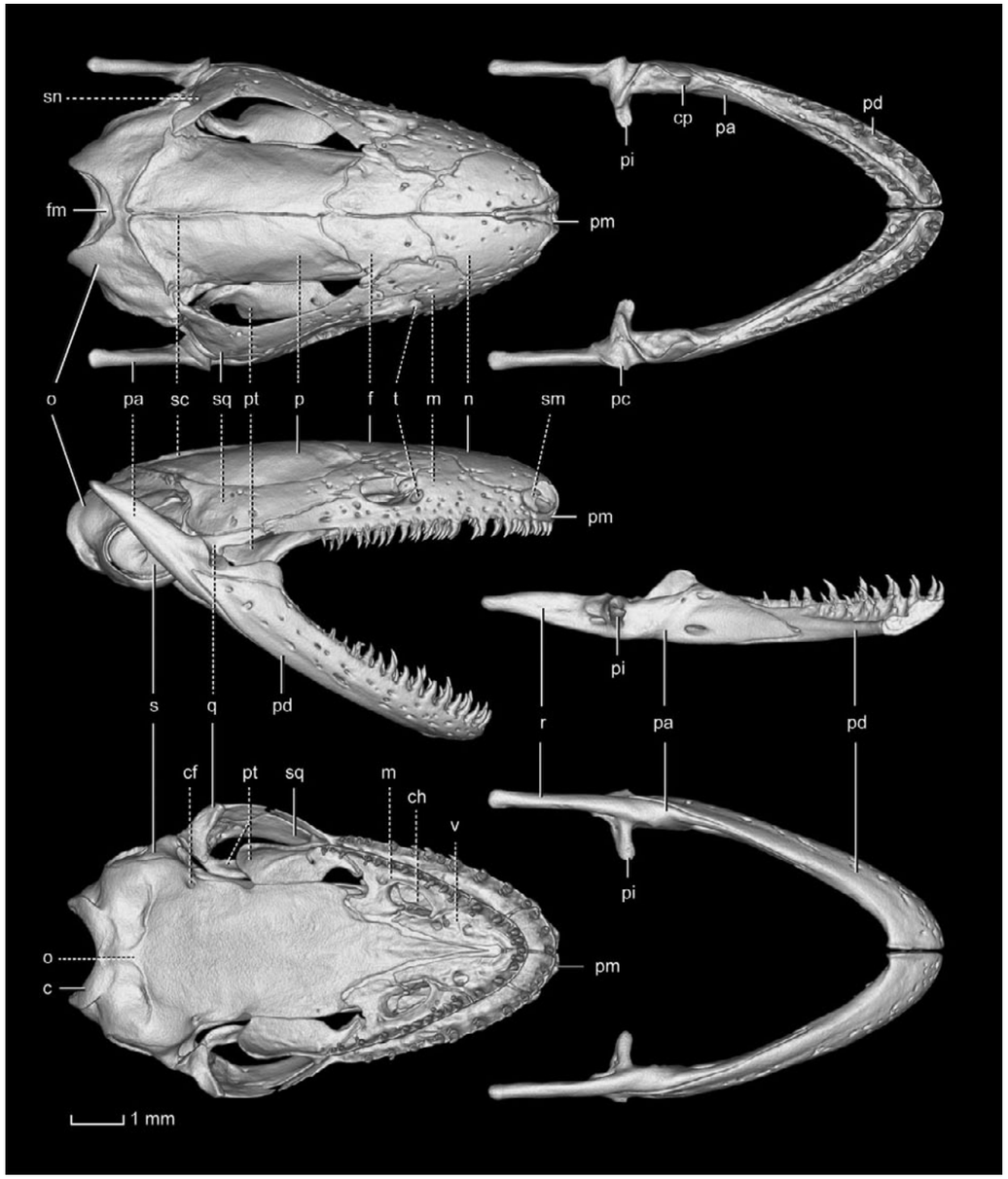

Figure 3. CT scan of skull USNM 320729, holotype of *Amazops amazops* sp. nov. in dorsal (top), right lateral (middle) and ventral (bottom) views. c = occipital condyle; cf = carotid foramen; ch = choana (internal nostril); cp = canalis primordialis; f = frontal; fm = foramen magnum; m = maxillopalatine; n = nasal; o = os basale; p = parietal; pa = pseudangular; pc = processus condyloides; pd = pseudodentary; pi = processus internus; pm = premaxilla; pt = pterygoid; q = quadrate; r = retroarticular process of the pseudoangular; s = stapes; sc = sagittal crest; sm = septomaxilla; sn = squamosal nothch; sq = squamosal; t = foramen for tentacular ducts; v = vomer.

Although not identical, numbers of teeth determined from CT scans are reassuringly similar to direct counts.

Tongue with more or less longitudinal plicae over entire surface, margin free not covering any inner mandibular teeth, sides forming an angle of c. 100° anteriorly. Choanae elongate, much longer than wide, distance between them more than eight times their maximal transverse diameters, posterior limit about level with middle of the eye. Palate without plicae.

First groove in the collar region (interpreted as first nuchal groove) poorly marked on dorsum and dorsolaterally, not visible in ventral view. Second nuchal

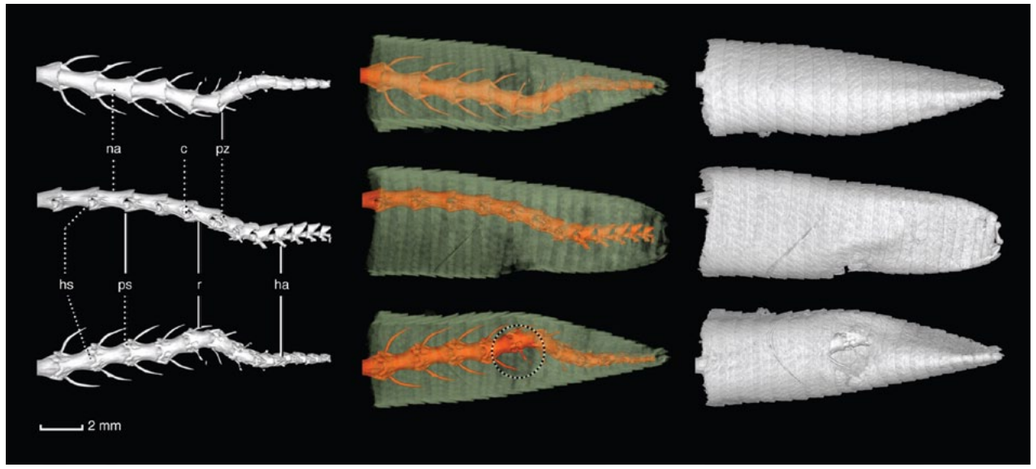

Figure 4. CT scan of tail end of USNM 320729, holotype of *Amazops amazops* sp. nov. revealing vertebrae (left), scales (right) and relationship between scales (green) and vertebrae (orange), in dorsal (top) lateral (middle) and ventral (bottom) views. C = centrum; ha = haemal arch; hs = hyposphene; na = neural arch; ps = parasphene; pz = prezygapophysis; r = rib. Dotted circle highlights the scale free region surrounding the vent.

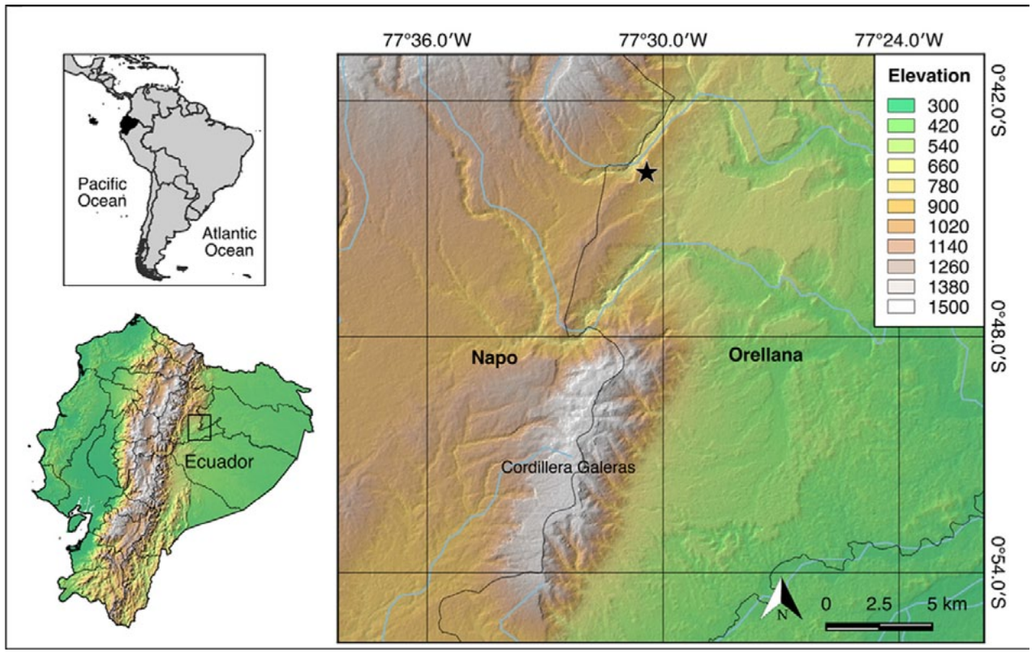

Figure 5. Map showing type locality (black star) of *Amazops amazops* sp. nov.

groove faint dorsally, pale and clearly marked laterally and ventrally. Third nuchal groove complete, bows slightly anteromedially on the dorsum and resembles subsequent annular grooves. First nuchal collar much shorter (1.2 mm) than the second (4.5 mm). Four regularly spaced dorsal transverse grooves on second collar bowing slightly anteromedially and of slightly increasing length all ending dorsolaterally. Behind the collars 247 annular grooves, those in the first third and last sixth orthoplicate, otherwise slightly angulate (curving posteromedially) on venter except last complete annular groove before the vent which curves anteromedially mirroring the anterior limit of the disc. First and last (c. 15-20) annuli are a little longer than others. Annular grooves are complete ventrally except for two interrupted by the vent and disc, tail (area behind the vent) with ten complete and one

(the last) dorsoventrally incomplete annular grooves.

A single row of small subcircular scales present in shallow pockets (about one quarter the length of a midbody annulus) below the dorsal transverse grooves on the second nuchal collar. At midbody and posteriorly, scales occur in two well-defined rows, a posterior row of larger scales (e.g., 1.1 x 1.0 mm) and an anterior row of slightly smaller scales, in pockets about as deep as the length of an annulus at midbody, about one and a half times the length of an annulus posteriorly. Additional scattered scales may lie below (posterior to) the row of larger scales. The two rows of scales in posterior annuli can be clearly discerned in the CT scan of the tail end (Fig. 4), which reveals that apart from the last rows, scales mostly form complete transverse rings around the body and tail except where these and the associated annuli are interrupted by the vent. Scales in a single row overlap with their neighbours. Midventral scales are superficial to the proximal edges of the adjacent scales on each side, the distal edges of which are superficial to the proximal edges of the succeeding adjacent scale and so on until some mid-dorsal scale with edges that are deep to both its neighbours. Successive scale rows are offset in a brick layout (i.e., shifted half a scale in the transverse direction).

Vent slightly longitudinal, bordered by an irregular array of partially subdivided denticulations, perhaps two pairs and one posteromedial denticulations posteriorly, posterior denticulations pigmented and glandular like the adjacent skin especially peripherally, anterior denticulations, pale, with shorter interdenticular grooves. There is no disc other than what is delimited by the denticulations around the vent, approximately egg-shaped with the narrow apex anterior formed by the unpigmented denticulations. No indication of papillae. Very small ovarian eggs (largest c. 0.8 mm diameter) are visible towards the front of the ventral incision. There are no melanophores in the viscera.

Almost uniformly dark, brownish lavender, slightly paler on the head, more so on throat. Pale areas around eye, a slightly paler snout tip encompassing nares. Pale, narrow, faint paramandibular stripes (inset from lips) on ventral surface of head, distinctive pale second nuchal groove on ventral collar region. Paler around vent, especially anteriorly. Annular grooves with a light posterior margin and typically slightly longer anterior dark, aglandular area.

