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Front cover: Female and radio-telemetered male *Gopherus agassizii* in the Sonoran Desert of California. See article on opposite page. Photo taken by Shellie R. Puffer, U.S. Geological Survey.

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Micro-geographic variation in burrow use of Agassiz's desert tortoises in the Sonoran Desert of California

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Little has been published regarding the burrowing habits of Agassiz's desert tortoises (*Gopherus agassizii*) in the Sonoran Desert of California. We monitored the interactions of tortoises with their burrows, and other tortoises, via radio-telemetry at two nearby sites between the Cottonwood and Orocopia Mountains, from 2015-2018. We examined how annual cycles of drought and non-drought years, behaviourally affected how tortoises use their burrows (i.e., burrow fidelity, cohabitation, and location), including the timing of the tortoise brumation period. Burrow locations were strongly dependent on local geology and topography, with a tendency to orientate in conformance with the general aspect of the landscape. The timing of brumation was similar to records for *G. agassizii* throughout their range (with a few exceptions). There was no difference in the estimated number of burrows used per 30 days between the active seasons (2017 and 2018) at the Orocopia site, despite the occurrence of drought in 2018.

Keywords: *Gopherus agassizii*, Mojave Desert, hibernation, brumation, drought

INTRODUCTION

The threatened Agassiz's desert tortoise (*Gopherus agassizii*) occupies portions of the Mojave and Sonoran Deserts, north and west of the Colorado River. The habitat of *G. agassizii* has historically been described as areas without a great deal of topographic diversity including valleys, alluvial fans, and bajadas (outwash plains) interspersed with desert washes (Berry et al., 2002; Ernst & Lovich, 2009; Murphy et al., 2011). In contrast, its congener, Morafka's desert tortoise (*Gopherus morafkai*), that occupies other parts of the Sonoran Desert, south and east of the Colorado River in Arizona and into northern Mexico, tends to utilise rocky hill slopes and deeply-incised washes with caliche layers that extend out onto valley floors (Germano et al., 1994; Murphy et al., 2011; Riedle et al., 2008). Both species show a high degree of site fidelity to their burrows with relatively small home ranges (Averill-Murray et al., 2002b; Freilich et al., 2000). We compared burrow use of *G. agassizii* between two sites in the Sonoran Desert of California.

Underground refugia are extremely important for the survival of *G. agassizii*, and the species spends as much as 98 percent of their annual cycle sheltering in self-constructed burrows (Nagy & Medica, 1986) to avoid environmental extremes that characterise the region (Bulova, 2002; Mack et al., 2015; Zimmerman et al., 1994). Burrow use varies annually and seasonally. Tortoises typically occupy multiple burrows in a given year, in different substrates, and burrows are usually characterised by their half-dome-shaped entrance and large mound of excavated material at the opening (Burge, 1978; Luckenbach, 1982). Tortoises are believed to select burrows based on a variety of factors that affect their suitability for survival including slope, aspect, and proper soil type for digging and stability (Anderson et al., 2000; Bulova, 2002; Ernst & Lovich, 2009; Lovich & Daniels, 2000; Stager et al., 2017). Proper placement and construction of a burrow has important fitness consequences for a tortoise because they provide protection from predators, thermal extremes, floods, fires, and other mortality factors (Kinlaw, 1999; Lovich & Daniels, 2000).

Burrows are not just important to the desert tortoises; burrows also provide shelter for many other species, some of which cannot dig burrows of their own, like burrowing owls (*Athene cunicularia*) (Agha et al., 2017; Crowe & Longshore, 2013; Ernst & Lovich, 2009; Lovich et al., 2018a; Luckenbach, 1982). Burrow commensals of tortoises include a variety of animals such as lizards, snakes, birds, rodents, spiders, and insects (Agha et al., 2017; Burge, 1978; Kinlaw, 1999; Kinlaw & Grasmueck, 2012; Lovich et al., 2018a; Luckenbach, 1982; Woodbury

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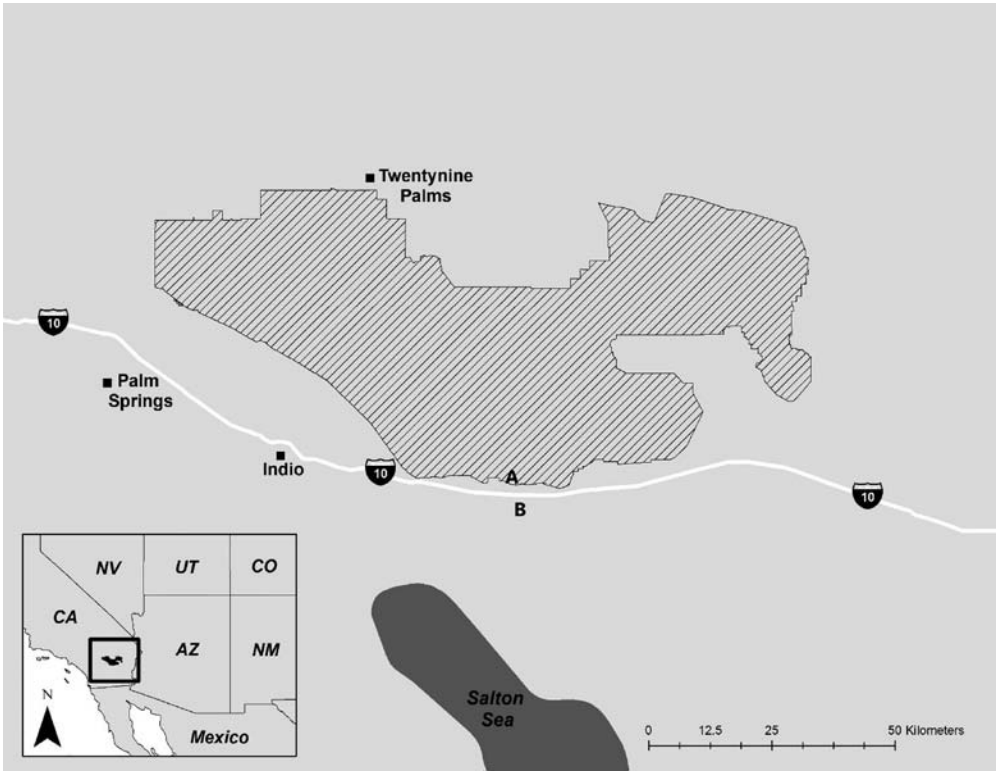

Figure 1. The Cottonwood (A) and Orocopia (B) study sites in reference to Joshua Tree National Park (lined) and the Salton Sea (shaded). Interstate 10 runs east to west between the two sites. The study area is highlighted in the box in California. CA=California, AZ=Arizona, NM=New Mexico, CO=Colorado, UT=Utah, NV=Nevada, USA.

& Hardy, 1948). Commensal species utilise tortoise burrows in numerous ways, sometimes contributing to a trophic bottom-up effect in active tortoise burrows, thus attracting other species to the burrow (Agha et al., 2017; Currylow et al., 2016; Lips, 1991; Walde et al., 2015).

Because drought is an overriding driver in desert ecosystems, burrow use should be affected under drier than usual conditions. Noy-Meir (1973) stated that "desert ecosystems are water-controlled ecosystems with infrequent, discrete, and largely unpredictable water in-puts". All trophic levels are affected during times of decreased precipitation on both micro- (i.e., individual organisms) and macroecological (i.e., large scale ecosystems) spatial scales (Noy-Meir, 1973; Parmesan et al., 2000). During drought conditions, limited water availability causes a decrease in plant biomass that affects the movements, reproduction, and overall behaviour of herbivores (Noy-Meir, 1974; Parmesan et al., 2000). Many animals adapt to drought conditions through behaviour modification (i.e., prolonged time spent sheltering in burrows) or migration to areas less affected by drought or with greater resource availability (Barrows, 2011; Noy-Meir, 1974; Parmesan et al., 2000). Generally, tortoises opt for the former. There are several studies documenting tortoise behaviour during times of decreased precipitation and food availability (Barrows, 2011; Bulova, 2002; Duda et al., 1999; Peterson, 1996). Most of these studies document decreased movements and increased time in burrows in an attempt to conserve resources until conditions improve (Duda et al., 1999; Freilich et al., 2000); however, some report little behavioural change (Rautenstrauch et al., 2002). Our study encompassed periods of both increased and decreased plant productivity and precipitation, including severe drought.

The burrowing characteristics of *G. agassizii* have been studied extensively in the Mojave Desert (e.g., Burge, 1978; Duda et al., 2002; Germano et al., 1994; Luckenbach, 1982). However, very little is published on the burrow use patterns of *G. agassizii* in the Sonoran Desert of California (Agha et al., 2015a; Agha et al., 2015b; Agha et al., 2017; Lovich & Daniels, 2000). Populations of *G. agassizii* occur in and around the Orocopia and Cottonwood Mountains in the western Sonoran Desert of California (Berry & Murphy, 2019; Dimmitt, 1977; Luckenbach, 1982). We examined burrow location and use, of radio-telemetered *G. agassizii* at two study sites between these mountains from 2015–2018. We also describe the burrows used by tortoises at both sites during the cold season inactivity or brumation period. These burrows are referred to as brumacula. We hypothesised that 1) burrow use would be similar between both sites because of their proximity, despite topographic differences, and 2) that tortoises would use fewer burrows during drought years of decreased precipitation and plant productivity because of their need to conserve water resources by decreasing activity levels and remaining in burrows until conditions improve.

METHODS

Study Sites

Data were collected from two study sites (Fig. 1) on

opposing sides of the valley between the Cottonwood and Orocopia Mountains in the Sonoran Desert of southern California. Two major xeroriparian washes drain the valley: Maniobra Wash and Shavers Wash. The study sites are separated by Interstate 10 (a heavily trafficked multi-lane highway extending west to east from Santa Monica, California to Jacksonville, Florida). The Orocopia study site is located on the south side of Interstate 10 approximately 4–5 km south-east of the Cottonwood study site. The highway and distance between sites effectively isolate the two populations from the possibility of intermingling. The Cottonwood study site (north of Interstate 10) extends over an area of approximately 5.75 km^2 in the southern part of Joshua Tree National Park (JTNP) in the area drained by Cottonwood Wash (a tributary of Shavers Wash). This site abuts the southern side of the Cottonwood Mountains and ranges in elevation from about 520 m AMSL on the bajada to over 800 m AMSL in the rocky, mountainous slopes. Topography varies from gentle, sloping, sandy, bajadas with washes, to steep, boulder-covered terrain on the mountain slopes where the washes originate. The geology is dominated by granitic boulders and outcrops in the Cottonwood Mountains. Typical vegetation throughout is creosote scrub (*Larrea tridentata*) interspersed with brittlebush (*Encelia farinosa*), smoketrees (*Psorothamnus spinosus*), ironwoods (*Olneya tesota*), blue palo verde (*Parkinsonia florida*), and ocotillo (*Fouquieria splendens*). Additional site information is presented in Lovich et al. (2018b).

The Orocopia study site covers approximately 21 km^2, from near the south side of Interstate 10 to the northern edge of the Orocopia Mountains, just west-south-west of Chiriaco Summit, a minimum of about 2–3 km away. The site is relatively flat, with a gentle uphill grade to the east and south towards the Orocopia Mountains. Elevations range from 482 to 618 m AMSL. This site is characterised by areas of desert pavement interrupted by sandy to gravelly soil with tank and jeep track scars from WWII training activities at nearby Camp Young in the early 1940s (Henley, 2000; Lathrop, 1983; Prose, 1985). There are a few scattered, small hills and many variably sized washes flowing northerly throughout the site, exposing occasional caliche layers. Orocopia schist is the dominant geology with relatively few granitic boulders in comparison with the Cottonwood site. The vegetation is dominated by creosote scrub (*Larrea tridentata*) interspersed with brittlebush (*Encelia farinosa*), burrobush (*Ambrosia dumosa*), and widely scattered ocotillo (*Fouquieria splendens*) and blue palo verde (*Parkinsonia florida*).

Climate

Southern and central California experienced the worst drought in as long as 1,200 years from 2012–2014 (Diaz & Wahl, 2015; Griffin & Anchukaitis, 2014). Even though the drought continued into 2016 (Flint et al., 2018), the effects were less devastating and allowed enough precipitation to fall for adequate germination of annual food plants for tortoises in 2015 and 2016 at the Cottonwood site due to spatial variation in rainfall. The year 2017 at the Orocopia site also proved to be a reasonably productive year for germination. However, conditions in the 2018 field season returned to severe drought, as a result of the extremely dry winter of 2017–2018 with only one small rainfall event in December. We used the Westmap (https://cefa.dri.edu/Westmap/) pixel function to calculate estimated mean annual precipitation. Estimated mean annual water year (WY) precipitation at the Cottonwood site was 7.84 cm in 2015 and 9.88 cm in 2016. The estimated mean annual precipitation at the Orocopia site was 13.15 cm in WY2017 compared to 1.39 cm in WY2018. No germination of winter annual tortoise food plants was observed at the site in 2018. Long-term precipitation data for the Mojave and Sonoran Desert regions are summarised by Hereford et al. (2006) and Woodhouse (1997), respectively.

Data Collection

At the Cottonwood site, from March 2015 to July 2016, we walked line transects with 2–4 people spaced approximately 12–25 m apart as described in Lovich et al. (2014) looking for all evidence of tortoises and their burrows. Similar techniques were employed at the Orocopia site from February 2017 to August 2018. As tortoises were located throughout the study site, adult males and females were outfitted with radio transmitters (models R1850, R1860; Advanced Telemetry Systems, and model HLPR 2850; Wildlife Materials radio) that weighed no more than 45g. Radios were attached following the procedures outlined in Boarman et al. (1998). All radio-telemetered tortoises were tracked approximately every 14 days during the egg production season from April to July and females were X-radiographed to determine clutch size for another study. After the egg production season, tortoises were tracked once monthly for the remainder of the year while transmitters were still attached (tortoises generally were not handled unless they were out of their burrows or easily accessible). While it is not completely known if *G. agassizii* burrow fidelity is negatively affected by human handling interactions at the burrow, Kahn et al. (2007) did not find a difference in the number of burrows used by or in home range size of gopher tortoises (*Gopherus polyphemus*) that were handled versus tortoises that were not handled. We did not observe any obvious changes to behaviour associated with tortoise handling. As tortoises were radio-tracked, we noted their position and activity in or near the burrow, and the number of tortoises occupying the burrow. Only radio-tracked tortoises were included in the burrow use study because those tortoises were each located up to 30 times (Table 1). Rautenstrauch et al. (2002) suggested that 50 or more locations per tortoise were needed to eliminate the bias of study duration time, but we did not have that many locations for any tortoise. To offset this potential bias, we utilised a weighted estimate of unique burrows used per tortoise per time interval. We define a “unique burrow” as a burrow that is identified belonging to the tortoise(s) that inhabited it. Since individual tortoises were monitored

Table 1. Number of unique burrows used per tortoise at each study site during the entire radio-telemetered period. The identification number (ID) is the unique identifier for individual tortoises. Since the number of days is different for each tortoise due to later additions to the study and radio failures, a weighted estimate of burrow use was calculated. We calculated the number of unique burrows used for each individual tortoise every 30 days by dividing the total number of unique burrows used by the total number of days each tortoise was radio-transmittered, then multiplied that number by 30 days. The number of unique burrows reused is the number of burrows each tortoise used repeatedly non-consecutively.

Site	ID	Sex	Total # times located	Total # unique burrows used	Total # days radio-telemetered	Unique burrows/ 30 days	Total # unique burrows reused
Cottonwood	1	M	27	14	469	0.896	0
Cottonwood	2	F	29	12	469	0.768	3
Cottonwood	3	F	11	7	469	0.448	1
Cottonwood	7	F	25	9	456	0.592	1
Cottonwood	8	M	24	9	456	0.592	1
Cottonwood	9	F	11	3	100	0.9	1
Cottonwood	10	M	10	4	133	0.902	1
Cottonwood	11	F	25	8	454	0.529	3
Cottonwood	12	F	27	11	452	0.73	4
Cottonwood	13	M	25	12	452	0.796	3
Cottonwood	28	F	7	5	97	1.546	0
Cottonwood	31	F	6	4	81	1.481	0
Orocopia	33	M	30	7	489	0.429	3
Orocopia	34	F	28	11	509	0.648	2
Orocopia	35	M	28	8	476	0.504	2
Orocopia	37	M	24	7	449	0.468	2
Orocopia	39	M	24	7	448	0.469	2
Orocopia	40	F	28	9	531	0.508	2
Orocopia	43	M	20	5	407	0.369	1
Orocopia	45	F	26	9	428	0.631	1
Orocopia	51	F	15	3	263	0.342	1

Table 2. Mean number of burrows used throughout the range of *G. agassizii*. Tortoises at the Orocopia and Cottonwood study sites used a similar number of burrows when compared to *G. agassizii* at other sites. The term "burrows" refers to all categories of burrow substrate. Mean burrows is the number of burrows that tortoises used during the time frame specified. Time is the time frame as indicated in each study. Time frame is variable for each study listed as there is little consistency amongst publications. It is assumed that only burrows found housing a tortoise were used since authors did not specify otherwise. Burrows were only counted once each season regardless of the number of times a tortoise(s) repeatedly used a burrow and then counted once again the next season if they were used, unless specified otherwise below.

Citation	Location	$\bar{x}$ Burrows	Time	Additional Notes
Our site [a]	Orocopia site	8.3; 9.1	365 Days	February–December 2017; January–August 2018, Estimated potential mean
Our site [a]	Cottonwood site	7.5; 12.5	365 Days	March–December 2015; January–July 2016, Estimated potential mean
Rautenstrauch et al. (2002)	Mojave Desert of Nevada	11.7	Year	Over all years (January 1992–February 1995)
Freilich et al. (2000) [b]	Mojave Desert of California	5.6; 7.9; 12.6; 3.8	Year (tortoise activity season)	Each year respectively (April 1993–May 1996), a year was considered the tortoise activity season of March or April until May (1994 & 1996 were drought years)
Duda et al. (1999) [b]	Mojave Desert of California-(MCAGCC)	6.9/6.2; 3.8/3.1	Year	Male/female respectively per year. March–December 1995; March–November 1996 (drought year), Marine Corps Air Ground Combat Center
Duda et al. (1999) [b]	Mojave Desert of California-(JTNP)	13.8/11.6; 4.8/4.4	Year	Male/Female respectively per year. March–December 1995 & 1996 (drought year), Joshua Tree National Park

[a] Estimated number of burrows used per individual radio-tracking interval multiplied by 365

[b] Unspecified if burrows were only counted once yearly despite burrow reuse

for differing time periods from when they were first and last outfitted with radio transmitters, we calculated the weighted estimate of burrow use as the number of unique burrows used by an individual tortoise divided by the total number of days each tortoise was radio-transmittered. We then multiplied that number by 30 or 365 to generate an estimate of the number of individual burrows used per 30 (Table 1) or 365 days (Table 2), respectively. The Spearman correlation coefficient was used to measure the strength of association between the number of burrows tortoises used with the number of days each tortoise was monitored since monitoring duration in days was not normally distributed. The yearly (365 days) calculation was only used as a comparison with other studies and represents the potential number of burrows that each tortoise is capable of using every year.

At both sites, some burrows were re-occupied by the same or different tortoises throughout the year. For the count of unique burrows, each burrow was counted one time each calendar year even though the tortoise may have used that burrow two calendar years in a row (i.e., if a tortoise used a burrow repeatedly for two consecutive calendar years, it was counted as a unique burrow used once each year). Each year was analysed separately due to the dramatic difference in precipitation amongst years. Tortoises at both sites reused burrows, defined as the repetitive non-consecutive use of a burrow by an individual (e.g., a tortoise used a unique burrow, left for another burrow(s), and then returned to the first burrow one or more times). Since we were not locating tortoises on a daily basis, we made the assumption that tortoises located in the same burrow on consecutive trips, remained in that burrow in between detections. We compared and contrasted our data with other published data. While our burrows are all considered to be unique to the tortoise that inhabits them, other authors do not specify this criterion, and instead may count the total number of times tortoises were found in burrows, potentially counting a unique burrow multiple times; this is noted as appropriate in our comparisons (Table 2).

All burrows used in this study were given an individual identification number, marked with an aluminum tag, and a GPS point was taken using a Garmin Oregon 550T GPS unit (accurate to +/- 3 m). Only burrows that were observed to be occupied by radio telemetered tortoises were included in our analyses. Descriptions of each burrow were recorded, including the general burrow location relative to obvious landmarks and burrow type. Burrow type was not always recorded at every burrow at the Cottonwood site since the protocol for recording burrow type was established after the Cottonwood study had begun for other reasons (e.g., collecting demographic and reproductive data). Burrow type was classified into three categories: soil burrow, pallet, or rock shelter. We define a soil burrow as a burrow that is excavated in a mostly soil substrate and that is as long or longer than a tortoise's shell length. A pallet is a shallow indentation, often under the canopy of a shrub where a tortoise can be partially or completely concealed. A rock shelter is an opening in or under one or more rocks that form a "cave". We use the term "burrow" loosely to represent all shelter types in this manuscript for simplification unless noted otherwise. Burrow mouth orientation was noted at the Orocopia site for each burrow but not always noted at the Cottonwood site for the reason given above. Therefore, landscape aspect, slope, and elevation were determined for each burrow location through geospatial analysis (ARCGIS 10.5.1) at both sites for consistency. A 10-metre digital elevation model obtained from the National Map (U.S. Geological Survey: https://www.usgs.gov/core-science-systems/national-geospatial-program/national-map) was first used to create aspect and slope raster layers prior to extracting terrain values at each burrow location. Each burrow in the geospatial analysis was only included once, even though the burrow may have been used by a tortoise or tortoises many times. We ran a Watson-Williams F-Test for circular statistics to compare the landscape aspect vectors determined from the geospatial analysis.