Other than the osteological features that distinguish *Amazops amazops* sp. nov. from the species of *Rhinatrema* and *Epicrionops*, the skull and mandibles are typically rhinatrematid (Nussbaum, 1977). Thus they are zygokrotaphic, with the characteristic squamosal notch and associated process of the os basale and medial parietal ridges providing part of the origin of the primary adductor musculature. There are separate septomaxillae but prefrontals and postfrontals are lacking. The os basale forms a continuous bony dorsal margin of the foramen magnum and a well-developed parasphenoidal rostrum separates the vomers. The retroarticular processes of the mandibles are relatively short and straight, not curving medially or dorsally. Different from what has been reported for other rhinatrematids (Nussbaum, 1977; Reiss, 1996), the pterygoids are large and single on each side with posterodorsal processes closely adpressed to the quadrates and there is no indication of any pterygoid process of the quadrate. There are 77 vertebrae of which the last seven are entirely posterior to the vent and differ from more anterior vertebrae in bearing some indication of haemal arches associated with the parasphenes. Bony ribs are absent from the last four of these caudal vertebrae.

Remarks. That the species is known from a single specimen is sufficient reason to suggest that the IUCN conservation status of the species should be data deficient. Effort is needed to identify populations of this distinctive lineage as a precursor to any meaningful study of its natural history. Based on it being a rhinatrematid it is assumed that it will share the reproductive mode of the other rhinatrematids, as far as is known, in being oviparous with an aquatic larval stage (San Mauro et al., 2014, Müller, 2020) and thus being dependent on water bodies for its reproduction.

Etymology. As for the genus. For nomenclatural purposes the specific epithet is considered to be a genderless noun in apposition.

DISCUSSION

Wilkinson et al. (2011) provided a minimalistic most recent diagnosis of the Rhinatrematidae as the only caecilians with the primary adductor musculature originating dorsomedially and passing through the upper temporal fenestrae, a feature discovered by Nussbaum (1977) and argued to provide evidence for Rhinatrematidae being the sister group of all other caecilians. Although not observed directly in *Amazops*, osteological correlates of this condition (the substantial upper temporal fenestra and a sagittal crest mid-dorsally on the parietals) are apparent from CT scans. Also apparent is the squamosal notch receiving a process of the os basale that Nussbaum (1977) identified as a synapomorphy of the family. Nussbaum's (1977) original diagnosis of the Rhinatrematidae, and the subsequent diagnoses of Nussbaum & Wilkinson (1989) and Wilkinson & Nussbaum (2006), also included three osteological features (fronto-squamosal contact, quadrate-maxillopalatine contact laterally, and orbit entirely within the maxillopalatine) that do not pertain in *Amazops*. As well as setting *Amazops* apart from other rhinatrematids, these differences would need to be taken into account in any less minimalistic rediagnosis of the family.

Nussbaum (1977:16) noted that "the quadrate articulates with the maxillary portion of the maxillopalatine in *Epicrionops* and *Rhinatrema*, a condition which is apparently unique to these two genera among living vertebrates and is, therefore, an important diagnostic feature of the Rhinatrematidae". Nussbaum (1977) considered this to be an ancestral condition within caecilians based on comparison with labryinthodonts, though one might reasonably doubt that assessment given that labyrinthodonts typically have

a jugal separating the quadrate and maxilla and because no such contact is reported in a putative stem caecilian (Jenkins et al., 2007). The lack of contact between the maxillopalatine and quadrate along the ventrolateral margin of the cranium in the type of *Amazops* might perhaps reflect incomplete maturity of that specimen. This articulation is not present in larvae but develops at metamorphosis (Reiss, 1996) and the notch-like gap between the elements is small and would represent an unusual feature in a mature animal. On the other hand similar sized representatives of other species including the slightly smaller type of *Epicrionops colombianus* have extensive contact between the squamosal and maxillopalatine, there are no other traces of larval features in the holotype that would suggest incomplete metamorphosis, and other metamorphic fusions (of the maxillae and palatines) or expansions (of the squamosals) are not incomplete. Thus the condition of *Amazops* is seemingly unique among metamorphosed rhinatrematids and it remains plausible though quite uncertain that this is an ancestral character state within the family and evidence that *Amazops* as the sister of the other rhinatrematids.

Amazops differs from other rhinatrematids in having more elongate squamosals occupying areas anteriorly that are occupied by the maxillopalatine in other rhinatrematids, and contributing to the bony orbits and making contact with the frontals at the anterior ends of the upper temporal fenestrae. Comparisons with fossils are of limited utility in determining character polarity because the various conditions within Rhinatrematidae and within the extant Gymnophiona as a whole all appear derived with respect to the fossil and living outgroups. That these features do not occur elsewhere within rhinatrematids suggest they might be derived conditions under the criterion of common equals primitive (Estabrook, 1977). However, these features are common (nearly universal) in non-rhinatrematid caecilians, implying either convergence with these or that they are instead ancestral for Gymnophiona. The latter implies that *Amazops* represents one side of the basal divergence within known rhinatrematids and is the sister taxon to all other known rhinatrematids. Other characters do not seem to preclude that possibility. Reiss (1996) reported that the pterygoids divide into two at metamorphosis in *Epicrionops* and considered that to be a derived condition, implying that the undivided pterygoids of *Amazops* represent an ancestral condition within caecilians.

Given that caecilian taxonomy can be challenging because of the paucity of external characters and of specimens (Wilkinson, 2020), non-destructive CT scanning may be particularly helpful for revealing details of character systems that would remain otherwise undetermined. Externally, *Amazops* is very similar to *Epicrionops* and, in the absence of the knowledge of its cranial morphology provided by CT scanning, would probably have been described as a species of that genus. Traditional examination of caecilian scales is limited by the necessity of opening scale pockets and the difficulty of visualising scale rows within the scale pockets, but CT scanning can also provide very helpful visualisation of scales in situ. Similarly, counting teeth may be easier, more accurate, and more informative (by yielding separate counts for each tooth bearing element, rather than composite counts for multi element series) from CT scans.

After Taylor's (1968) monographic revision of caecilian taxonomy, there was a hiatus of more than 40 years before the description of any new species of rhinatrematid caecilians. That hiatus was ended by the relatively rapid descriptions of four new species of *Rhinatrema* from the Guianas and lowland Amazonian Brazil (Gower et al., 2010; Wilkinson & Gower, 2010; Maciel et al., 2018). In contrast, the majority of described rhinatrematid species are from the Andes and description here of a new Andean rhinatrematid is the first such for over 50 years. This highlights how incompletely known and poorly surveyed are the rhinatrematid caecilians of the Andes and the possibility that there may be many undescribed species awaiting discovery.

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Make like a glass frog: In support of increased transparency in herpetology

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Across many scientific disciplines, direct replication efforts and meta-analyses have fuelled concerns on the replicability of findings. Ecology and evolution are similarly affected. Investigations into the causes of this lack of replicability have implicated a suite of research practices linked to incentives in the current publishing system. Other fields have taken great strides to counter incentives that can reward obfuscation –chiefly by championing transparency. But how prominent are pro-transparency (open science) policies in herpetology journals? We use the recently developed Transparency and Openness Promotion (TOP) Factor to assess the transparency promotion of 19 herpetology journals, and compare the TOP scores to broader science. We find promotion of transparent practices currently lacking in many herpetological journals; and encourage authors, students, editors, and publishers to redouble efforts to bring open science practices to herpetology by changing journal policy, peer-review, and personal practice. We promote an array of options –developed and tested in other fields– demonstrated to counter publication bias, boost research uptake, and enable more transparent science, to enrich herpetological research.

Keywords: accountability, herpetology, journal policy, open science, peer review, reproducibility, transparency

INTRODUCTION

Across scientific disciplines replication efforts have revealed marked deviation from previously observed results (Freedman et al., 2015; Kelly, 2019; Open Science Collaboration, 2015); this lack of replicability, as shown in medical fields, can incur huge costs and impact human health (Freedman et al., 2015). Despite relatively infrequent efforts to test replicability in ecology and evolution (Kelly, 2019; Schnitzer & Carson, 2016), several examples exist of apparently well-documented effects being brought into question by larger scale replications and meta-analyses (Clark et al., 2020; Roche et al., 2020; Sánchez-Tójar et al., 2018; Wang et al., 2018) suggesting that ecology and evolution must be similarly wary of irreplicable results.

The research community has begun questioning the causes behind observed inconsistencies in results, and exploring options on how to guarantee a more verifiable body of findings. Building a robust body of literature avoids wasting limited research resources (Grainger et al., 2019), correctly informs decisions, and maintains wider trust in science (Anvari & Lakens, 2018).

Partly as a result of fears over irreplicability, we are seeing a change in how researchers and publishers work, namely a shift towards greater transparency and openness in published results. Shifts to greater transparency can counter a suite of incentivised questionable research practices –ranging from the seemingly benign, through questionable, to the demonstrably unethical (Ware & Munafò, 2015)– that are suggested to amplify the variation in, and undermine the replicability of, results. The publishing system incentivises researchers to produce novel, significant results that can be presented within a clean single-article narrative (Brembs, 2019; Fanelli, 2012; O'Boyle et al., 2014). In particular we see prestigious journals prioritising exciting, novel studies with large effects, rather than those undertaking rigorous and replicable science (Barto & Rillig, 2012; Brembs, 2019).

Despite near universal desire to follow best practice in research, the system's prioritisation of significant results (inadvertently) encourages detrimental research practices such as p-hacking, HARKing, and cherry-picking (Cairo et al., 2020; Forstmeier et al., 2017; Fraser et al., 2018). P-hacking is repeatedly testing the same data using different methods until a "significant" result is obtained, paired with failure to report repeated testing. HARKing (Hypothesising After Results Known) is reporting an unexpected result as expected, a way of ensuring a confirmatory result, possibly rationalised by "hindsight bias" on the part of the researchers (Forstmeier et al., 2017). Cherry-picking covers scenarios where researchers fail to report all variables or data

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tested, consigning non-significant variables or outliers to a file-drawer. All three practices are implicated in causing irreplicable results in other fields. Ecology and evolution, and by extension herpetology, are not insulated from the incentives encouraging detrimental research practices (Fraser et al., 2018).

Open science (transparency) provides a way of countering these questionable practices, while also presenting an opportunity to increase the value of scientific articles. Scientific articles often serve as currency for research careers (Rice et al., 2020), and play a central role in incentivising research practices good and bad (Brembs, 2018; Fanelli, 2010; Nosek et al., 2012). Implementing transparent practices in articles benefits researchers by building a more efficient workflow, boosting citation rates, increasing publication chances, and enhancing research reputation (Allen & Mehler, 2018; Markowetz, 2015; Piwowar & Vision, 2013).

Integrating more transparent practices into research is feasible without journal support, but journal policy can enhance normalising transparency (Nilsen et al., 2020; Roche et al., 2015). Aligning journal policy to normative ends may not be new, but recent efforts to combat opaque practices are exemplified by the Transparency and Openness Promotion (TOP) guidelines (Nosek et al., 2015); http://cos.io/top). The guidelines aim to help journals implement transparent policies, while also providing a framework (TOP Factor) for assessing journals' adherence to transparent policies. Although moves towards more transparent practices are new, the implemented solutions do indeed appear to be reducing publication bias and associated false positive rates (Allen & Mehler, 2018; Kaplan & Irvin, 2015; Scheel et al., 2020; Toth et al., 2020).

Herpetology—the study of reptiles and amphibians—covers a broad spectrum of disciplines, from anatomy and physiology, to genetics, evolution, and ecology. If there are replicability issues in the broader fields, then they likely exist in herpetology, yet journal practices targeted at mitigating reproducibility issues have yet to be examined. To understand to what extent herpetology journals currently promote open science practices, we explored how current journal policies adhere to the TOP Factor framework. We then use these findings to promote a number of changes, demonstrated by other fields, to improve openness and hopefully enrich herpetology.

METHODS

We produced a list of herpetology journals by searching for herpeto* using Scimago Journal and Country Rank (https://www.scimagojr.com/ accessed 2020-02-21). We attempted to retrieve author guidelines from each journal's website. For those journals with accessible author guidelines, we scored them according to the TOP Factor (final n = 19; 3 exclusions).

TOP Factor scores journals on their adherence to ten transparency policies (http://cos.io/top). All polices are scored on a 0 to 3 scale, with the exception of policy 10 that has a maximum score of 2. Journals are scored 0 on a policy if they only encourage adherence, or say nothing regarding the practice (original scoring rubric: https://osf.io/t2yu5/).