Brumation events, the ectotherm analogue of hibernation, were monitored from the end of the spring/summer activity season until the beginning of the following spring activity season. The brumation entrance date was the date tortoises discontinued moving from one burrow to another and remained in one burrow until the month they were located either out of their burrow or in a new burrow in the late winter or spring season of the following calendar year (i.e., brumation emergence date; with one exception for a tortoise that changed burrows in the winter). We hand-measured brumaculum depth whereas aspect was determined through geospatial analysis (ARCGIS 10.5.1). Brumaculum depth was measured from the entrance of the burrow to the farthest point reached with a flexible tape measure to the back of the burrow. The tape measure was not always able to reach the very end of some burrows due to burrow curves and side tunnels, so measurements represent a minimum depth. Because the study duration was less than two full years at each site, we collected one complete season of winter dormancy data for brumacula use at each.

RESULTS

Burrow Use

Relocations of radioed tortoises ranged from 6–30 events over both studies (Table 1). At the Cottonwood site, tortoises used a range of 3–14 unique burrows each during the 16-month study, and tortoises at the Orocopia site occupied a range of 3–11 unique burrows each during the 19-month study (Table 1) for a total of 164 unique burrows for all tortoises across both sites. Tortoises at the Orocopia site used an estimated mean of 0.485 unique burrows per 30 days over the study duration (Table 1). Estimated potential mean number of burrows used at the Orocopia site per 365 days was 8.3 in 2017 and 9.1 in 2018 (Table 2). Tortoises at the Cottonwood site used an estimated mean of 0.848 unique burrows per 30 days over the study duration and an estimated potential

mean number of burrows per 365 days of 7.5 in 2015 and 12.5 in 2016 (Tables 1; 2). The Orocopia tortoises utilised 61 soil burrows, 3 pallets, and 2 rock shelters. The burrows used by the Cottonwood tortoises included 27 soil burrows, 1 pallet, and 28 rock shelters. The number of unique burrows occupied by tortoises throughout our study at both sites combined increased with the number of days that individual tortoises were monitored (Fig. 2). Monitoring duration explained 70 % of the variation in number of unique burrows occupied. At the Orocopia site, the median estimated number of unique burrows used per tortoise per 30 days did not differ between the non-drought ($\tilde{x}$ = 0.7) and drought-affected years ($\tilde{x}$ = 0.7) of 2017 and 2018 (Mann-Whitney U Test Statistic = 33.0, *P* = 0.5, Chi-Square approximation = 0.4, df = 1).

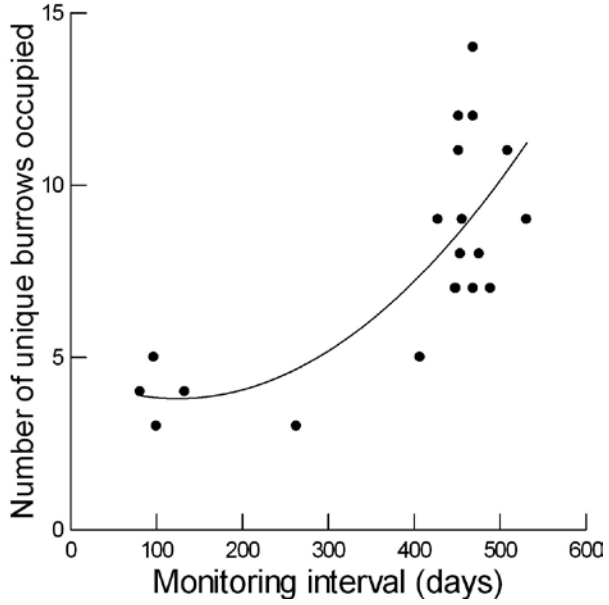

Figure 2. The number of unique burrows used by each tortoise within the radio-telemetered monitoring period. A quadratic smoother has been applied to demonstrate that the increase in the number of burrows occupied is directly proportional to the number of days that tortoises are radio-telemetered. A quadratic smoother appears to fit the data well. Burrow occupancy increases with time.

Topography

Overall, mean burrow landscape aspect vectors between sites were statistically different (Watson-Williams F-Tests, F=222.4 ˚, df = 1, 147 ˚, p < 0.001) based on our geospatial analyses. The topography at the Orocopia site had a predominately northerly aspect while the Cottonwood site had a south-westerly aspect, and that was reflected in the aspects of burrow locations (Orocopia mean aspect vector = 356.8˚ ± SD 22.7˚, n = 65; Cottonwood mean aspect vector = 231.1˚ ± SD 63.5˚, n = 84; Fig. 3). Burrows at the Cottonwood site ranged from 588 m–762 m AMSL in elevation (= 663.7 m, SD = 49.0) and a slope range of 1.9 % – 33.4 % (= 10.7 %, SD = 10.6). Tortoise burrows at the Orocopia site ranged in elevation from 502 m–574 m AMSL (= 537.6 m, SD = 16.2) with a slope range of 0.6 %–2.4 % (= 1.6 %, SD = 0.4).

Burrow Reuse

Each tortoise at the Orocopia site reused previously inhabited burrows, whereas, some tortoises at the Cottonwood site did not (Table 1). The number of burrows reused by tortoises increased with the number of days since radios were attached. For Cottonwood, the Pearson correlation coefficient between those two variables was 0.53 (P=0.08). The value for Orocopia was 0.67 (P=0.05). For both sites combined the value was 0.55 (P=0.009). At the Orocopia site, some tortoises also showed increased burrow fidelity for extended periods of time. Female tortoise #51 was located in only three burrows during the 263-day tracking period including one burrow that was used for 10 months straight (from brumation into the following summer). Male tortoise #43 was only located in five burrows during the 407 days he was tracked. The burrow where he was most often located was used for eight months leading up to and during brumation (two of those months were during the summer season).

Cohabitation

Tortoises at both sites used burrows that appeared to be inhabited only by that individual, but on several occasions, tortoises used burrows that originally sheltered a different tortoise. For example, at the Orocopia site, a burrow was originally found sheltering male #35. Thereafter, male #33 was found twice in that burrow, as well as female #40 who was also found twice in that same burrow (cohabitating each time with either male #35 or male #33). All the cohabitation events that occurred over both sites (n = 7) involved male/female combinations, and five of these events occurred after the egg production season for the region (Lovich et al., 2018b) (April–June) between the months of August and October, while two events occurred in April. At the Orocopia site, a total of six cohabitation events were recorded. One event in October involved three tortoises simultaneously using a burrow (two males [#39 & #50] and one female [#51]). At the Cottonwood site, there was only one possible cohabitation event where a marked male tortoise was sitting on the apron of a radio-telemetered female tortoises burrow while she was inside. Although we never saw the male tortoise in the burrow, he could have been in the burrow before or after our observation.

Brumation

Seven tortoises were included in the brumation data at the Cottonwood site. Four radio-telemetered tortoises at the Cottonwood site were not included in the brumation study either because they were not located prior to the brumation period or they had a radio failure prior to the beginning of the brumation period. Eighty-six percent (n = 6) of the tortoises at the Cottonwood site entered brumation in November of 2015, and the earliest brumation entrance date was October 2015 by one female (#11) (Table 3). Eighty-six percent (n = 6) of the tortoises also exited brumation in February 2016. The same female tortoise (#11) that entered brumation earlier than all other tortoises emerged in March 2016, later than all other tortoises. Nine tortoises were included

Table 3. Brumacula data for both the Orocopia and Cottonwood study sites. ID is the individual tortoise identification number assigned to each tortoise. Depth is the length of the burrow from the mouth to the farthest point reached with the tape measure at the back of the burrow. One tortoise at the Orocopia site used two burrows during one brumation season creating an extra burrow for the Orocopia data. Some radio-telemetered tortoises included in the overall burrow study were not located prior to the brumacula study, accounting for the decreased number of tortoises included in this list. We were unable to collect burrow depth on tortoises #2 and #8 due to burrow collapse.

Site	ID	Sex	Month/Year of Brumation Duration	Depth (cm)	Mouth Orientation (degrees)	Landscape Aspect (degrees)
Cottonwood	1	M	November 2015-February 2016	117	170	236
Cottonwood	2	F	November 2015-February 2016		180	246
Cottonwood	7	F	November 2015-February 2016	35	80	152
Cottonwood	8	M	November 2015-February 2016		224	179
Cottonwood	11	F	October 2015-March 2016	100	140	236
Cottonwood	12	F	November 2015-February 2016	90	280	322
Cottonwood	13	M	November 2015-February 2016	84	274	320
Orocopia	33	M	November 2017-March 2018	100	55	37
Orocopia	34	F	September 2017-February2018	96	230	6
Orocopia	35	M	November 2017-March 2018	56	220	41
Orocopia	37	M	November 2017-March 2018	107	340	53
Orocopia	39	M	November 2017-March 2018	48	90	0.9
Orocopia	40	F	November 2017-May 2018	62	250	347
Orocopia	43	M	October 2017-March 2018	87	160	253
Orocopia	45	F	November 2017-January 2018	140	300	335
			January 2018-April 2018	53	155	337
Orocopia	51	F	November 2017-May 2018	120	270	323

in the Orocopia brumation study. Seventy-eight percent of the tortoises (n = 7) entered brumation in November 2017 and fifty-six percent (n = 5) emerged in March 2018 (Table 3). All males entered brumation between October (n = 1) and November (n = 4) in 2017 and emerged in March 2018. Of the four radio-telemetered female tortoises at the Orocopia site, three entered brumation in November 2017 and emerged in April and May of 2018 (Table 3). The fourth female (#34) went into her brumaculum early in September 2017, and she was also the earliest to emerge in February 2018. We do know that females #34 and #40 emerged between February and March the previous year in 2017, since female tortoise #34 was originally found basking on 16 February 2017 and female #40 was discovered courting with male #35 on 15 March 2017. At the Orocopia site, one female (#45) used two brumation burrows in a single brumation period. She entered her first brumaculum in November 2017, was found in her second brumaculum in January 2018, and finally emerged from brumation in April 2018 (Table 3). These were both considered brumacula due to the seasonal timing.

At Cottonwood, brumacula mouth orientation ranged from 80–280 degrees, with a general southerly preference for all tortoises (Fig. 4; Table 3). Fifty-seven percent (n = 4) of the brumacula were soil burrows while the remainder (n = 3) were rock shelters. Brumacula at the Orocopia site had a burrow mouth orientation ranging from 55–340 degrees, with the greatest number of brumacula mouth openings (n = 5) facing south-westerly (Fig. 4; Table 3). Eight of ten brumacula were in soil burrows (one was in a caliche layer); the two remaining burrows were a pallet and a rock shelter. Burrows used for brumation at both sites tended to have southerly mean burrow mouth opening orientation vectors (± circular SD; Fig. 4) but sample sizes were too small for further statistical comparison (Cottonwood mean vector = 196.0˚ ± 72.0˚, n = 7; Orocopia mean vector = 231.2˚ ± 95.0˚, n = 10). Mean depth of brumacula were as follows: Orocopia: 86.9 cm, range 48–140 cm; Cottonwood: 85.2 cm, range 35–177 cm (Table 3).

DISCUSSION

Burrow Use and Selection

In this study, we documented different aspects of the burrowing habits (including brumation and cohabitation) of two proximate but separate populations of *G. agassizii* in the Sonoran Desert of California and how they use the geologic and topographic terrain within their home ranges during varied periods of precipitation. After analysis of the data, our initial two hypotheses were rejected. First, while tortoises used similar substrates in which to construct burrows, burrow types were different as a result of site-specific geologic and topographic characteristics. Second, tortoises did not use fewer burrows during a year of greatly decreased precipitation and plant productivity as expected. Previous research suggests that *G. agassizii* actively select burrow and shelter sites based on certain environmental characteristics (Duda et al., 2002; Lovich

& Daniels, 2000; Sah et al., 2016). Tortoises at both sites utilised rock shelters, soil burrows, and/or pallets at one time or another and used new burrows as well as previously used burrows, and over a range of elevational relief.

Traditionally, Agassiz's desert tortoises (*G. agassizii*) have been characterised as occupants of desert flats, while its congener, Morafka's desert tortoises (*G. morafkai*) prefers rocky, mountainous terrain (Germano et al., 1994). However, previous studies reveal reciprocal use of habitats by both species depending on habitat availability and tortoise preference (Averill-Murray & Averill-Murray, 2005; Bury et al., 1994; Germano et al., 1994). Several of the tortoises at Cottonwood exhibited shelter site use comparable to *G. morafkai* in Arizona (Germano et al., 1994; Riedle et al., 2008). Tortoises at Cottonwood often used granitic boulder rock shelters on hillsides (when a rocky hill slope was available within the tortoise's home range), whereas some utilised soil burrows on the nearby flat topography, and several tortoises utilised both forms of burrows within their home range. At the Orocopia site, we located most of the tortoises and burrows on the flats and in washes, possibly due to the rarity of steep boulder-strewn terrain in the area. Burrow use at the Orocopia site is consistent with other reports of *G. agassizii* behaviour in the Mojave Desert of California (Berry et al., 2002; Ernst & Lovich, 2009; Murphy et al., 2011).

The locations of burrows at both sites tended to match the overall aspect of the landscape. For example, on the north-facing Orocopia landscape there were topographic features (washes and small hills) with aspects facing other directions but most burrows were located on north-facing aspects (Fig. 3), even though tortoises had the option to select otherwise. Alternatively, Cottonwood tortoises utilised burrows primarily located on a south-westerly landscape aspect, which was also the overall aspect of the landscape. Other studies identify landscape aspects which tortoises tend to utilise most (Anderson et al., 2000; Bulova, 1994), however, they do not state the overall landscape aspect of the study site as a comparison.

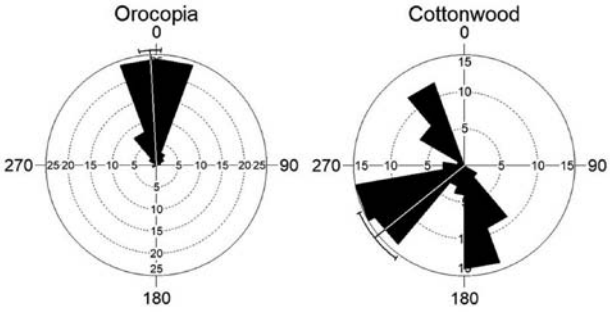

Figure 3. Site aspect orientation roses based on data determined through geospatial analysis (ARCGIS 10.5.1). The mean vector is shown with the associated 95 % confidence interval. Orocopia tortoises utilised landscapes with north-facing aspects and Cottonwood tortoises primarily utilised landscapes with south-western facing aspects overall throughout radio-telemetered periods. Burrows at both sites tended to orientate in conformance with the landscape aspects created by topography.

Burrow Reuse

Tortoises demonstrate a preference for certain burrows, returning to these burrows for subsequent "reuse", possibly as a means of energy conservation. It is energetically less expensive to reuse a previous burrow than to dig a new one. Other studies show that tortoises have varying levels of burrow fidelity. Burge (1978) reported that 73 % of tortoises used a burrow for 2–15 days and 19 % for 16–46 days, while Duda et al. (1999) noted three tortoises did not move from their burrows for the entirety of a single season during a drought year. At the Orocopia site, all tortoises reused either one or two burrows during the study period. Our results indicate that, generally, the detection of burrow reuse increases with time tortoises are monitored. Due to the intermittent observation of tortoises, it is assumed that they may reuse burrows more frequently than documented (Burge, 1978). At Cottonwood, three tortoises did not reuse any burrow during the study. Two of the afore-mentioned three tortoises were radio-transmittered for the shortest duration of time (81 and 97 days), which may account for the lack of subsequent observed burrow use. However, the third tortoise (male #1) was the first marked tortoise on the site and had the longest radio-telemetered duration (469 days) (Table 1). One tortoise (#12) reused four different burrows during 452 days at the Cottonwood site, which is the greatest number of burrows reused at either site. In a previous study on the northern side of the Cottonwood mountains, in the Pinto basin, tortoises were noted reusing previous burrows consistently, only adding new burrows occasionally (Freilich et al., 2000).

Cohabitation

Tortoise cohabitation is documented in the wild; however, they are more often found individually in burrows (Bulova, 1996; Burge, 1978). Woodbury & Hardy (1948) noted that although tortoises have small home ranges, they often overlap with the home ranges of other tortoises, increasing the odds of cohabitation. Our study documents cohabitation during the spring as well as the summer and fall seasons and involving mostly opposite sex associations. We did not observe any cohabitations during the brumation period. Other studies have documented cohabitation events, especially during the reproductive season (Bulova, 1994; Burge, 1978). Woodbury & Hardy (1948) discovered up to 17 tortoises in one burrow simultaneously during the brumation period and estimated an average cohabitance of 3.11 tortoises per burrow at a study site in Utah.

Drought

Tortoises can tolerate high concentrations of biological wastes and conserve water in urinary bladders in times of

Table 4. Number of unique burrows used by radio-telemetered tortoises per 30 days during each individual year 2017 and 2018 at the Orocopia site only. The identification number (ID) is the unique identifier for individual tortoises. A mean of 0.7 burrows were used per 30 days each year. The drought year (2018) did not influence the number of burrows used.

		Unique Burrows/30 days	
ID	Sex	2017	2018
33	M	0.590	0.774
34	F	0.492	1.371
35	M	0.616	0.774
37	M	0.643	0.643
39	M	0.753	0.643
40	F	0.755	0.536
43	M	0.455	0.789
45	F	0.807	0.682
51	F	1.017	0.514

drought (Nagy & Medica, 1986; Peterson, 1996). Burrows provide a safe refuge and minimise physiologic water loss through underground moisture content (Bulova, 2002; Peterson, 1996). During drought years, tortoises can be very difficult to find, and high-density tortoise areas can appear to be largely deserted due to these water conservation mechanisms (Anderson et al., 2000; Freilich et al., 2000). We experienced this phenomenon during the 2018 drought year (1.39 cm precipitation) since only one new tortoise was found and marked that year. The year 2017 provided good germination for tortoise annual food plants with the yearly precipitation total of 13.15 cm. However, 2018 had no germination of winter annual plants. Surprisingly, the mean number of unique burrows used by tortoises per 30 days between 2017 and 2018 at the Orocopia site were identical (0.7 burrows) (Table 4). Similarly, Rautenstrauch et al. (2002) noted that tortoises at his study site did not demonstrate a change in burrow use (the number of burrows used and the amount of time tortoises spent in their burrows) during a period of decreased precipitation of 11.7 cm from the site average of 13.9 cm annually, even though two years of the five year study had above average precipitation of 26.9 cm and 26.6 cm. They suggested that perhaps the precipitation decrease was not dramatic enough for a behavioural response. However, Duda et al. (1999) and Freilich et al. (2000) reported a significant decrease in the number of burrows tortoises used during drought (1.2 cm winter precipitation and less than 5.5 cm yearly precipitation, respectively), increasing the amount of time spent in a particular burrow and decreasing home range size. With extended periods of time inactive in burrows and less time above ground, tortoises conserve energy by reducing their metabolic rates, allowing them to tolerate poor conditions for longer periods of time (Henen, 1997; Henen, 2002).

Brumation

We had one complete season of monitored data on brumacula use at each site (Table 3). Our results are comparable to other brumation data reported. Most tortoises at other sites have been reported entering brumation between October – December and are reported exiting between February and, at the latest, June (Bailey et al., 1995; Burge, 1978; Nussear et al., 2007; Rautenstrauch et al., 1998). All tortoises at the Orocopia site exhibited later emergence in 2018 than tortoises at the Cottonwood site in 2016 overall (Table 3). Bailey et al. (1995) noted that female *G. morafkai* emerged earlier than males at two sites, but we did not observe this phenomenon at either of our sites. Females may remain in their burrows for extended periods of time in the spring during drought conditions to conserve resources for reproduction (Henen, 1997; Henen, 2002). Three of the four Orocopia female tortoises emerged from brumation later than all of the male tortoises, in April and May of 2018.

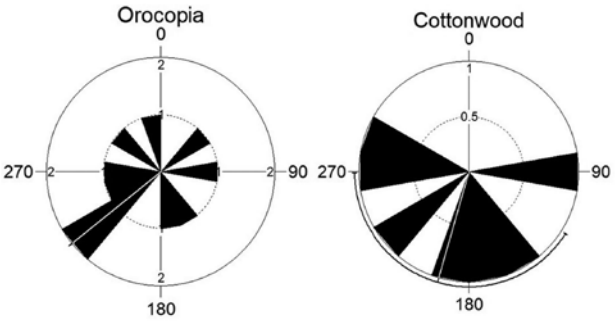

Figure 4. Orocopia (left) and Cottonwood (right) brumacula burrow mouth orientation roses. This dataset was hand-measured in the field with a compass, not with geospatial analysis software. The mean vector is shown with the associated 95 % confidence interval. Tortoises predominantly favoured a south-western mean burrow mouth orientation vector for brumacula. There was no obvious relationship between the burrow mouth orientation vectors and topography aspect at the Orocopia site, whereas, at the Cottonwood site, burrow mouth openings tended to orientate in conformance with the general aspect of the landscape.