1. Data citation - Journals are required to supply guides on how to cite data. Scores of 2 for stricter enforcement measures, and 3 for delaying article publication until citations standards are met.
2. Data transparency - Journal articles must state whether data is available and where. Scores of 2 for stricter enforcement measures such as requiring data is stored in an approved data repository, and 3 for delaying article publication until data is openly available and analysis results are independently reproduced.
3. Analytic code transparency - Journal articles must state whether code is available and where. Higher scores for stricter enforcement measures, following the standards of data transparency.
4. Materials transparency - Journal articles must state whether materials are available and where. Higher scores for stricter enforcement measures, following the standards of data transparency.
5. Design and analysis guidelines - Journal must clearly state design transparency standards. Scores for 2 for requiring adherence to transparency standards, and 3 for enforcing adherence, both during review and publication.
6. Study preregistration - Journal articles must state whether a preregistration exists and where it can be accessed. Scores of 2 if the journal also mandates access to preregistration during peer-review, and 3 for requiring preregistration (with associated link) and adding a pre-registration "badge" to articles.
7. Analysis plan preregistration - Same standards as study preregistration, but limited to the analysis phase.
8. Replication - Journal encourages replication studies. Scores of 2 for encouraging replication while implementing results blind review, and 3 for facilitating the submission of registered reports.
9. Registered reports - Journals state that an article's publication chance is not impacted by significance or novelty. Scores of 2 for implementing results blind reviews, and 3 for facilitating the submission of registered reports.
10. Open science badges - Journal awards articles with one or two open science badges. Higher score if all three badges are implemented. Maximum score of 2. N.b. open science "badges" are labels journals apply to individuals articles (both in html and pdf versions) that signify that an article adheres to certain open science practices (Blohowiak et al., 2020; https://osf.io/tvyxz/).

We also obtained the TOP scores pertaining to the 346 journals so far assessed (top-factor.csv (v. 11) data obtained from https://osf.io/kgnva/, accessed on 2020-03-24). We compared our scores for herpetology journals to the overall presence of open and transparent practices in broader science.

We used R v.3.5.3 (R Core Team, 2019) and R Studio v.1.2.1335 (R Studio Team, 2019), in conjunction with the dplyr v.0.8.4 (Wickham et al., 2019) and reshape2 v.1.4.3 (Wickham, 2007) packages for data manipulations, and ggplot2 v.3.2.1 (Wickham, 2016) for data visualisation. We made illustrative diagrams using Affinity Designer v.1.8.3.641 (Serif, 2020).

RESULTS

Nineteen journals listed on Scimago Journal and Country Rank had accessible author guideline URLs (see https://osf.io/j4fyr/ [DOI: 10.17605/OSF.IO/J4FYR] for search and assessment results). Our assessment of these 19 journals revealed that herpetology journals fare poorly by TOP metrics. Sixteen of 19 journals assessed join 22 % (75/346) of journals (so far assessed) failing to score a single TOP Factor point (Fig. 1). The overall mean (0.53 ±0.37; n = 19; all ± denote standard error) and median (0; n = 19) total scores for herpetology journals are lower than those derived from the TOP dataset (mean = 4.92 ±0.28; median = 3; n = 346; Fig. 2). The highest scores achieved by herpetology journals were in the design and analysis guidelines.

DISCUSSION

While herpetology journals fare poorly regarding transparency policies, we have a distinct advantage in tackling practices that compromise replicability. We can quickly adopt methods, infrastructure, and guidelines from other fields; a wealth of options are available with transparency at their centre (Hampton et al., 2015; Parker et al., 2016), and we can prioritise the most successful and effort-effective solutions (Nuijten, 2019; Parker & Nakagawa, 2014; Seigel, 2016; Voelkl et al., 2020). A concerted effort by the entire research community can maximise transparency, countering questionable research practices, while boosting the value of every study. Longer term changes will come with journal support, but authors can lead the charge irrespective of slow institution-level changes.

Complete reporting

Consistent and complete reporting of results is a simple way of improving transparency. Reporting practices often prove inadequate, missing key information (Archmiller et al., 2020; Cassey et al., 2004; Parker et al., 2016), but there are clear guides for what information to provide and how best to present that information (Fidler et al., 2018; Percie du Sert et al., 2019).

- Make a study discoverable (including full indexing by journals)
- Report a measure of spread with all means or medians
- Ensure all measures have units
- Include study location, use coordinates and state the coordinate system

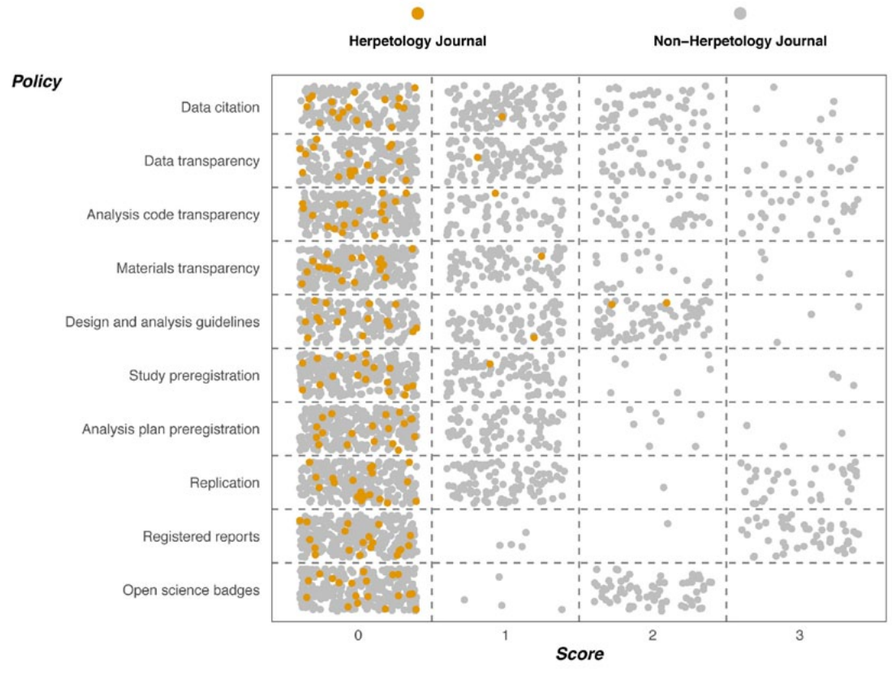

Figure 1. TOP scores of all 346 journals (grey points) plus 19 herpetology journals (orange points). Point locations are jittered within each score category; therefore, point position within the category is not indicative of decimal score.

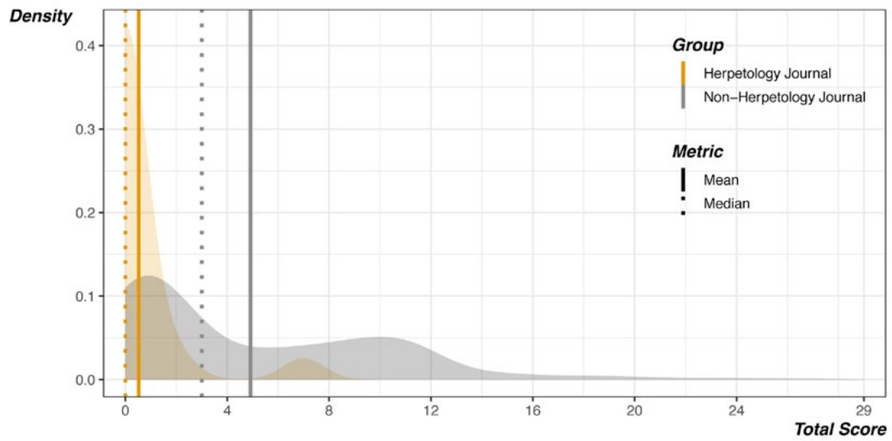

Figure 2. Density of total TOP scores of 19 herpetology journals (orange fill) and 346 non-herpetology journals (grey fill). Vertical lines indicate the mean (solid) and median (dotted) values for herpetology journals (orange) and non-herpetology journals (grey).

- Include spatial and temporal scale of study (i.e., study site and plot extents, study duration –be as specific as possible)
- Provide clear details on how means/medians are calculated from the total sample or subsets
- Report sample size and confidence/credible intervals for each statistical analysis
- Report all results regardless of outcome/significance
- Further suggestions can be found in Fidler et al. (2018) and Gerstner et al. (2017)

By following best practice for reporting we can maximise studies' utility and thus optimise for meta-analysis inclusion (Hillebrand & Gurevitch, 2013; Nichols et al., 2019). Ensuring that methods and statistics are fully reported boosts reach and citations (Gerstner et al., 2017). Low sample sizes result in underpowered tests, variable effect sizes, and unreliable results that exacerbate false positives (Barto & Rillig, 2012; Christie et al., 2019; Forstmeier et al., 2017; Jennions & Møller, 2002). However, being able to combine these results from small studies will be even more valuable in cases where samples are limited by low-detection rates (Boback et al., 2020; Durso & Seigel, 2015; Steen, 2010), technological limitations (Wolfe et al., 2018), and logistical obstacles (Christie et al., 2019). When fully and transparently reported, smaller studies expand and refine broader knowledge (Lemoine et al., 2016).

Full reporting through supplementary material can support meta-analyses. Many journals have restrictions on article length, often prompting us to prioritise the most pertinent findings and to deprioritise others. Making liberal use of supplementary material to report null results, visualise data distributions, and report fruitless exploratory analysis will keep null results present in the literature (Forstmeier et al., 2017). Without such results, meta-analyses can be biased towards positive significant findings (Jennions & Møller, 2002), thus undermining the maturation of knowledge.

Open data

Data availability is the foundation of full reporting and expands study legacy (Gerstner et al., 2017). Extremely concerning findings from other disciplines connect the resistance to data sharing (even at review) to wider replication concerns; lack of data sharing hinders the detection of fabricated data (Czarnitzki et al., 2015; Miyakawa, 2020).

Sharing data on journal websites, or any website without long-term storage infrastructure, leaves data vulnerable to loss, change, and closure (e.g., Applied Herpetology ceased 2010, Hamadryad ceased 2012). The alternative is using dedicated data repositories (Whitlock, 2011). Data repositories (such as datadryad.org, Zenodo.org and OSF.io) are flexible regarding file types and size, as well as being considerably more durable, preserving data well after journals disappear (Whitlock, 2011).

The monetary cost of using these repositories is low to zero (Dryad: US$120 but subject to waivers, Zenodo: Free, OSF: Free; Mislan et al., 2016). Researchers may be reticent to spend the time depositing data. But following existing guidance on data sharing (and ensuring its use by others) can substantially reduce the effort (Borer et al., 2009; Whitlock, 2011; Wilkinson et al., 2016). Using platforms like OSF during the data collection and collaborative phases can streamline final publication. In a few clicks researchers can switch repositories' visibility from private to public, immediately achieving two key points of open data:

- Store data on a stable repository
- Make data citable

Supplying adequate metadata (i.e., information describing the dataset) and storing data in non-proprietary formats (e.g., for datasheets use .csv versus .xlsx) maximises data utility. Metadata should fully explain all data columns, provide details on missing data values, and describe categorical variable codes, to fully enable

third party data use. Using non-proprietary formats maintains data readability and boosts accessibility for researchers without expensive software. Improve data sheet readability by:

- Providing clear documentation
- Avoiding special characters (e.g., %, $, £, @)
- Restricting columns to a single data-type (e.g., binary, interval, categorical, continuous)
- Standardising date formats (ideally the international standard (ISO 8601) YYYY-MM-DD)
- Using descriptive file names
- Following further guidance by Borer et al. (2009).

Many data sharing best practices mirror those for data management, and easier to implement prior to data collection (Alston & Rick, 2020; see Supplementary Table 1). As ecological datasets grow in size and complexity, metadata generation and data management are becoming increasingly necessary skills (Hernandez et al., 2012; Lewis et al., 2018). The British Ecological Society provides concise guidelines and checklists to promote a full suite of good data management practices (British Ecological Society, 2014).

Open code

Open data benefits are further bolstered by open reproducible analyses. The rise of programmable/code-based analyses has enabled entirely recreatable workflows, from data curation to publication (Alston & Rick, 2020); but providing open code is still lagging behind (Culina et al., 2020). A reproducible workflow is a phenomenal resource for reviewers and future researchers (Poisot, 2015), providing both transparency and guidance. Open data paired with code-based analyses enable reviewers to detect errors prior to publication, form the backbone of future studies, and facilitate replications (Mislan et al., 2016). When supplied alongside a paper, code offers a supplementary and more precise description of analysis that avoids the ambiguous language of prose methods sections (Archmiller et al., 2020; Ince et al., 2012). Open data and open code are both necessary for full computational reproducibility and the highest TOP scores for data and analytical transparency.