Warmer brumacula environments may provide thermal buffering, providing a physiologic benefit for reproduction (Averill-Murray et al., 2002a; Bailey et al., 1995). South-facing slopes tend to have increased soil temperatures and solar radiation with a decreased 24-hour temperature swing (Bailey et al., 1995; Nobel & Linton, 1997; Warren, 2008) creating a consistently warmer brumaculum environment during the winter. At both of our sites, brumacula openings tended to orientate toward the south-west even though the general aspect of the Orocopia landscape is north and overall, most burrows (including brumacula) at the Orocopia site had a north facing landscape aspect (Table 3; Fig. 4). Tortoises at the Cottonwood site predominantly inhabited brumacula with southerly facing openings on southerly facing landscape aspects (Fig. 4). Bailey et

al. (1995) noted that tortoises in the Sonoran Desert demonstrated a preference for southern facing slopes during two brumation seasons. Brumacula depth may also provide some thermal buffer. The deeper the burrow, the more consistent the ambient temperature is over a 24-hour period (Mack et al., 2015; Rosenberg et al., 1983), protecting tortoises from reaching their critical thermal minimum of 4.4 ^{o}C (Averill-Murray et al., 2002a; Lowe et al., 1971; Woodbury & Hardy, 1948).

The information presented here demonstrates that *G. agassizii* utilise habitats differently depending on the availability of local resources within the geological and environmental constraints of their home ranges. Our study provides additional insight to the limited information for tortoise behaviours in association with burrowing habits of *G. agassizii* in the Sonoran Desert of California. Tortoise burrow use varies due to the interactions of precipitation, season, and landscape characteristics, compelling tortoises to adjust to climates and environmental landscapes, even over small spatial and temporal scales.

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REFERENCES

Agha, M., Augustine, B., Lovich, J.E., Delaney, D., Sinervo, B., Murphy, M.O., Ennen, J.R., Briggs, J.R., Cooper, R. & Price, S.J. (2015a). Using motion-sensor camera technology to infer seasonal activity and thermal niche of the desert tortoise (*Gopherus agassizii*). *Journal of Thermal Biology* 49, 119–126.

Agha, M., Delaney, D., Lovich, J.E., Briggs, J., Austin, M. & Price, S.J. (2015b). Nelson's big horn sheep (*Ovis canadensis nelsoni*) trample Agassiz's desert tortoise (*Gopherus agassizii*) burrow at a California wind energy facility. *Bulletin, Southern California Academy of Sciences* 114, 58–63.

Agha, M., Smith, A.L., Lovich, J.E., Delaney, D., Ennen, J.R., Briggs, J., Fleckenstein, L.J., Tennant, L.A., Puffer, S.R., Walde, A., Arundel, T.R., Price, S.J. & Todd, B.T. (2017). Mammalian mesocarnivore visitation at tortoise burrows in a wind farm. *The Journal of Wildlife Management* 81, 1117–1124.

Anderson, D.R., Burnham, K.P., Lubow, B.C., Thomas, L., Corn, P.S., Medica, P.A. & Marlow, R.W. (2000). Field trials of line transect methods applied to estimation of desert tortoise abundance. *The Journal of Wildlife Management* 65, 583–597.

Averill-Murray, R., Martin, B., Bailey, S. & Wirt, E. (2002a). Activity and behavior of the Sonoran desert tortoise in Arizona. In *The Sonoran desert tortoise: natural history, biology, and conservation*, 135–158. Van Devender, T.R. (ed.). Tucson, Az, USA: The University of Arizona Press.

Averill-Murray, R.C. & Averill-Murray, A. (2005). Regional-scale estimation of density and habitat use of the desert tortoise (*Gopherus agassizii*) in Arizona. *Journal of Herpetology* 39, 65–72.

Averill-Murray, R.C., Woodman, A.P. & Howland, J.M. (2002b). Population ecology of the Sonoran desert tortoise in Arizona. In *The Sonoran desert tortoise: natural history, biology, and conservation*, 109–134. Van Devender, T.R. (ed.). Tucson, Az, USA: The University of Arizona Press.

Bailey, S.J., Schwalbe, C.R. & Lowe, C.H. (1995). Hibernaculum use by a population of desert tortoises (*Gopherus agassizii*) in the Sonoran Desert. *Journal of Herpetology* 29, 361–369.

Barrows, C. (2011). Sensitivity to climate change for two reptiles at the Mojave–Sonoran Desert interface. *Journal of Arid Environments* 75, 629–635.

Berry, K.C. & Murphy, R.W. (2019). *Gopherus agassizii* (Cooper 1861) – Mojave desert tortoise, Agassiz's desert tortoise. In *Chelonian Research Monographs. Conservation Biology of Freshwater Turtles and Tortoises: A Compilation Project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group*, 109.101–109.145. Rhodin, A.G.J., Iverson, J.B., van Dijk, P.P., Stanford, C.B., Goode, E.V., Buhlmann, K.A., Pritchard, P.C.H. & Mittermeier, R.A. (Eds.). Chelonian Research Foundation and Turtle Conservancy.

Berry, K.H., Morafka, D.J. & Murphy, R.W. (2002). Defining the desert tortoise(s): our first priority for a coherent conservation strategy. *Chelonian Conservation and Biology* 4, 249–262.

Boarman, W.I., Goodlett, T. & Goodlett, G. (1998). Review of radio transmitter attachment techniques for chelonian research and recommendations for improvement. *Herpetological Review* 29, 26–33.

Bulova, S.J. (1994). Patterns of burrow use by desert tortoises: gender differences and seasonal trends. *Herpetological Monographs* 8, 133–143.

Bulova, S.J. (1996). Burrow use by desert tortoises (*Gopherus agassizii*): social factors. *Proceedings of the Desert Tortoise Council Symposium* 1993, 23–25.

Bulova, S.J. (2002). How temperature, humidity, and burrow selection affect evaporative water loss in desert tortoises. *Journal of Thermal Biology* 27, 175–189.

Burge, B. (1978). Physical characteristics and patterns of utilization of cover sites used by *Gopherus agassizii* in southern Nevada. *Proceedings of the Desert Tortoise Council Symposium* 1978, 80–111.

Bury, R.B., Esque, T., DeFalco, L. & Medica, P. (1994). Distribution, habitat use and protection of desert tortoise in the Eastern Mojave Desert. *Fish and Wildlife Research* 13, 57–72.

Crowe, D.E. & Longshore, K.M. (2013). Nest site characteristics and nesting success of the western burrowing owl in the eastern Mojave Desert. Journal of Arid Environments 94, 113–120.

Currylow, A., Walde, A.D. & Walde, A.M. (2016). *Gopherus agassizii* (Mojave desert tortoise) and *Coluber flagellum piceus* (red racer). Burrow associates. In *Natural History Notes*, 122–123. *Herpetological Review* 47.

Diaz, H.F. & Wahl, E.R. (2015). Recent California water year precipitation deficits: A 440-year perspective. *Journal of Climate* 28, 4637–4652.

Dimmitt, M.A. (1977). Status of the desert tortoise in the Colorado Desert. *Proceedings symposium: The Desert Tortoise Council* 1977, 46–53.

Duda, J.J., Krzysik, A.J. & Freilich, J.E. (1999). Effects of drought on desert tortoise movement and activity. *The Journal of Wildlife Management* 63, 1181–1192.

Duda, J.J., Krzysik, A.J. & Meloche, J.M. (2002). Spatial organization of desert tortoises and their burrows at a landscape scale. *Chelonian Conservation and Biology* 4, 387–397.

Ernst, C.H. & Lovich, J.E. (2009). *Turtles of the United States and Canada*. John Hopkins University Press.

Flint, L.E., Flint, A.L., Mendoza, J., Kalansky, J. & Ralph, F. (2018). Characterizing drought in California: new drought indices and scenario-testing in support of resource management. *Ecological Processes* 7, 1–13.

Freilich, J.E., Burnham, K.P., Collins, C.M. & Garry, C.A. (2000). Factors affecting population assessments of desert tortoises. *Conservation Biology* 14, 1479–1489.

Germano, D.J., Bury, R., Esque, T., Fritts, T. & Medica, P. (1994). Range and habitats of the desert tortoise. *Biology of North American Tortoises* 13, 73–84.

Griffin, D. & Anchukaitis, K.J. (2014). How unusual is the 2012–2014 California drought? *Geophysical Research Letters* 41, 9017–9023.

Henen, B.T. (1997). Seasonal and annual energy budgets of female desert tortoises (*Gopherus agassizii*). *Ecology* 78, 283–296.

Henen, B.T. (2002). Reproductive effort and reproductive nutrition of female desert tortoises: essential field methods. *Integrative and Comparative Biology* 42, 43–50.

Henley, D.C. (2000). The land that God forgot: the saga of General George Patton's desert training camps: Western American History Series. Western Military History Association.

Hereford, R., Webb, R.H. & Longpré, C.I. (2006). Precipitation history and ecosystem response to multidecadal precipitation variability in the Mojave Desert region, 1893–2001. *Journal of Arid Environments* 67, 13–34.

Kahn, P.F., Guyer, C. & Mendonça, M.T. (2007). Handling, blood sampling, and temporary captivity do not affect plasma corticosterone or movement patterns of gopher tortoises *(Gopherus polyphemus*). *Copeia* 2007, 614–621.

Kinlaw, A. (1999). A review of burrowing by semi-fossorial vertebrates in arid environments. *Journal of Arid Environments* 41, 127–145.

Kinlaw, A. & Grasmueck, M. (2012). Evidence for and geomorphologic consequences of a reptilian ecosystem engineer: the burrowing cascade initiated by the gopher tortoise. *Geomorphology* 157, 108–121.

Lathrop, E.W. (1983). Recovery of perennial vegetation in military maneuver areas, In *Environmental Effects of Off-Road Vehicles*, 265–277. Webb, R.H. & Wilshire, H.G. (Eds.). New York, New York, USA: Springer Verlag New York.

Lips, K.R. (1991). Vertebrates associated with tortoise (*Gopherus polyphemus*) burrows in four habitats in south-central Florida. *Journal of Herpetology* 25, 477–481.

Lovich, J.E. & Daniels, R. (2000). Environmental characteristics of desert tortoise (*Gopherus agassizii*) burrow locations in an altered industrial landscape. *Chelonian Conservation and Biology* 3, 714–721.

Lovich, J.E., Ennen, J.R., Agha, M. & Gibbons, J.W. (2018a). Where have all the turtles gone and why does it matter? *Bioscience* 68, 771–781.

Lovich, J.E., Puffer, S.R., Agha, M., Ennen, J.R., Meyer-Wilkins, K., Tennant, L.A., Smith, A.L., Arundel, T.R., Brundige, K.D. & Vamstad, M.S. (2018b). Reproductive output and clutch phenology of female Agassiz's desert tortoises (*Gopherus agassizii*) in the Sonoran Desert region of Joshua Tree National Park. *Current Herpetology* 37, 40–57.

Lovich, J.E., Yackulic, C.B., Freilich, J., Agha, M., Austin, M., Meyer, K.P., Arundel, T.R., Hansen, J., Vamstad, M.S. & Root, S.A. (2014). Climatic variation and tortoise survival: Has a desert species met its match? *Biological Conservation* 169, 214–224.

Lowe, C.H., Lardner, P.J. & Halpern, E.A. (1971). Supercooling in reptiles and other vertebrates. *Comparative Biochemistry and Physiology Part A: Physiology* 39, 125–135.

Luckenbach, R.A. (1982). Ecology and management of the desert tortoise (*Gopherus agassizii*) in California. North American Tortoises: Conservation and Ecology. *US Fish and Wildlife Service, Wildlife Research Report* 12, 1–38.

Mack, J.S., Berry, K.H., Miller, D.M. & Carlson, A.S. (2015). Factors affecting the thermal environment of Agassiz's desert tortoise (*Gopherus agassizii*) cover sites in the central Mojave Desert during periods of temperature extremes. *Society for the Study of Amphibians and Reptiles* 49, 405–414.

Murphy, R.W., Berry, K.H., Edwards, T., Leviton, A.E., Lathrop, A. & Riedle, J.D. (2011). The dazed and confused identity of Agassiz's land tortoise, *Gopherus agassizii* (Testudines, Testudinidae) with the description of a new species, and its consequences for conservation. *ZooKeys* 113, 39–71.

Nagy, K.A. & Medica, P.A. (1986). Physiological ecology of desert tortoises in southern Nevada. *Herpetologica* 42, 73–92.

Nobel, P.S. & Linton, M.J. (1997). Frequencies, microclimate and root properties for three codominant perennials in the northwestern Sonoran Desert on north- vs south-facing slopes. *Annals of Botany* 80, 731–739.

Noy-Meir, I. (1973). Desert ecosystems: environment and producers. *Annual Review of Ecology and Systematics* 4, 25–51.

Noy-Meir, I. (1974). Desert ecosystems: higher trophic levels. *Annual Review of Ecology and Systematics* 5, 195–214.

Nussear, K.E., Esque, T.C., Haines, D.F. & Tracy, C.R. (2007). Desert tortoise hibernation: temperatures, timing, and environment. *Copeia* 2007, 378–386.

Parmesan, C., Root, T.L. & Willig, M.R. (2000). Impacts of extreme weather and climate on terrestrial biota. *Bulletin of the American Meteorological Society* 81, 443–450.

Peterson, C.C. (1996). Anhomeostasis: seasonal water and solute relations in two populations of the desert tortoise (*Gopherus agassizii*) during chronic drought. *Physiological Zoology* 69, 1324–1358.

Prose, D.V. (1985). Persisting effects of armored military maneuvers on some soils of the Mojave Desert. *Environmental Geology and Water Sciences* 7, 163–170.

Rautenstrauch, K., Rakestraw, D., Brown, G., Boone, J. & Lederle, P. (2002). Patterns of burrow use by desert tortoises (*Gopherus agassizii*) in southcentral Nevada. *Chelonian Conservation and Biology* 4, 398–405.

Rautenstrauch, K.R., Rager, A.L. & Rakestraw, D.L. (1998). Winter behavior of desert tortoises in southcentral Nevada. *The Journal of Wildlife Management* 62, 98–104.

Riedle, J.D., Averill-Murray, R.C., Lutz, C.L. & Bolen, D.K. (2008). Habitat use by desert tortoises (*Gopherus agassizii*) on alluvial fans in the Sonoran Desert, south-central Arizona. *Copeia* 2008, 414–420.

Rosenberg, N.J., Blad, B.L. & Verma, S.B. (1983). Microclimate: the biological environment. John Wiley & Sons.

Sah, P., Nussear, K.E., Esque, T.C., Aiello, C.M., Hudson, P.J. & Bansal, S. (2016). Inferring social structure and its drivers from refuge use in the desert tortoise, a relatively solitary species. *Behavioral Ecology and Sociobiology* 70, 1277–1289.

Stager, R.D., Roundy, E., Brackley, G., Leonard, S. & Lato, L. (2017). Case study: using soil survey to help predict Sonoran desert tortoise population distribution and densities. *Rangelands* 39, 97–111.

Walde, A.D., Currylow, A. & Walde, A.M. (2015). Discovery of a new burrow associate of the desert tortoise (*Gopherus agassizii*), the long-nosed leopard lizard (*Gambelia wislizenii*). *Herpetology Notes* 8, 107–109.

Warren, R.J. (2008). Mechanisms driving understory evergreen herb distributions across slope aspects: as derived from landscape position. *Plant Ecology* 198, 297–308.

Woodbury, A.M. & Hardy, R. (1948). Studies of the desert tortoise, *Gopherus agassizii*. *Ecological Monographs* 18, 145–200.

Woodhouse, C.A. (1997). Winter climate and atmospheric circulation patterns in the Sonoran Desert region, USA. *International Journal of Climatology: A Journal of the Royal Meteorological Society* 17, 859–873.

Zimmerman, L.C., O'Connor, M.P., Bulova, S.J., Spotila, J.R., Kemp, S.J. & Salice, C.J. (1994). Thermal ecology of desert tortoises in the eastern Mojave Desert: seasonal patterns of operative and body temperatures, and microhabitat utilization. *Herpetological Monographs* 8, 45–59.

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Revisiting the generic position and acoustic diagnosis of *Odontophrynus salvatori* (Anura: Odontophrynidae)

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Herein we evaluate the phylogenetic position, and revisit the generic allocation of *Odontophrynus salvatori*, which has for long been considered controversial because it exhibits intermediate morphological features between *Odontophrynus* and *Proceratophrys*. By assessing a fragment of the 16S mitochondrial gene from topotypical specimens, we confirm that *O. salvatori* is a member of the genus *Proceratophrys* and sister to *P. moratoi*, also forming a clade with *P. concavitympanum* and *P. ararype*. Therefore, we formally transfer *O. salvatori* to the genus *Proceratophrys* [*Proceratophrys salvatori* (Caramaschi 1996) comb. nov.]. Additionally, the calls of *Proceratophrys salvatori* and *P. moratoi*, formally compared for the first time, are shown to exhibit similar structures: they both emit single multi-pulsed notes that differ mainly in pulse repetition rate and dominant frequency. Finally, we summarise occurrence records for *P. salvatori* and *P. moratoi* and provide a new record of *P. moratoi* in Mato Grosso State, extending its distribution about 490 km to the north-west.

Keywords: Advertisement call, geographic distribution, phylogenetic position, *Proceratophrys*, species diagnosis, taxonomy

INTRODUCTION

Odontophrynus salvatori Caramaschi 1996 was described based on two adult males collected at Chapada dos Veadeiros National Park, Alto Paraíso de Goiás Municipality, Goiás State, Brazil (Caramaschi, 1996). Compared to its congeners, this species is morphologically more similar to "*Odontophrynus*" *moratoi* (Jim & Caramaschi, 1980), which was reallocated to the genus *Proceratophrys* based on multilocus molecular data (Amaro et al., 2009), being sister to the clade formed by *P. concavitympanum* and *P. ararype* (Mângia et al., 2018). Despite its currently undoubtful phylogenetic position within *Proceratophrys*, *P. moratoi* was tentatively allocated in the genus *Odontophrynus* by having the optic ramus of the squamosal bone not touching the maxillae (not seen in species of *Proceratophrys*; Jim & Caramaschi, 1980), while also lacking some external morphological characteristics exhibited by species in this genus. At that time, such combination of features rendered it an intermediate position between *Odontophrynus* and *Proceratophrys* and posed some doubts about its correct generic allocation until the work of Amaro et al. (2009).

Accordingly, the same osteological condition was observed in *Odontophrynus salvatori*, leading Caramaschi (1996) to allocate this species as a congener of "*Odontophrynus*" *moratoi* (as originally published). However, the hypothesis that *O. salvatori* is a member of *Proceratophrys* has been for long suggested either because of the phylogenetic position of *P. moratoi* (the most likely sister taxa of *O. salvatori*, Caramaschi, 1996; Amaro et al., 2009) and its overall larval morphology, which resembles mostly *Proceratophrys* rather than *Odontophrynus* (Rossa-Feres & Jim, 1996; Brandão & Batista, 2000). Nevertheless, *O. salvatori* was never included in any molecular phylogenetic analysis and its generic position is still considered uncertain.

Advertisement calls of frogs are primarily involved in mating recognition and have been employed as an important taxonomic tool to diagnose morphologically cryptic anuran species (Köhler et al., 2017), especially within *Proceratophrys* (Cruz & Napoli, 2010; Mângia et al., 2010). Both *Odontophrynus salvatori* and *P. moratoi* have had their calls described (Brasileiro et al., 2008; Bastos et al., 2011; Martins & Giaretta, 2012), but were never compared, as they were not considered congeners. Additionally, Bastos et al. (2011) did not report pulse repetition rate of *O. salvatori* calls, a key

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acoustic diagnostic feature among species of the family Odontophrynidae (Martino & Sinsch, 2002; Cruz & Napoli, 2010; Nascimento et al., 2019). Because there are few morphological characters that diagnose *O. salvatori* from *P. moratoi* (Caramaschi, 1996), acoustic comparisons (including pulse rate) are highly desirable to improve their diagnosis and test the utility of calls in anuran taxonomy.

Herein, we present the phylogenetic position of *Odontophrynus salvatori* based on a segment of the 16S rRNA mitochondrial gene (16S mtDNA) from topotypical specimens, clarifying the position of this species within Odontophrynidae (sensu Pyron & Wiens, 2011). Additionally, we revisited the previously published calls of *O. salvatori* and *Proceratophrys moratoi* and provide new acoustic information from *O. salvatori* topotypical specimens aiming to evaluate acoustic diagnosis between these species based on standardised analysis. Finally, we summarise occurrence records for both *O. salvatori* and *P. moratoi* across the Cerrado biome in central Brazil and provide a new record for *P. moratoi* in Mato Grosso State.