Version control is an added benefit of coding analysis: a way of recording all changes to files, with the ability to restore previous versions, and make changes simultaneously via branches (simultaneously existing versions of the same file). Online repositories further support version control such as GitHub (https://github.com/), Bitbucket (https://bitbucket.org/), and GitLab (https://www.gitlab.com/). These online platforms provide an additional back-up of files and joint work space for collaborators. Once analysis is completed, researchers can share analysis code via citable repositories with both reviewers and readers (Cooper et al., 2017; Poisot, 2015; White, 2015). However, GitHub and others are non-permanent, final code storage requires long-term solutions mirroring data storage options (Culina et al., 2020). In its most reproducible form, code repositories can combine with Docker (https://www.docker.com/) or Binder (https://mybinder.org/) ensuring a re-runnable workspace (workspace being the environment and files associated with the code) capable of displaying analysis and results independent of the original researchers' workspace (Alston & Rick, 2020).

Doubts over one's coding ability can make us reluctant to share. But realising that most code is cobbled together until it works counters this fear: "...if your code is good enough to do the job, then it is good enough to release." (Barnes, 2010). Errors in analysis are inevitable, even for organisations like the Met Office or NASA (Barnes, 2010; Ince et al., 2012). Sharing at the earliest opportunity offers the simplest solution to find and mitigate analytical errors. There is a wealth of options for learning and finding coding support (Carey & Papin, 2018; Cooper et al., 2017; White, 2015; Wilson et al., 2014; Software Carpentry (http://software-carpentry.org/; see Supplementary Table 1).

Resistance to sharing

There is resistance to sharing, namely due to fears of scooping or competition (Anderson et al., 2007; Blumenthal et al., 2006), high cost-to-benefit, and exposing sensitive species. We feel these concerns can be easily mitigated within current frameworks.

Short-term data embargoes can counter scooping, where data is available to reviewers, and then opened once researchers have completed further desired analyses. Piwowar & Vision (2013)'s findings suggest that one year embargoes would likely be adequate to guarantee the original researchers exclusive access, as third-party citations tend to occur at least two years after publishing. We still discourage most cases of embargoes. Immediate unfettered data access leads to immediate benefits. The costs to researchers are low, but the benefits are massive: boosting citation rates (Piwowar & Vision, 2013), opening doors for new collaborations, and enabling large scale synthesis projects (Hampton et al., 2015; see Tucker et al. (2019) for an example of a study enabled by data storage). Sharing generates new questions, increases study reach, and informs future study/analysis design –resulting in better questions, quicker.

Protecting sensitive species' locations is a legitimate concern, as publication of new species localities has been implicated in the damaging exploitation of herpetofauna (Auliya et al., 2016; Stuart et al., 2006). Such concerns can be a legitimate reason for withholding geographic information. However, we encourage researchers to explicitly state limitations in data availability statements (as per TOP suggestions), while censoring the minimum amount of geographic information and leaving remainder accessible. As data accessibility becomes the default and statements on access mandatory (Aalbersberg et al., 2018), more authors will benefit from open data.

Normative peer-review

Peer review is a critical avenue for these practices to

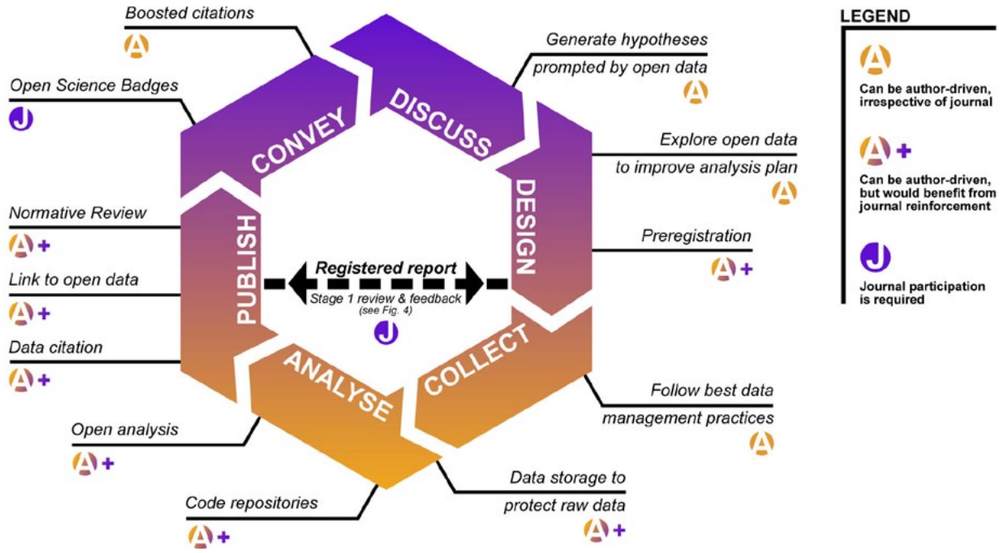

Figure 3. Diagram illustrating where practices and benefits of open science fit within the research cycle.

become more accepted and eventually standard (Morey et al., 2016; Poisot, 2015). We are provided with repeated opportunities to help each other refine manuscripts, and maximise studies' contributions. Referring back to guidelines for statistical reporting, data access, and reproducible analysis can ensure a consistent normative push towards a better body of literature (Morey et al., 2016). By leveraging checklists we improve the thoroughness of reviews and minimise the effort (Grey et al., 2020; Parker et al., 2018; Percie du Sert et al., 2019). The checklists compliment the improved reporting and transparency guidelines (paraphrased checklist from Parker et al., 2018):

- All sample sizes (and sample subsets) are fully reported
- Methods are sufficiently detailed for repeated analysis
- Statistical results are reported completely (e.g., all variables, tests, and transformations)
- Efforts taken to reduce unconscious biases
- Sample stopping rule stated (i.e., predefined sample size justification)
- Analysis designed prior to observing the data (e.g., a preregistration exists), otherwise described as exploratory
- Suitable research methods irrespective of results
- Sample size are capable of supporting authors' conclusions (e.g., tests are sufficiently powered)
- Estimated effect (and uncertainty) is considered in relation to the biological context
- Unexpected results are supported by strong evidence

Deficiencies in any of the above can highlight areas to improve reporting or where authors must acknowledge potential biases, rather than simply necessitating rejection (Parker et al., 2018). By increasing transparency our ability to assess research quality is improved (Aalbersberg et al., 2018); open data and analysis enable us to suggest more targeted solutions, boosting the potency of the peer review process. Ideally journals can source specific code reviewers, who are fluent and familiar with the analysis code; code reviewers' impact would be bolstered if combined with a two-stage review system (see registered reports below).

Reinforce with journal policy

Journals can promote transparent policies by modifying their author guidelines. Journal-level enforcement (Roche et al., 2015), and clear guidelines for editors and authors (Christian et al., 2020), are required because data access requests are rarely fulfilled without enforcement (Archmiller et al., 2020). Similar enforcement will likely improve transparency in data citation and analysis code (Culina et al., 2020).

Other journals have demonstrated how policy changes can rapidly modify publishing practice (Nosek et al., 2012). Several herpetology journals already require mandatory data deposition for genetic data (e.g., *African Journal of Herpetology*, *Copeia* [renamed to *Ichthyology and Herpetology*], *Herpetology Notes*, *Salamandra*, and *South American Journal of Herpetology*). Therefore, improving TOP scores only requires expanding existing policy rather than introducing new rules. Once journal policy has expanded to target pro-transparency practices, authors and peer reviewers can be supplied with checklists to guide best practices (e.g., those provided by *PeerJ*, *Herpetological Conservation and Biology*, *Nature Communications*).

The discipline of psychology has led the charge toward open science practices, showing successful implementations drove normative change. For instance, the journal *Psychological Science* is one of a number of journals that began using "open science badges" to label papers that implemented practices such as data archiving. These badges not only help readers identify transparent and reliable papers, but they may incentivise authors to pursue open science to earn the badges (Blohowiak et al., 2020; Kidwell et al., 2016).

Publish negative results and replications

All herpetology journals scored zero in the replication TOP policy (Fig. 1). Following the recommendations of TOP, journals must be open to publishing replications and negative results (Nakagawa & Parker, 2015), dispensing with statements encouraging novelty. If journals can relieve the pressure for positive/significant results, the incentives to undertake questionable practices will cease to exist (Nilsen et al., 2020), and counter publication biases (Nichols et al., 2019). Replications do not suffer the lower citations rates that journals fear (Forstmeier et al., 2017), and researchers welcome them (Fraser et al., 2019). We argue that replication studies in herpetology would accumulate citations faster (along with original study) as they further validate or refute findings.

Registered reports are the most direct way to counter publication bias (Allen & Mehler, 2018). Registered reports remove the results from the assessment of publication-worthiness, via a two-stage peer review: stage 1 assesses the study methodology prior to interacting with the data, stage 2 assesses whether the researchers followed their proposed methodology (Fig. 4). Critically, the journal decides whether to accept the publication at stage 1, via an "in principle acceptance", meaning any decision is only contingent on a solid study design, not novel findings or significant results. The stage 1 peer review has vast benefits for researchers: we can correct weaker methods and analyses, we can identify journal guideline conflicts, and we can improve methods based on overlooked literature, prior to expending time and money on experimentation (Dirnagl, 2020).

Disassociating the results from publishing decisions is especially valuable for countering questionable research practices (p-hacking, HARKing, and cherry-picking). As the methods are decided prior to seeing the data, researchers' analytical flexibility becomes limited and the distinction between hypothesis testing versus exploratory results becomes clearer. In addition, obtaining a peer review prior to data collection can generate new hypotheses, approaches for analysing the data, and new useful covariates.

While registered reports require journal collaboration, authors can begin restructuring their research workflow with preregistrations. Preregistrations lack registered reports' level of rigor (namely the stage 1 peer review), and the protection against journals' prioritisation of positive/significant results. But they do present a powerful framework considering study design flexibility. Preregistrations produce a record of time-stamped a priori hypotheses that aids later peer-reviewers to identify exploratory analysis and selective reporting (Parker et al., 2019; Toth et al., 2020). Herpetology journal adoption of registered reports is currently non-existent, so authors can leverage preregistration to improve their own practice.

The lack of incentives to review can create an environment where submitted publications outstrip available reviewers (Fox & Petchey, 2010; Peres-Neto, 2016); therefore, an additional review stage may seem like a further burden. Using a wider more diverse pool of career researchers would lessen the pressure on individual peer reviewers (e.g., the Early Career Reviewer Database created by Susan Perkins, Curator & Professor, AMNH: https://sites.google.com/view/ecrdatabase/home; Garisto, 2020; Seigel, 2016), and multi-stage review will catch issues earlier, thus reducing the overall effort of review during publishing (Parker et al., 2019).

Closing remarks

We highlight three key avenues to enhance replicability in herpetology: personal practice, open science, and journal policy. The first requires author awareness. Only by recognising the incentives that promote questionable practices can we actively shun them. Second, share widely and freely, promote openness and reward transparent reporting. If we make transparency core to publishing and peer review, we can steer practices towards a system that amplifies error detection, provides more

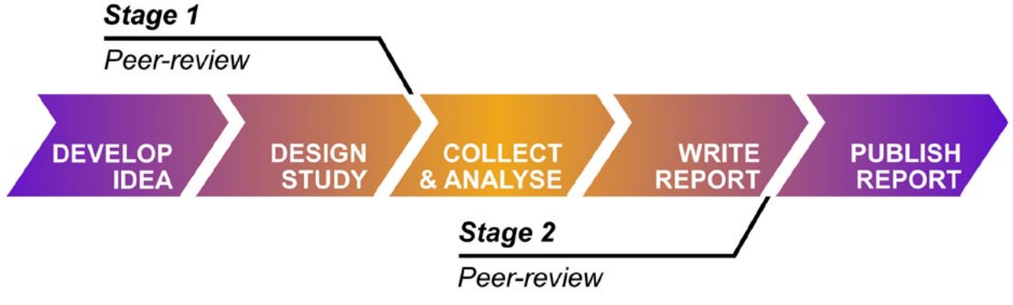

Figure 4. Diagram illustrating the registered report timeline from idea to final publication. Redrawn from OSF summary on registered reports: https://osf.io/rr/.

insightful refinements, and builds stronger foundations for future studies. Finally, support journals promoting transparency, but do not allow enforcement deficiencies to prevent us from following our own best practice to maximise transparency. The publishing system may be slow to change –we, as authors, editors and reviewers, must lead. We can all enhance herpetology by adopting and benefiting from open science.

Data and code availability

Supplementary Table 1 contains open science resources (Supplementary Table 1 - Open science resources.csv), the Scimago Journal and Country Rank search results (SJR - Journal Search Results 2020-02-21.pdf), assessment results of herpetology journals (top-herp-assessment_2020-03-16.csv & top-herp-assessment_metadata_2020-03-16.csv), the overall TOP dataset used (top-factor_2020-03-24.csv & top-factor_metadata_2020-03-24.csv), R code to summarise the data and produce figures (Summary of TOP scores.R), and diagrams of the research cycle and registered reports are available at https://osf.io/j4fyr/, DOI: 10.17605/OSF.IO/J4FYR.

Contributions

Conceptualisation - B.M.M. and C.T.S., Formal analyses - B.M.M., Investigation - B.M.M., Writing - Original Draft - B.M.M., Writing - Review & Editing - B.M.M. and C.T.S., Visualisation - B.M.M.

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Habitat use and age structure of the Fer-de-Lance (*Bothrops asper*, Viperidae) in Braulio Carrillo National Park, Costa Rica

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The Fer-de-lance or terciopelo (*Bothrops asper*) inhabits a wide range of environmental conditions and habitats across Central America. While much information on the species is based on anecdotal observations and museum specimens, data collected under natural conditions are more limited. To better document its natural history, this study sought to determine the habitat use and age structure of *B. asper* in the Quebrada Gonzalez sector of Braulio Carrillo National Park, Costa Rica. Snake surveys were conducted from January 2015 to August 2017 and used to evaluate the population age-class distribution and sex ratio. To evaluate macrohabitat use, surveys were conducted in four habitat types (mature forest, late succession, early succession, and human infrastructure). Microhabitat use was determined by recording several structural variables at each snake location and at random sites. Amphibians were sampled in each habitat type to evaluate the available prey base. Fifty-five individuals were captured, mostly females and juveniles, with five recaptures. Snakes were encountered in all habitat types but most often in early succession forests, which have dense vegetation cover and high prey availability. Snakes selected areas with heavy understory cover when resting, and more exposed sites, often closer to bodies of water, when ambushing prey or moving. Human-disturbed sites were used least. Although snake encounters did tend to correlate with higher amphibian abundance, other factors such as mammalian prey abundance could also influence snake distribution.

Keywords: occupancy, population ecology, habitat selection, behaviour, predator-prey relationship

INTRODUCTION

The venomous snake genus *Bothrops* (Serpentes: Viperidae) is well-known for its widespread geographic distribution across Latin America, occurrence in a wide variety of habitats, diversity of species, and for being the primary cause of snakebites throughout the region (Oliveira, 2001; Valdujo et al., 2002; Nogueira et al., 2003; Hartmann et al., 2005). In addition to natural habitats, some species such as *B. asper*, *B. atrox*, and *B. jararaca* frequently display a willingness to utilise areas modified and used by humans (e.g. plantations, rural villages), resulting in extensive interactions with humans and therefore correspondingly a high number of snakebites (Martins et al., 2001; Oliveira, 2001). This is particularly true for *B. asper*, popularly known as the "terciopelo" or "barba amarilla" in its local range, and "Fer-de-Lance" or "lancehead" in the English-speaking world. This snake has strongly cryptic colouration, potent venom, and often-high local abundance in various macro- and microhabitats (including primary and secondary forests, swamps, pastures, agricultural plantations, and human settlements) across a variety of vegetation types (Bolaños, 1972; Janzen, 1980; Cisneros-Heredia & Touzet, 2004; Sasa et al., 2009). Consequently, it is also the species responsible for the majority of snakebites and snakebite fatalities in Central and northern South America (Otero-Patiño, 2009).

Understanding *B. asper's* ecology and behaviour is an essential component of managing snakebite risk within its range. However, while a good deal of research has focused on the species' toxicology, reproduction, and systematics, relatively little has addressed its natural history or behaviour (Sasa et al., 2009). The majority of available natural history information was collected at La Selva Biological Station in Costa Rica (Wasko & Sasa, 2009; Wasko & Sasa, 2010; Wasko & Sasa, 2012), to date the only comprehensive studies to address the behavioural ecology of *B. asper* under natural conditions. Although these studies were carried out at a single lowland-rainforest site at a somewhat fragmented and highly-visited field, their results have been used to propose snakebite-management measures throughout a wide region of Costa Rica (Hansson et al., 2013). Further

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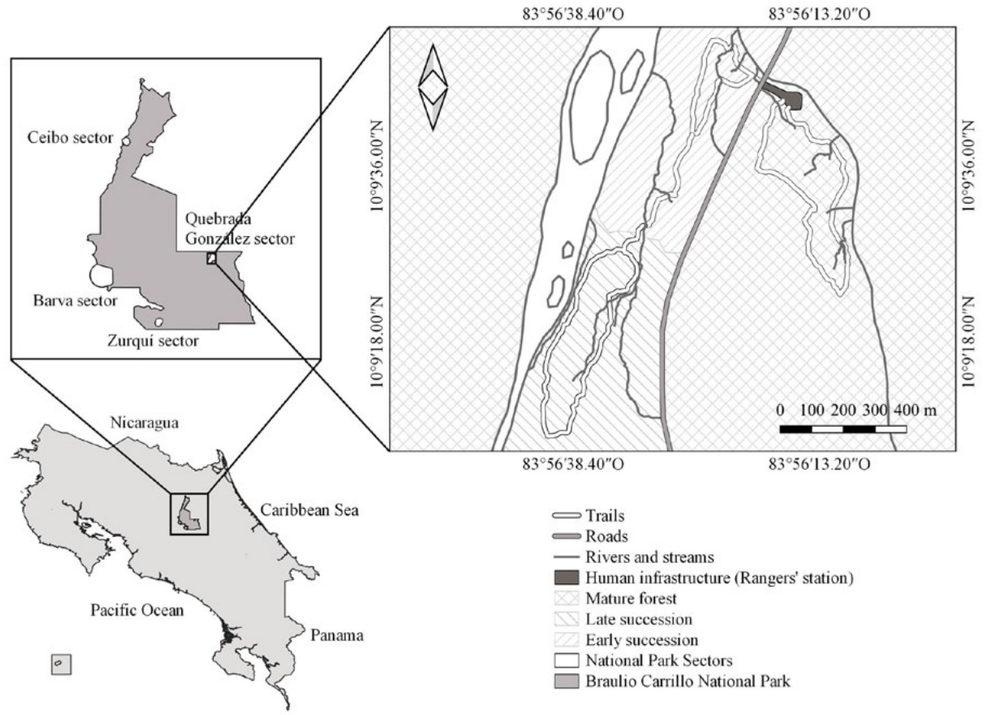

Figure 1. Map of Quebrada González sector of Braulio Carrillo National Park, Costa Rica, showing the location of habitat types, trails, ranger station, roads, rivers and streams. Boundaries between habitat types are approximate and show some transition. River and road data taken from ATLAS 2014 of the Instituto Tecnológico de Costa Rica (ITCR), Cartago, Costa Rica.

study in other natural and rural areas would be useful to further generalise our understanding of *B. asper´*s ecology and to manage its snakebite risk (Brito, 2003; Pearson et al., 2005; Morrison et al., 2006).

We conducted an ecological survey of *B. asper* in the Quebrada González sector of Braulio Carrillo National Park in Costa Rica, an area that is contiguous with La Selva Biological Station but differs in size, elevational gradient, habitat diversity, degree of fragmentation, and rate of visitation by humans. No quantitative data on the ecology of *B. asper* exist for the site, but it is reported as common (Ramírez-Arce et al., 2019). Our specific goals were to; 1) determine the age-class distribution and sex ratio for the *B. asper* population, 2) evaluate macrohabitat and microhabitat use, and 3) assess the relationship between prey availability and *B. asper* abundance.

METHODS

Study area

Braulio Carrillo National Park is a large (47,683 ha) reserve located along the Cordillera Central mountain range of Costa Rica (Tenorio, 1993). Quebrada González sector is a 72 ha region on its eastern side, consisting largely of pluvial premontane forest transitioning to basal humid tropical forest, with an average elevation of 514.40 ± 81.30 m and steep topography (Oviedo-Pérez & Fournier-Gutierrez, 2008) (Fig. 1). The mean annual temperature is 24° C and mean annual precipitation is 6,375.50 mm, with a distinct wet (May-December) and dry season (January-April; Guyer & Donnelly, 1990). Vegetation cover includes mature forest, secondary forest, and a broad open area of riverbed with some grasslands (Lücking, 1999; Oviedo-Pérez & Fournier-Gutierrez, 2008; Vásquez-Acosta, 2009) (Fig. 1). Major river tributaries and smaller streams maintain a constant flow, providing abundant water resources throughout the year (Tenorio, 1993; Schelhas & Sánchez-Azofeifa, 2006).

We classified the area into four distinct habitat types: Mature forest, Late succession, Early succession and Human infrastructure (Fig. 1). Mature forest, Late succession and Early succession comprised natural forested areas, while Human infrastructure a ranger station and surrounding disturbed areas (0.92 ha). Each habitat classification was determined using the literature (Lücking, 1999; Oviedo-Pérez & Fournier-Gutierrez, 2008; Vásquez-Acosta, 2009) and by quantifying structural cover in natural areas. Ten vegetation sampling plots were randomly distributed along each natural area using QGIS 2.16.3 (QGIS, 2016). At each 10 x 10 m plot we determined tree density, total basal area covered by trees, and diameter at breast height (DBH) of each tree with a diameter more than 5 cm, while for trees with a diameter less than 5 cm we obtained only tree density (Table 1). Three unpaved trails with similar lengths (1.50-2.00 km)

Table 1. Density of trees and basal area covered by trees for natural habitat types of Quebrada González sector, Braulio Carrillo National Park, Costa Rica. DBH = Diameter at breast height.

Habitat type	Tree diameter				
	DBH < 5cm	5 cm ≤ DBH < 10 cm		DBH ≥ 10 cm	
	No. /ha	No./ha	Basal area (m^2/ha)	No./ha	Basal area (m^2/ha)
Early Succession	1380	470	1.77	510	19.92
Late Succession	1420	400	1.62	500	28.01
Mature Forest	1150	290	1.23	730	33.77

traverse the sector's natural areas, each predominantly through only one of these habitats. Trails were therefore used to access and assess natural habitats, while Human infrastructure was assessed by searching in its entire area.

Snake surveys and age structure

This study was conducted from January 2015 to August 2017. As tropical snakes may demonstrate seasonal variation in habitat use (Savage, 2002; Morrison et al., 2006), we surveyed both rainy and dry seasons each year. We conducted monthly field trips, each lasting three days, for a total of 28 field trips and 84 days of fieldwork. As *B. asper* shows a marked difference in behaviour and microhabitat use during day and night (Wasko & Sasa, 2009; Wasko & Sasa, 2010), we carried out 54 diurnal and 69 nocturnal surveys. The duration of each survey was 3 to 5 hours, carried out by two people, for a total survey effort of 350.80 person-hours during the day and 470.60 person-hours at night.

Snakes were located using standard visual-encounter surveys of about 3-5 hours each. For natural habitats, each survey was performed by searching either along their respective trails or along established 50 x 2 m perpendicular rectangular transects that were 10 m from the actual trail. Transects were separated by 100 m, resulting in 15 transects in Early succession, 20 in Late succession, and 16 in Mature forest. Trail length was 1,500 m for Early succession, 2,000 m for Late succession and 1,600 m for Mature forest, and we searched within 1.5 m of each side of the trail. For Human infrastructure, size and shape of the area didn't allow us to establish linear transects as in natural areas, so surveys were performed by searching throughout its area for 3-4 hours, with a search effort roughly equivalent to each natural habitat. The total survey effort in person-hours and total search area for each habitat type, is presented in Table 2.

Individual *B. asper* were manually captured using standard field tools and marked with PIT tags (Biomark®, 8.40 mm 134.20 kHz ISO FDX-B). Each snake was measured for total length (following the methods of Solórzano & Cerdas, 1989) with a measuring tape and sex was determined by eversion of hemipenes (McDiarmid et al., 2012). Individuals were classified as juveniles

Table 2. Survey effort performed in each habitat type in Quebrada González sector, Braulio Carrillo National Park, Costa Rica.

Habitat type	Total survey effort		
	Search area (ha)	Person-hours	
		Day	Night
Mature Forest	0.64	91.00	122.40
Late Succession	0.80	103.80	117.60
Early Succession	0.60	92.00	140.60
Human Infrastructure	0.92	64.00	90.00

if their total length was less than 99.50 cm (males) or 115 cm (females), and classified as adults otherwise, in accordance with the species size at sexual maturation (Solórzano & Cerdas, 1989). From these data, we calculated the a) age-class distribution, b) overall sex ratio, and c) sex ratio within each age class. The specific point of capture was recorded with a GPS unit (Garmin 62s). Following data collection, snakes were released at the point of capture.