METHODS

Voucher specimens of molecular samples used in this study are housed at the Herpetological collection from Universidade de Brasília, Brasília Municipality, Brazil (CHUNB), Amphibian collection from Universidade Estadual Paulista, São José do Rio Preto Municipality, São Paulo State, Brazil (DZSJRP) and Amphibian collection from Universidade Federal de Uberlândia, Uberlândia municipality, Minas Gerais State, Brazil (AAG-UFU). Some specimens only have field number tags associated (Reuber Albuquerque Brandão–RAB, or Guarino Colli–GRCOLLI). We sequenced two adult specimens (RAB3148, 3149) and a tadpole (DZSJRP 3206.1) of *Odontophrynys salvatori* collected at the type locality (Caramaschi, 1996) in Chapada dos Veadeiros National Park, Alto Paraíso de Goiás Municipality, Goiás State, central Brazil (14°9'35.60"S, 47°36'14.14"W; 1180 m above sea level [a.s.l.], DATUM WGS84). To assure taxonomic identification, we also sequenced a specimen collected at Nova Xavantina Municipality, Mato Grosso State (14°38'49.42"S, 52°4'37.21"W; 312 m a.s.l. DATUM WGS84) that morphologically resembles *Proceratophrys moratoi* (GRCOLLI20295). We also provide additional sequences of *Proceratophrys* species from the Cerrado biome: *P. branti* from Mateiros municipality, Tocantins State (CHUNB27366, 27376 and 23378), *P. cururu* from the Espinhaço mountain range, Minas Gerais State (RAB3225 and 3290), topotypical *P. goyana* from Chapada dos Veadeiros, Goiás State (RAB3285), and *P. cristiceps* from western Bahia State (AAG-UFU1984). We extracted genomic DNA from specimen's livers and tadpole tail muscle using the salt precipitation method described by Bruford et al. (1992). We amplified a ~550 base pair (bp) segment of the 16S mtDNA using primers pair 16Sar-L (CGCCTGTTTATCAAAAACAT) and 16Sbr-H (CCGGTCTGAACTCAGATCACGT) of Palumbi et al. (1991) and PCR protocols described in Amaro et al. (2009). We sent PCR products to Macrogen Inc. (Seoul, South Korea) for purification and sequencing. We checked sequencing quality and edited chromatograms in Geneious v1.8.7 (Kearse et al., 2012).

To confirm the phylogenetic position of *O. salvatori*, we assembled 16S mtDNA segments available in GenBank (mainly those originally provided by Amaro et al., 2009), encompassing representatives from all genera in the family Odontophrynidae (sensu Pyron & Wiens, 2011), such as *Proceratophrys* (42 individuals, including our 11 sequenced individuals), *Odontophrynus* (16 individuals), and *Macrogenioglottus* (2 individuals), plus *Thoropa miliaris* and *Cycloramphus acangatan* as outgroups, totaling 62 terminals (see Supplementary Materials Appendix I for GenBank accession numbers). We aligned sequences using the default settings of MAFFT algorithm (Katoh et al., 2002) also available in Geneious v.1.8.7, resulting in a 516 bp final alignment (including gaps). We estimated the 16S gene tree with BEAST v1.10.4 software (Suchard et al., 2018), implementing the GTR+I+G nucleotide substitution model as suggested by jModeltest version 2.1.6 (Darriba et al., 2012), and birth-death speciation model as the tree prior. We ran BEAST analysis for 10 million generations sampling every 1000 steps. We assessed run convergence (Effective Sample Size > 200) with Tracer v1.7, generated the maximum credibility tree with TreeAnnotator v1.10.4 (https://beast.community/treeannotator), and drew it using FigTree v1.4.2 (Rambaut, 2014). Additionally, we used the Tamura-Nei (Tamura & Nei, 1993) corrected p-distances implemented in Molecular Evolutionary Genetics Analysis (MEGA) v7.0 software (Kumar et al., 2015) to compute between-group mean genetic distances of *O. salvatori* in comparison to species among the three genera within Odontophrynidae, which is shown in Table 1. Prior to this analysis, we trimmed our alignment to fit the shortest sequence available, resulting in a 448 bp alignment employed to calculate distances.

We recorded advertisement calls of two *Odontophrynus salvatori* topotypical males on 16 December 2012 (recording vouchers: RAB3147, SVL 26.5 mm; and RAB3149, SVL 25.5 mm) using a Marantz PMD 660 coupled with a Sennheiser ME66 directional microphone. To evaluate if there are acoustic diagnostic traits between *O. salvatori* and *Proceratophrys moratoi*, we gathered call information previously published for these species (Brasileiro et al., 2008; Bastos et al., 2011; Martins & Giaretta, 2012) and also reanalysed available recordings deposited at Fonoteca Neotropical Jacques Vielliard (FNJV) in a standardised approach (Supplementary Materials Appendix II). We also compare calls of *O. salvatori* and *P. moratoi* with those of *P. concavitympanum* and *P. ararype* (data from Santana et al., 2010; Mângia et al., 2018), considering their close phylogenetic relationship (see results). We summarised call parameters in Table 2. We analysed advertisement calls in Raven Pro 1.5 (Center for Conservation Bioacoustics, 2014) with the following spectrogram settings: Hann window type, Fast Fourier Transform window width = 256 samples, frame = 100,

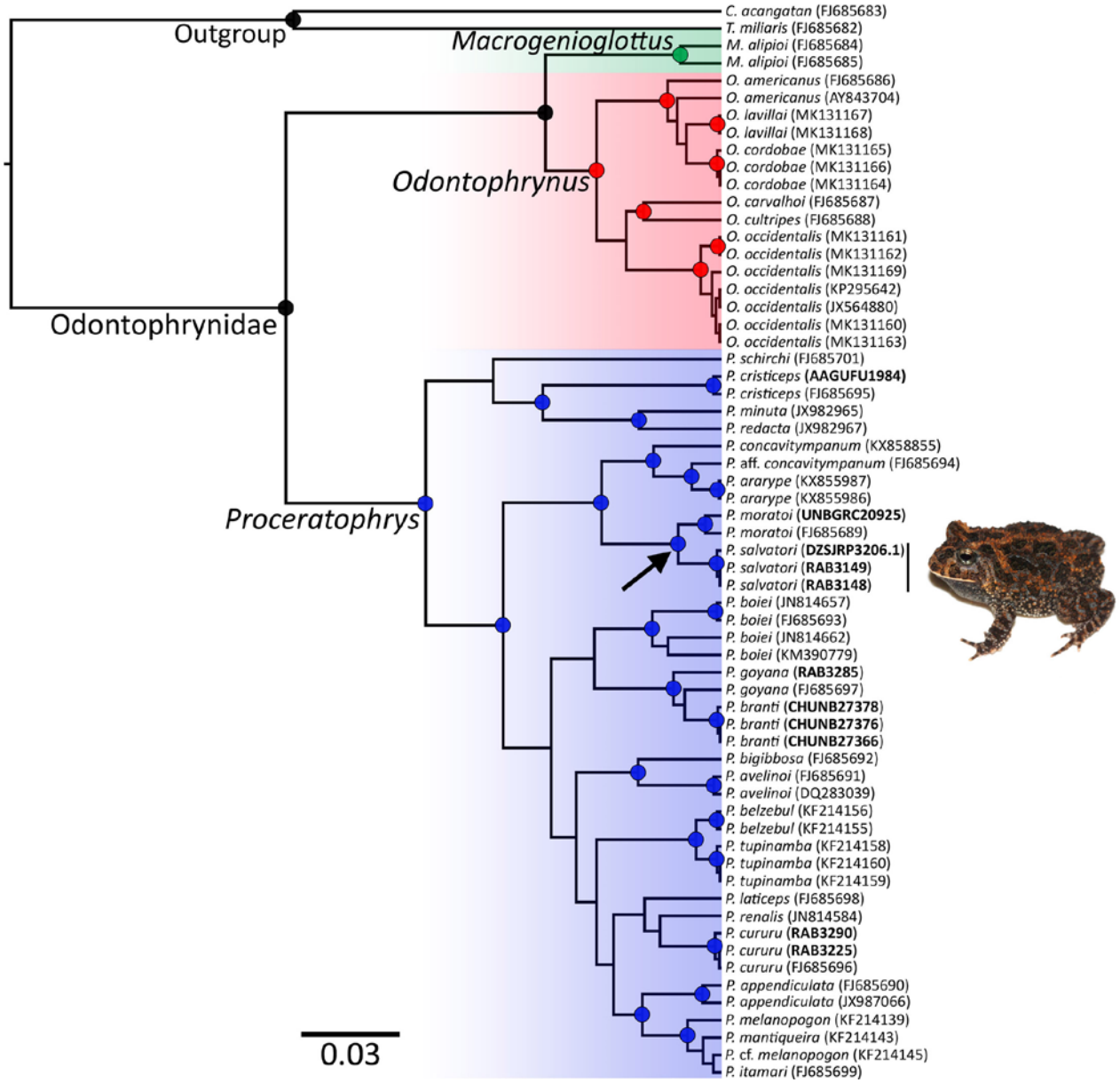

Figure 1. Maximum clade credibility 16S rRNA mitochondrial gene tree as inferred from a Bayesian analysis in BEAST. Circles on nodes denote significant posterior probability (pp = 0.95–1.0), and are coloured according to genus. All nodes recovered with non-significant support (pp < 0.95) are omitted. Scale indicates rate of base substitutions per site. Arrow indicates the phylogenetic position of *P. salvatori*. Sequences provided by us are highlighted in bold.

Table 1. Tamura-Nei corrected pairwise distances (average p-values in %) of *P. salvatori* and phylogenetically related *Proceratophrys* species estimated from a 448 base pair segment of the 16S rRNA mitochondrial gene. The remaining species of *Proceratophrys* and species of *Odontophrynus* were pooled to facilitate visualisation. For these, we reported the minimum and maximum values of genetic distance.

	Species	1	2	3	4	5	6	7	8
1	*P. salvatori*	–							
2	*P. moratoi*	2.4	–						
3	*P. ararype*	5.7	5.7	–					
4	*P. concavitympanum*	6.8	7.8	4.5	–				
5	*P.* aff. *concavitympanum*	6.8	6.7	1.6	4.8	–			
6	pooled remaining *Proceratophrys*	6.9–11.6	6.7–12.2	6.7–10.8	7.3–11.5	7.0–11.5	–		
7	pooled *Odontophrynus*	11.1–12.7	12.4–14.3	10.8–13.4	11.1–13.1	10.7–13.6	9.3–15.8	–	
8	*Macrogenioglottus alipioi*	12.3	13.8	12.8	12.3	12.4	10.3–14.5	4.7–8.4	–

overlap = 50 %, and DFT size = 256 samples. All other settings followed the 'default' of Raven. We constructed audio spectrograms with the R package Seewave 1.7.3 (Sueur et al., 2008) in R 3.6.1 platform (R Development Core Team, 2018) using the following settings: Hanning window, 256 points resolution (Fast Fourier Transform), and 70 % of overlap. We follow the terminology for call descriptions proposed by Köhler et al. (2017). Recordings were stored as uncompressed wav files at the Arquivos Sonoros da Universidade Federal do Rio Grande do Norte (ASURFN679–680).