We also noted the snake's behaviour at the time it was found. Behaviours were classified as either resting (lying coiled or uncoiled with the head on the ground or on its own body), ambushing (lying coiled with the head raised and alert), or moving (if the snake was actually in motion horizontally or vertically).

Habitat use and availability

We assessed macrohabitat use by comparing the number of captures by habitat types (Mature forest, Late succession, Early succession and Human infrastructure). We assessed microhabitat use by recording a series of variables at each snake capture that have been documented as relevant to snakes (Blouin-Demers & Weatherhead, 2001; Charland & Gregory 1995; Wasko & Sasa, 2010): Substrate type, canopy cover, ground cover, height, distance to nearest permanent water body, distance to nearest fallen log ≥ 10 cm diameter, and distance to nearest tree ≥ 25 cm DBH. Substrate type was the substrate on which the snake was located (e.g. soil, leaf litter, log, paved cement) and height the distance from the ground to the element of the habitat were the snake was encounter, such as if resting in a fallen log. Canopy cover was determined with a spherical crown densiometer and ground cover was calculated as the total percentage of the ground area covered by anything other than exposed soil/leaf litter, typically structural elements like low vegetation, following Charland & Gregory (1995). Distances over 50 m were considered "not available" at that location based on typical movements of *B. asper* (Wasko & Sasa, 2009).

In order to investigate habitat selection by snakes, we compared habitat use to availability, with non-random use considered active selection (Blouin-Demers & Weatherhead, 2001; Tozetti & Martins, 2008). Macrohabitat availability was quantified as the proportion of each habitat type across the search area, determined using QGIS 2.16.3 (QGIS, 2016). For this purpose the "search area" was defined as the area

searched for snakes (trails, transects, and the entire Human infrastructure, Table 1). For microhabitat, sixty random points distributed across all habitat types were generated at the study site using QGIS 2.16.3 (QGIS, 2016), they were treated like snake locations and the same microhabitat variables were recorded.

Amphibian availability

To evaluate a possible relationship between snake abundance and prey availability, we measured amphibian abundance in each habitat type. Amphibian surveys were conducted in the same area and following the same methodology as snake surveys. From January 2015 to September 2016, we counted the abundance of amphibians but did not record species identity. From October 2016 to August 2017, we also recorded species identity, using this information to assess which potential prey species were most abundant. Many amphibians are a common prey type for juvenile *B. asper* (Sasa et al., 2009), and some larger species for adults (Wasko & Sasa, 2010). Adults largely specialise on small mammals, but mammal sampling was unfortunately not possible during the study.

Data analyses

We determined whether mean body length differed between males and females using a Mann-Whitney U test. We assessed whether the population differed in proportions of a) juveniles vs. adults, b) overall males vs. females, and c) males vs. females within each age class using Chi-Square tests. We compared the number of snakes observed performing each recorded behaviour (resting, ambushing, or moving) during day vs. night observations and between habitat types.

We determined whether macrohabitat use differed from that predicted by random availability using a likelihood-ratio test. We then addressed snakes' relative use of each habitat using Manly selection ratios, calculated by the proportional use of a habitat divided by the proportional availability of that habitat (Manly et al., 2002). Selection ratios > 1 indicate habitats with greater use relative to their availability; ratios < 1 indicate habitats with lesser use. We also compared the number of individuals in each habitat type between wet and dry seasons and between juvenile and adult snakes using Pearson Chi-Square tests, in order to determine if macrohabitat use differed between seasons and age classes.

Microhabitat use was determined by comparing the values of microhabitat variables from actual snake locations with those from random points using MANOVA, with a difference between groups considered an indicator of active selection. Post-hoc Discriminant Function Analysis was used to determine which microhabitat variables contributed most to the difference between snake locations and random points, as it gives a coefficient of contribution for each variable. We similarly used MANOVA to compare microhabitats used by snakes for different activities, between seasons, and between age classes. Data from males and females were pooled for these analyses because microhabitat use does not seem to differ between sexes (Wasko & Sasa, 2009; Wasko & Sasa, 2010).

To evaluate the possible relationship between amphibian and snake abundances we used a Poisson regression model, with amphibian abundance as a continuous predictor variable. Juvenile and adult snakes were analysed separately. The coefficients produced by this model (positive, negative, or zero) indicate the directionality of the relationship between snake and amphibian abundance (positive, negative, or no relationship, respectively; Quinn & Keough, 2002). To estimate the magnitude of such a relationship, we used incidence rates ratios, which indicate the percent change in the incident rate of the dependent variable for every unit increase/decrease in the predictor variable. A deviance goodness of fit test assessed the overall fit of the model to our data.

Mann Whitney U tests were conducted in STATISTICA 8.0 (Weiß, 2007). All other analyses were conducted using program R 3.5.0 (R Core Team, 2018). For all tests, $\alpha = 0.05$.

RESULTS

Age structure and *B. asper* behaviours

A total of 55 individual *B. asper* were captured during the study, with five recaptures. Across all age classes, females had a mean total length of 95.16 ± 45.01 cm (range 31.50 – 174 cm) and males 53.20 ± 26.79 cm (range 29 – 136 cm; Fig. 2). Females were significantly larger than males

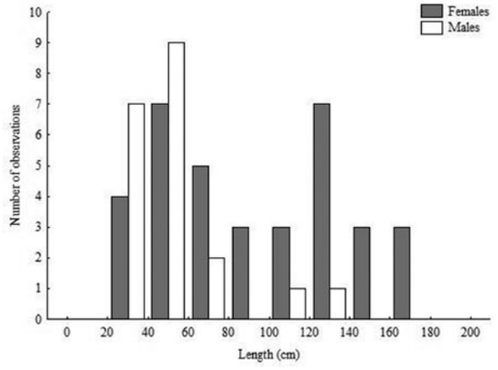

Figure 2. Size classes of *B. asper* males and females encountered at Quebrada Gonzalez sector of Braulio Carrillo National Park, Costa Rica.

(U = 158.50; p = 0.0008), conforming to the expected size dimorphism of *B. asper* (Savage, 2002).

Thirty-nine individuals were juveniles (70.90 % of captures) and 16 were adults (29.10 %); this age class distribution was significantly non-random ($\chi^2 = 9.62$; p = 0.002). Thirty-five snakes were female (63.63 %) and 20 were male (36.36 %), a 1.75:1 female-male ratio. The overall sex-ratio across all age classes was significantly non-random ($\chi^2 = 4.09$; p = 0.043). Within age classes, sex ratio did not differ significantly in juveniles ($\chi^2 = 0.23$;

p = 0.631), but more adults were female (χ^2 = 9; p = 0.002).

Snakes demonstrated significantly different behavioural patterns during the day and at night (χ^2 = 125.14; p < 0.0001). During the day, snakes were most often observed resting and inactive (73 % of observations) and were never seen moving, while at night snakes were more often ambushing (61 % of observations) or moving (39 % of observations), and no snake was observed resting. Behaviours also differed significantly between habitat types (χ^2 = 12.85; p = 0.002). In natural areas (Early succession, Late succession, Mature forest) snakes were more often observed ambushing, while in disturbed areas (Human infrastructure) they were most often seen moving (Fig. 3).

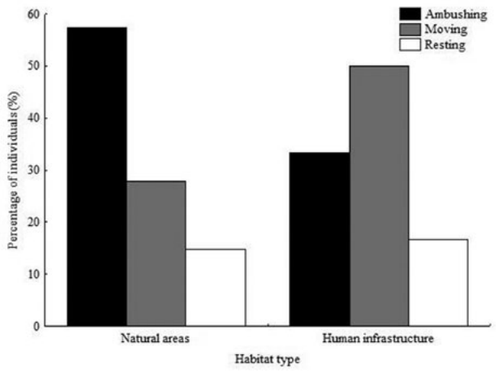

Figure 3. Percentage of *B. asper* individuals observed performing each recorded behaviour between natural and human-disturbed areas at Quebrada González sector, Braulio Carrillo National Park, Costa Rica. Data are pooled from both day and night observations.

Habitat use

Macrohabitat use by snakes was significantly non-random, indicating active selection (G^2 = 52.77; p < 0.0001) (Fig. 4). Early succession had selection ratios higher than 1, indicating a higher use of this habitat relative to its availability, while Mature forest, Late succession and Human infrastructure were lesser used relative to availability (Table 3). Of all 60 snake encounters in both wet and dry seasons, the proportion actually found in each macrohabitat type and the proportion predicted by availability of that habitat are presented in Table 3. Macrohabitat use did not differ between seasons (χ^2 = 7.48; p = 0.06) or between age classes (χ^2 = 6.15; p = 0.10).

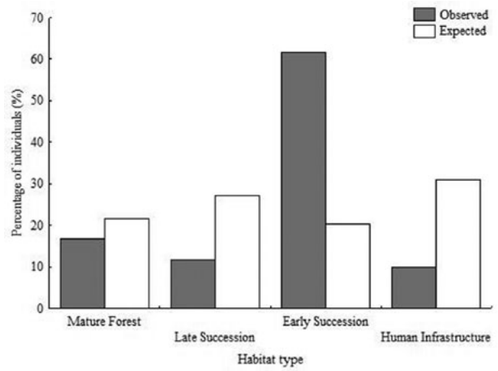

Figure 4. Percentage of *B. asper* individuals observed in each habitat type and percentage expected in each habitat type according to its availability at Quebrada González sector, Braulio Carrillo National Park, Costa Rica.

Across all natural macrohabitat types, nearly all snakes (98.30 %) were found on soil/leaf litter. Only one individual was found on any other substrate, the branch of a tree at a height of 150 cm. In Human infrastructure, all individuals were observed on pavement, except for one individual resting below fallen logs next to a building. All snakes encountered here were always near (12.50 ± 5.60 m) the edge of the forest or buildings, never being found completely in the open.

Snakes used significantly different microhabitat for the observed behaviours (Wilks' λ = 0.58; $F_{10,100}$ = 3.15; p = 0.001). Snakes resting were typically in more concealed areas with higher ground cover and close to fallen logs or trees, while snakes moving and ambushing were in more open areas with less ground cover, closer to bodies of water, and farther from fallen logs. However microhabitat use did not differ between age classes (Wilks' λ = 0.89; $F_{5,51}$ = 1.28; p = 0.29) or between seasons (Wilks' λ = 0.90; $F_{5,51}$ = 1.15; p = 0.35). Data from both age classes and both seasons were therefore pooled for MANOVA analysis, which found overall microhabitat used by snakes differed significantly from that at random unused points (Wilks' λ = 0.47; $F_{15,304}$ = 6.40; p < 0.0001). Discriminant Function Analysis revealed that percent structural ground cover, distance to nearest permanent water body, and distance to nearest fallen log contributed the most to this difference (Table 4).

Table 3. Proportional availability, proportional use by snakes and Manly's selection ratios for each habitat type on Quebrada González sector, Braulio Carrillo National Park, Costa Rica. Data is given for both wet and dry seasons, and pooled from both seasons. Selection ratios > 1 indicate greater use of that habitat relative to its availability; ratios < 1 indicate lesser use (Manly et al., 2002).

Habitat type	% Available	% Used			Selection Ratio		
		Wet season	Dry season	Overall	Wet season	Dry season	Overall
Mature Forest	0.22	0.19	0.14	0.17	0.86	0.64	0.77
Early Succession	0.20	0.56	0.68	0.62	2.80	3.40	3.10
Late Succession	0.27	0.06	0.18	0.12	0.22	0.67	0.44
Human Infrastructure	0.31	0.19	0.00	0.10	0.61	0.00	0.32

Table 4. Microhabitat variables coefficients from Discriminant Function Analysis used to differentiate random points from those actually used by snakes at the Quebrada Gonzalez sector, Braulio Carrillo National Park, Costa Rica. Variables marked with an asterisk (*) were significant at P < 0.05. DF = Discriminant function.

Variable	DF1	DF2	DF3
Ground cover	0.92*	-0.10	-0.15
Distance to nearest permanent water body	0.33*	0.64*	0.60*
Distances to nearest fallen log	-0.39*	0.22	0.30
Distance to nearest tree	0.14	-0.72*	0.68*
Eigenvalues	0.97	0.06	0.00
Cumulative Proportion	0.94	0.99	1

Amphibian availability

A Poisson regression revealed a positive significant relationship between amphibian and juvenile snake abundance (p = 0.0002, coefficient estimate = 0.07, incidence rate ratio = 1.07), indicating an approximately 7 % increase in snake abundance for every unit increase in amphibian abundance. Adult snake abundance was not significantly associated with amphibian abundance (p = 0.18).

We identified 18 species of amphibians, of which at least six represent potential prey of *B. asper* (Solórzano, 2004; Sasa et al., 2009): the frogs *Craugastor fitzingeri*, *Lithobates warszewitschii*, *L. vaillanti*, *Rhinella horribilis*, *Smilisca phaeota* and *S. sordida*. *Incilius melanochlorus* and *L. warszewitschii* were the most abundant species, representing 70 % of amphibian captures. We are unaware of reports of *B. asper* predating the toad species *I. melanochlorus*, however they are known feed on the similar toad *R. horribilis*.

Amphibian distributions along the Quebrada González sector was similar to snake distributions. Amphibians were more abundant in Early succession and Mature forest, and less abundant in Late succession and in Human infrastructure. Of 442 encounters, 44.8 % were in Early succession, 33.03 % in Mature forest, 17.4 % in Late succession, and 4.8 % in Human infrastructure.

DISCUSSION

Population structure

The Fer-de-Lance population comprised a high proportion of juveniles and relatively few adults. *B. asper* has a high fecundity (up to 80 offspring per clutch) and annual or biennial reproduction (Solórzano & Cerdas, 1989), so the large number of juveniles encountered is not surprising, even considering the greater difficulty of visually locating smaller snakes. While the species has a high survivorship in captivity (Sasa et al., 2009) and food resources such as rodents and amphibians are abundant at the study site (Ramírez-Arce, pers. obs.), greater mortality in the wild could explain the lower encounter rate of adults.

The population also demonstrated differences in sex ratio that varied with age class, with males and females encountered equally as juveniles, but the majority of adults being females. Litters are not known to be biased in sex ratio and both sexes have similar cryptic colouration, foraging behaviour, and home ranges (Wasko & Sasa, 2010). The observed sex distribution may therefore be due to increased male mortality with ontogeny, as males tend to be smaller and may periodically demonstrate greater movement rates in search of females, which could consequently lead to a higher predation rate (Savage, 2002; Wasko & Sasa, 2009). Males' smaller body size may also contribute to a lower detectability in visual surveys. Sex-ratio data may also be biased since the hemipenes-eversion technique can sometimes produces misidentification of large and heavy-bodied snakes (McDiarmid et al., 2012).

Lira-da-Silva (2009) reports the same pattern of a population becoming increasingly female-dominated with increasing age/size in the ecologically-similar *B. leucurus*, as a result of higher mortality rate among males. Sasa (2009) reported a population sample from agricultural regions of Pacific Costa Rica that was dominated by adult females rather than juveniles, but populations from other regions showed no bias in age or sex ratio. Thus, *B. asper* population structure may vary regionally depending on any number of ecological and anthropogenic factors (Seigel et al., 1987) or data may be biased by survey methodology and differential detectability of sex and age class (Solórzano, 2004; Morrison et al., 2006).

Habitat use, behaviour, and prey association

Bothrops asper were observed in all surveyed areas, with a higher proportion in Early succession. This habitat is characterised by dense herbaceous plants, shrubs, and small-diameter trees and a lower basal area typical of secondary forests, as opposed to the more open structure of primary forest seen in Mature forest (Guariguata et al., 1997; Guariguata & Ostertag, 2001; Morales-Salazar et al., 2012). Snakes in this study were found principally using understory vegetation like shrubs, herbs, and small palms for shelter, and so may utilise the abundant low-lying plant cover of Early succession forest for protection and concealment (Blouin-Demers & Weatherhead, 2001). Alternatively, a higher abundance of amphibians were observed in Early succession compare to the other habitat types, so snakes may preferentially use these areas due to higher prey availability. This could also explain why we saw more snakes resting and ambushing in natural areas than in Human infrastructure, since natural areas provide higher prey availability and shelter. Although snakes were found in human-disturbed areas less than expected, they were seen ambushing and resting on some occasions. This may be explained by *B.*

asper's overall behavioural plasticity, as it demonstrates a willingness to at least enter a wide variety of habitats with varying structural complexity, including forests, pastures, agricultural fields, and rural human settlements (Sasa et al., 2009). This seems to be a property of *Bothrops* in general, as other species in the genus such as *B. atrox*, *B. leucurus*, and *B. pubescens* demonstrate similar habits (Oliveira, 2001; Hartmann et al., 2005; Lira-da-Silva, 2009). However the majority of snakes observed in Human infrastructure were actively moving, and so may be interpreted as "crossing" these areas rather than actively "using" them, moving through a non-preferred habitat to one with higher structural cover and prey availability (Wasko & Sasa, 2010).

Nearly all individuals were located on the ground, which concurs with other studies and is unsurprising since adult *B. asper* are terrestrial ambush-foragers, fairly heavy-bodied, and lack the gap-bridging ability of arboreal snakes (Martins et al., 2001; Sasa et al., 2009). However juveniles are frequently reported using shrubs or fallen trees, and even adults have occasionally been observed using branches of trees at heights of more than 2 m (Sasa et al., 2009; Vega-Coto et al., 2015), as in this study.

Bothrops asper at the study site preferentially utilised areas with high plant cover and/or fallen logs while resting during the day, and ambushed and moved in areas with reduced structural cover at night. This pattern is similar to that observed for the nearby *B. asper* population at La Selva Biological Station and for the related *B. atrox* and *B. neuwiedi* (Oliveira, 2001; Valdujo et al., 2002), as well as for the tropical rattlesnake *Crotalus durissus* in Brazil (Tozetti & Martins, 2008). Such habits are typically explained as snakes utilising vegetation as refuge while inactive during the day, then selecting open areas for nocturnal ambush foraging, where they can more easily detect prey (Seigel et al., 1987). This may also explain why most snakes observed during the study were seen ambushing and moving, since *B. asper* have been documented as spending most of their time resting in concealment, conditions in which visual detection is difficult or impossible (Wasko & Sasa, 2009, 2010).

Snakes at the study site actively selected for proximity to bodies of water when they were ambushing or moving. Since juvenile snake abundance and distribution associated positively with amphibian abundance and distribution in our study, we can infer *B. asper*'s selection of water may be explained by greater availability of amphibian prey. Juvenile *B. asper* feed heavily on amphibians (Martins et al., 2002), including the most frequently-encountered species at the study site. A similar pattern was described by Wasko & Sasa (2010) and Wasko & Sasa (2012) where *B. asper* demonstrated a strong association with water in order to feed on large amphibians, as a result of a low availability of mammalian prey at that time. Proximity to bodies of water may also be explained as snakes using water itself as a resource (such as for drinking), snakes using rivers and streams to move from one ambush site to another, or snakes searching for others types of prey like small mammals. Similar water-association is seen among other *Bothrops* species (Oliveira, 2001; Valdujo et al., 2002; Nogueira et al., 2003).

Unlike juveniles, adult snakes were not associated with amphibian abundance. *Bothrops asper* shows an ontogenetic change in diet, with a shift towards small-mammals specialisation as an adult (Martins et al., 2002). We were unable to methodically sample small mammals during this study, but numerous individuals were observed opportunistically. We confirmed the presence of a number of rodent species including *Heteromys desmarestianus*, *Melanomys chrysomelas*, *Mus musculus*, *Nyctomys sumichrasti*, *Proechimys semispinosus* and *Rheomys* sp. Adult *B. asper* responded strongly to an experimental availability of rodent prey at the La Selva Biological Station in Costa Rica (Wasko & Sasa, 2012), so further research is needed to evaluate the relationship of *B. asper* to mammalian food resources under natural conditions.

Snakebite management

Bothrops asper is a highly-venomous species responsible for the highest number of snakebites to humans in Central and northern South America, and is thus of great medical significance (Otero-Patiño, 2009). Studies such as this provide information important to understanding *B. asper* distributions and behaviour, which is critical to managing snakebite risk for people working or residing in natural, rural, or agricultural areas where encounters with *B. asper* is most likely (Hansson et al., 2013). For example, this information may allow park rangers to provide recommendations that help visitors to protected natural areas avoid snakebite accidents. Institutions like Costa Rica's Ministerio de Ambiente y Energía also utilise such information in educational outreach programs intended to minimise snakebite risk in rural areas. Additional studies of venomous snakes under natural conditions further our understanding of the factors explaining presence, abundance, and activity of these animals, as well as to manage the risk they can present to humans.

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SHORT NOTE

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Comparisons of image-matching software when identifying pool frog (*Pelophylax lessonae*) individuals from a reintroduced population

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Photographic identification of individual animals is a non-invasive and cost-effective method that can provide demographic information on wild populations. This study aims to compare two photo-matching algorithms (Wild-ID and I3S-Spot) using a reintroduced population of pool frogs (*Pelophylax lessonae*) in the UK as a case study. We compared the following parameters 1) sex and age, 2) image quality, 3) image collection size and 4) processing time to evaluate successful image match rates. There were no significant differences in successful match rates found between sex and age groups. Wild-ID was more sensitive to image quality than I3S-Spot. There was a significant negative relationship between image collection size and successful match rates for I3S-Spot, however, no such relationship for Wild-ID. The findings of our study can be used by conservation practitioners to reduce workload and improve accuracy during population monitoring activities.

Keywords: Amphibia, capture-recapture, I3S, photo identification, population monitoring, Wild-ID

Amphibians are experiencing global population declines due to threats such as climate change, habitat loss and infectious diseases (Antwis et al., 2014) and there is a need for adequate monitoring of wildlife populations to guide conservation activities. Methods including capture-mark-recapture (CMR) and capture-recapture (CR), are commonly used to assess population parameters in naturally occurring and reintroduced populations (Lagrange et al., 2014). Individuals are either identified by unique skin markings or artificial markers such as pit-tags, however, these methods can be time-consuming and cost ineffective (Bendick et al., 2013; Sannolo et al., 2016, Guimaraes et al., 2014; Griffiths et al., 2015; Chevalier et al., 2017).

The pool frog (*Pelophylax lessonae* - previously *Rana lessonae*) Camerano, 1882 is distributed across Europe but went extinct in the United Kingdom during the 1990s. Since then a reintroduction program has established a viable population at sites in England using individuals from the northern-clade in Sweden (Zeisset & Hoogesteger, 2018). Globally, the species is listed as 'Least Concern' on the IUCN Red List of Threatened Species but has a declining population trend and a need for conservation intervention has been recognised (Kuzmin et al., 2009). Pool frogs have distinctive and variable spotted skin patterns with a pale dorsal stripe, and exhibit some sexual dimorphism whereby adult males have paler dorsal base-colours than females (Hoogesteger et al., 2013). The presence of distinguishable morphological features and a need for adequate and minimally invasive population monitoring, mean that pool frogs are a suitable species for non-invasive photographic monitoring techniques. Photographic records of individuals from the reintroduced UK population are currently captured throughout the year, from which population estimates are currently calculated based on CR techniques (John Baker, pers. comm). However, this process is very time consuming for conservation practitioners.

There are now several automated software algorithms available that can aid this process and have been employed across a range of taxa, including amphibians (Elgue et al., 2014; Sannolo et al., 2016; Matthé et al., 2017; Speybroeck & Steenhoudt, 2017; Patel & Das, 2020), reptiles (Treilibs et al., 2016), large terrestrial mammals such as the Thornicroft's giraffe (*Giraffa cameleopardalis* thornicroft) (Halloran et al., 2014) and several species of elasmobranch (Gonzalez-Ramos et al., 2017; Navarro et al., 2018). However, such algorithms require varying amounts of input by the user and image quality (Yoshizaki et al., 2009; Halloran et al., 2014; Urian et al., 2015; Treilibs et al., 2016; Gonzalez-Ramos et al., 2017; Matthé et al., 2017; Gatto et al., 2018), data-set parameters (Matthé et al., 2017), morphological features of the study species (Yoshizaki et al., 2009; Urian et al., 2015; Matthé et al., 2017) and performance rates differ between software types.

This study aims to compare two commonly used photo-matching algorithms on a reintroduced population of pool frogs. We quantify differences between the algorithm types by 1) sex and age, 2) image quality, 3) image collection size and 4) processing time with respect

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to successful image match rates. Temporal efficiency is important for population monitoring activities (Iijima et al., 2013) and we aim to make inferences on which algorithm practitioners should use to achieve maximum accuracy with the least amount of user effort.

To achieve this, a total of 1255 pool frog images taken between May and August from 2010 to 2017, were visually examined and 37 % (465) were deemed suitable as they had previously been identified to the individual-level by a species expert and for the purposes of this study, are accepted as being known matches. These images were also visually examined prior to analysis ensuring all were of appropriate quality and key morphological features were identifiable (examples of good quality and poor quality photographs available, see Supplementary Material Fig. S1). Reasons for poor quality images included poor lighting, light reflection from the skin of the individual and obstruction due to submersion in water. Image matches were confirmed and collated by year, sex (male/female) and age (adult/juvenile), thus forming 'image collections'. To identify individuals, we used the characteristic spotted dorsal pattern which is common amongst the species and is unique at the individual-level (Hoogesteger et al., 2013). To control for potential operator error, we repeated the image matching process three times for all image collections and each software. We recorded the number of successful first-time matches along with the time taken to process each collection. Time-per-image was calculated by dividing collection processing time by image collection size. We defined 'image collection size' as the number of images in each collection. We used the quantitative measure dots-per-inch (DPI) to assess image quality (Zhang & Gourley, 2008) and basic summary statistics (mean, standard deviation and standard error) were calculated for each image collection. Successful match rates between the two algorithms were compared based only on images that presented with the highest match-likelihood score, as determined by each algorithm. We used a similar method to Sannolo et al. (2016) and calculated successful match rates as a percentage of known matches in each collection.

We compared the algorithms Wild-ID (Bolger et al., 2012; http://www.teamnetwork.org:8080/Wild.ID) and I3S-Spot (Hartog & Reijns, 2014; http://www.reijns.com/i3s) as they have previously been used in similar studies (see Bendick et al., 2013; Sannolo et al., 2016). Technical differences between algorithms are available in the Supplementary Material and Figure S2, and a step-by-step overview of our methods are available in Figure S3. Images were confirmed as matches if the first image presented by the algorithm was an identifiable match to the image it was being compared to. Overall match rates and time-per-image means were compared between algorithms using a paired t-test. Average match rate variance between sexes and ages was tested using a One-Way ANOVA. This was also analysed by algorithm, however, for Wild-ID we used a Kruskal-Wallis test due to non-parametric data. Simple linear regression was used to test for significant linear relationships between image quality (DPI) and match rates (overall vs. Wild-ID vs. I3S-Spot), image collection size and match rates (overall vs. Wild-ID vs. I3S-Spot), and time-per-image and match rates (overall). All statistical tests were performed using SOFA Statistics version 1.4.6 using an α value of 5 %.

Overall, the mean successful match rate was significantly greater for I3S-Spot (54.1 % ± 0.1 (SE)) than Wild-ID (40.8 % ± 0.2) ($t_{1,50}$ = 4.528, P <0.001). The average processing time per image (seconds) was also significantly greater for I3S-Spot (35 ± 3.7) than for Wild-ID (6 ±1.5) ($t_{1,50}$ = 65.956, P <0.001).

There were no significant differences in successful match rates between images of adult females (49.7 % ± 0.2 (SD)), adult males (48.4 % ± 0.2) and juveniles (40.2 % ± 0.2) ($F_{1,2}$ = 2.327, P > 0.05). When filtered by software type, there were no significant differences between median successful match rates for images of adult males (43.8 %; range 16.7-55.6 %) than adult females (42.9 %; 25-77.8 %) and juveniles (33.3 %; 12.5-54.6 %) using Wild-ID ($H_{1,2}$ = 3.116, P > 0.05) or I3S-Spot (match rates for images of adult males 57.7 % ± 0.1 compared with adult females 53.7 % ± 0.2 and juveniles 46.7 % ± 0.1) ($F_{1,2}$ = 2.070, P > 0.05). An overview of pool frog demographics in each dataset is provided in Supplementary Table S1.

When filtered by algorithm, there was a siginificant positive relationship between mean DPI and successful match rate for Wild-ID ($R^2_{1,49}$ = 0.209, P <0.001; Fig. 1a). There was no significant relationship between mean DPI and successful match rate for I3S-Spot ($R^2_{1,49}$ = 0.018, P >0.05; Fig. 1b).

There was also no significant relationship between image collection size (number of images) and successful match rate for Wild-ID ($R^2_{1,49}$ = 0.007, P >0.05; Fig. 1c). However, there was a significant negative relationship between image collection size and successful match rate for I3S-Spot ($R^2_{1,49}$ = 0.065, P <0.05; Fig. 1d) with higher match rates for smaller collections.

When combining data from both algorithms, there was a significant positive relationship between processing time-per-image and successful match rate ($R^2_{1,100}$ = 0.083, P <0.01). Furthermore, there was no significant relationship between time-per-image and successful match rate for Wild-ID ($R^2_{1,49}$ = 0.019, P >0.05; Fig. 1e), however, there was a significant negative relationship for I3S-Spot ($R^2_{1,49}$ = 0.173, P <0.01; Fig. 1f).

This study is one of the first comparing between two commonly used photo-matching algorithms to monitor reintroduced pool frog populations. Our results are comparable to similar studies with sample sizes ranging from 92 to 852 (Elgue et al., 2014; Sannolo et al., 2016; Treilibs et al., 2016; Gonzalez-Ramos,et al., 2017; Navarro et al., 2018; Patel & Das, 2020). Unlike other studies, we applied minimal operator effort (considering only the first potential matches presented as correct or not and only selecting the minimum requirement of 12 feature points when using I3S-Spot) to achieve our findings whereas others used greater levels of user effort (Sannolo et al., 2016; Treilibs et al., 2016; Matthé et al., 2017). Whilst not compared directly in this work, it is recognised that automated image processing can achieve successful match rates at least equal to manual observation, with potential for an increase in accuracy in several cases (Bendick et al., 2013; Treilibs et al., 2016; Matthé et al., 2017; Pawley et al., 2018). When comparing processing

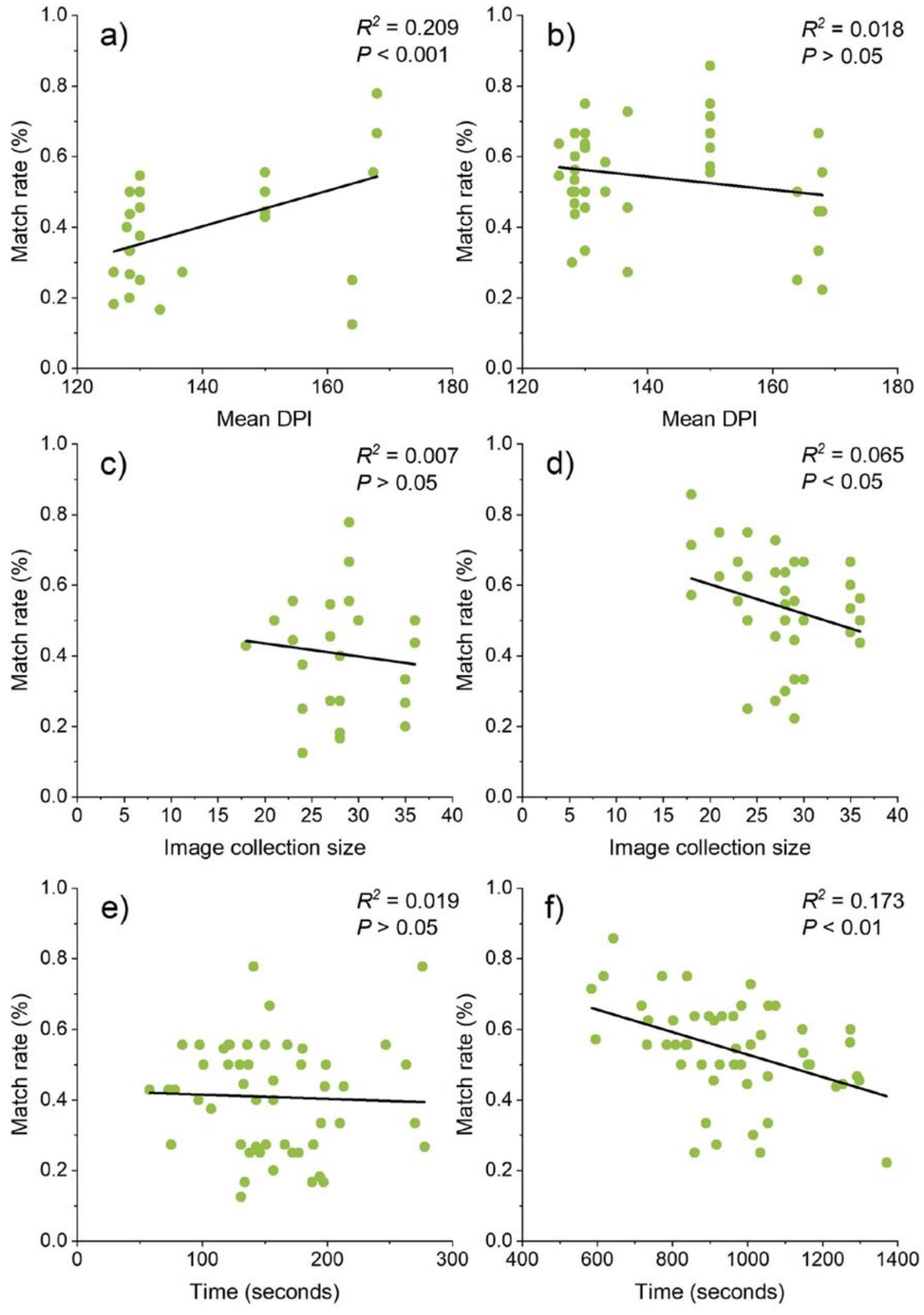

Table 1. Results from linear regression analyses a) Wild-ID match rate and mean image dots-per-inch (DPI), b) I3S-Spot match rate and mean image DPI, c) Wild-ID match rate and image collection size, d) I3S-Spot match rate and image collection size, e) Wild-ID match rate and time taken (seconds) and f) I3S-Spot match rate and time taken (seconds).

times, Wild-ID significantly outperformed I3S-Spot, with the latter taking more than six times longer on average to process images. However, the images used in this case were highly standardised due to a retrofit application of an existing image collection. It has been noted that feature-based algorithms, such as Wild-ID, benefit from image and pattern stability (Matthé et al., 2017) and it is likely that without such uniformity, the software would have performed less successfully. Despite this, we argue that in regards to time effciency both algorithms outperform manual efforts. This is similar to the findings of Halloran et al. (2014), who found that photo-id software could reduce processing time by up to 78 % compared with manual undertakings. We recommend users consider finding a balance between image processing time and successful match rates when using automated algorithms for accurate population assessments. Treilibs et al. (2016) recorded a 13 % increase in successful match rates when investing more time in image processing. We also found a positive relationship between image processing time and successful match rates for both algorithms.

Existing studies have revealed several influencing factors to be considered when using automated algorithms for photo-identification. Such aspects include changing features of indiviudals over time (Yoshizaki et al., 2009; Urian et al., 2015; Matthé et al., 2017), dataset size (Matthé et al., 2017) and image quality (Yoshizaki et al., 2009; Halloran et al., 2014; Urian et al., 2015; Treilibs et al., 2016; Gonzalez-Ramos et al., 2017; Matthé et al., 2017). Sex and age group did not significantly influence successful match rates. Other similar studies have found differences between the sexes, for example in Italian crested newts (*Triturus carnifex*) (Sannolo et al., 2016), however, there was no such difference in our study. This may be due to more extreme sexual dimorphism in Italian crested newts compared to pool frogs. There was a significant negative relationship between image collection size and successful match rates for I3S-Spot, which are similar to findings by Matthé et al. (2017). Our findings are also similar to other studies (Yoshizaki et al., 2009; Elgue et al., 2014; Halloran et al., 2014; Urian et al., 2015; Treilibs et al., 2016; Matthé et al., 2017; Gatto et al., 2018), however, these have been limited by qualitative approaches to assess image quality such as visual examination and manual scoring. It may be more appropiate to measure image quality via the user's ability to identify key morphological features, albeit this could be regarded as subjective and is dependent on user skill and effort. We used a quantitative approach in the form of DPI. Whilst DPI is a valid measurement of an image's quality, it does not classify an image based on the visibility of key features.

Our results have compared two existing automated photo-identification algorithms and highlighted differences between them. However, such algorithms are an asset when monitoring reintroduced populations allowing for the reduction in workload for conservation practitioners, but limited by differences between algorithms and identifying features between species. It is important that practitioners consider 1) an appropriate algorithm to use, 2) their project design and 3) balancing image processing time with successful match rates. This will improve accuracy and efficiency of in-situ conservation and offer automated tools as a viable, if not preferable, alternative to other more-invasive and time-consuming monitoring techniques.

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