RESULTS

The maximum clade credibility gene tree unequivocally recovered *Odontophrynus salvatori* as a member of the genus *Proceratophrys* and sister of *P. moratoi* with high support (posterior probability [pp] = 1.0; Fig. 1). Within the genus *Proceratophrys*, these two species are sister to the *P. concavitympanum* + *P. ararype* clade (pp = 1.0), as previously reported (Amaro et al., 2009; Mângia et al., 2018). Overall, our tree topology is generally similar to previous phylogenetic hypotheses (Amaro et al., 2009; Pyron & Wiens, 2011), with *Proceratophrys* being the sister clade to *Odontophrynus* + *Macrogenioglottus*, also with significant node support (pp = 1.0). Therefore, we formally place *O. salvatori* in the genus *Proceratophrys* [*Proceratophrys salvatori* (Caramaschi 1996) comb. nov.]. Additionally, *P. salvatori* exhibited 2.4 % genetic divergence in comparison to its closer relative (*P. moratoi*), and at least 5.7 % and 11 % in comparison to its other congeners and pooled *Odontophrynus* species, respectively (Table 1).

The advertisement call of *Proceratophrys salvatori* topotypical males (Fig. 2) is emitted in series of single multi-pulsed notes (complete pulses with silent intervals between each one; n = 65 analysed notes). Notes lasting from 0.297–0.413 s (mean 0.366 s; SD = 0.02) with 17–25 pulses (mean 21.2; SD = 1.5), and pulse repetition rate from 54–61 pulses per second (mean 58; SD = 2.1). Dominant frequency varied from 1688–1875 Hz (mean 1849 Hz; SD = 59). Our description matches that of Bastos et al. (2011), also based on calls from topotypical specimens, except for the lower mean dominant frequency reported (mean 1572 Hz; SD = 226). Accordingly, our data from the advertisement call reanalysis of some *P. moratoi* populations agrees with previous descriptions (Appendix II).

Based on the standardised analysis of advertisement calls, we identified that *Proceratophrys salvatori* calls are emitted with a lower pulse repetition rate and at a higher dominant frequency if compared to those of *P. moratoi* (see Table 2). Interestingly, although the dominant frequency of *P. salvatori* calls is higher than that of *P. moratoi*, these species do not differ in male size (Table 2). Considering mean values from different works, note duration is slightly longer in *P. salvatori* (mean ranging from 0.317–0.366 s) if compared to *P. moratoi* calls (mean ranging from 0.202–0.280 s), although raw values overlap (Table 2). Additionally, the lower pulse repetition rate and higher dominant frequency promptly distinguishes the calls of *P. salvatori* from those of *P. concavitympanum* and *P. ararype* (Table 2).

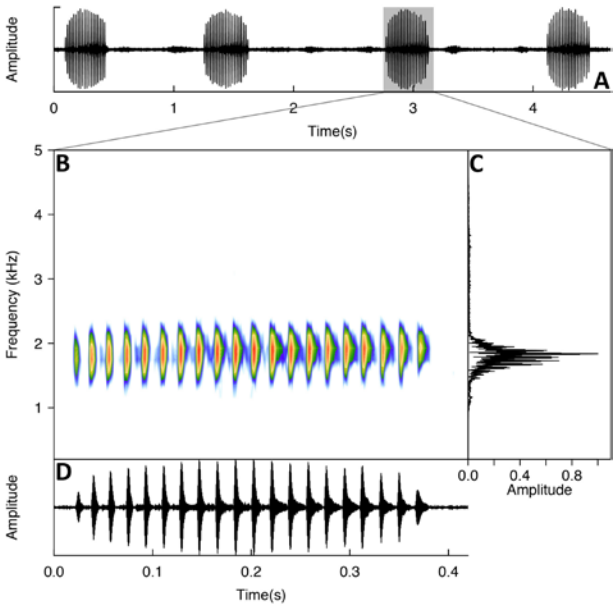

Figure 2. Advertisement call of *P. salvatori* (ASUFRN680, air temperature 23 °C, air humidity 78 %) recorded at Chapada dos Veadeiros National Park, Alto Paraíso de Goiás Municipality, Goiás State. A) Call sequence with 4 consecutive notes; and B) spectrogram C) power spectrum and D) oscillogram of one highlighted note.

DISCUSSION

As expected by previous non-molecular evidence (Caramaschi, 1996; Brandão & Batista, 2000), *Proceratophrys salvatori* was recovered as the sister taxa of *P. moratoi*. Both species lack ocular-dorsal ridge of warts, a feature that distinguishes them from remaining congeners, except from *P. vielliardi* (Brandão et al., 2013), and some populations of *P. cristiceps* (Mângia et al., 2020). Within Odontophrynidae, *P. salvatori* and *P. moratoi* share with their congeners the lack dorsal and tibial glands, interdigital membrane, and nuptial pads and exhibit tubercles on the thenar surfaces of hands and feet (Jim & Caramaschi, 1980; Caramaschi, 1996), characteristics not shared with species of *Odontophrynus*. Conversely, there is not much evidence that helps discriminate between these two species based on external morphology. For instance, Caramashi (1996) ranked as diagnostic characters the shorter squamosal bone on the optic branch (comparatively longer in *P. moratoi*; Jim & Caramaschi, 1980), dorsal coloration pattern and their presumably allopatric distribution (but see below). Recent works showed that dorsal coloration patterns are highly variable within *Proceratophrys* (Mângia et al., 2020), and a re-evaluation of this feature with a comprehensive sampling and genetic background is highly desirable to recognise species-specific traits.

Pulse repetition rate has been reported as an important acoustic component that discriminates species in *Proceratophrys* (e.g., Cruz & Napoli, 2010; Malagoli et al., 2016; Nascimento et al., 2019). Accordingly, the calls

Table 2. Summary of advertisement call parameters of *P. salvatori* and related species from all localities analysed and from literature (see also Supplementary Materials Appendix II). SVL reported represents the minimum and maximum values for adult males of each species. mm = millimetres; s = seconds; Hz = Hertz.

Species	male SVL (mm)	Note duration (s)	Pulses/note	Pulse rate (Pulses/s)	Dominant Frequency (Hz)
P. ararype	35.6–42.2[a]	0.374–0.648	38–65	96–103	1034–1378
P. concavitympanum	39.6–51.6[b]	0.178–0.500	19–51	100–119	754–1116
P. moratoi	24.7–31[c]	0.146–0.335	12–26	69–103	1153–1594
P. salvatori	25.5–27.8[d]	0.198–0.420	15–25	54–61	1572–1875

SVL data from: [a] Mângia et al., 2018; [b] Santana et al., 2010; [c] Jim & Caramaschi, 1980, Brasileiro et al., 2008; [d] our data, Caramaschi, 1996

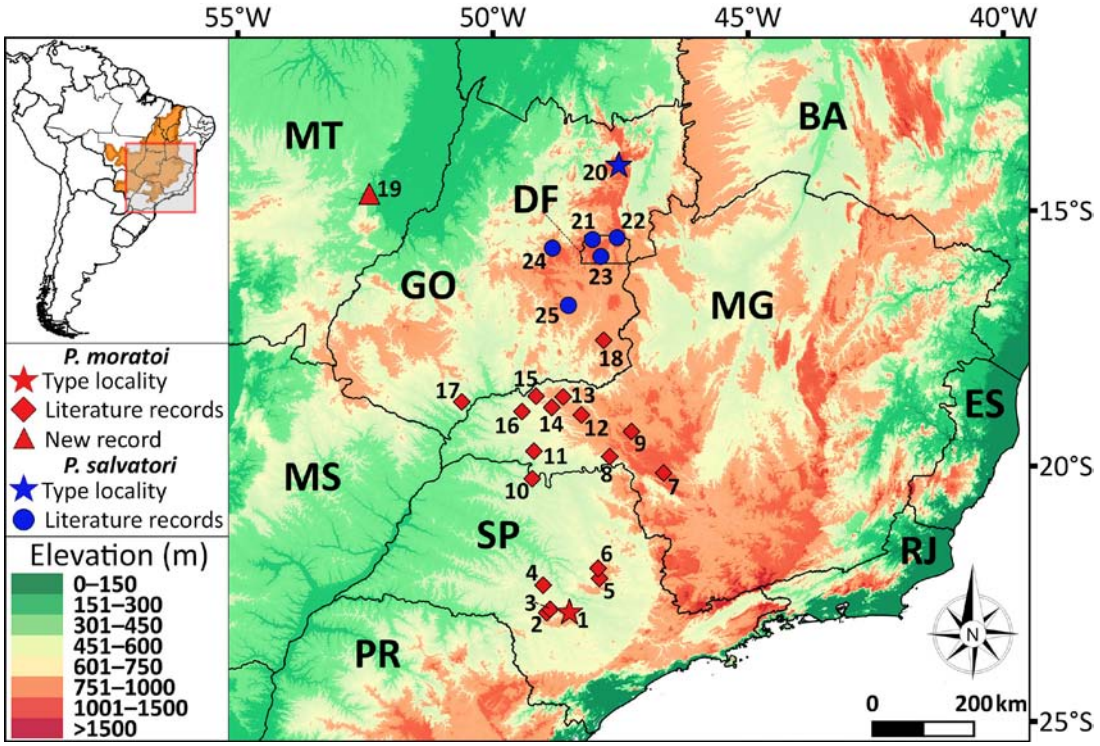

Figure 3. Distribution on topographic map of *P. moratoi* and *P. salvatori*. Abbreviations for Brazilian states: BA (Bahia), DF (Distrito Federal), GO (Goiás), MG (Minas Gerais), MS (Mato Grosso do Sul), MT (Mato Grosso), PR (Paraná), RJ (Rio de Janeiro), SP (São Paulo). The orange shading in South America inset represents the boundaries of Cerrado biome. Locality numbers from 1–25 refers to those in Table 3.

of *P. moratoi* and *P. salvatori* are mainly distinguished by pulse rate (values not overlapping) and dominant frequency (values barely overlap), also distinguishing the latter species from their phylogenetically closely related congeners (*P. concavitympanum* and *P. ararype*; Table 2). Moreover, it is well known that dominant frequency of anuran calls is constrained by male body size (being negatively correlated; Tonini et al., 2020), a pattern also observed for *Proceratophrys* (Nascimento et al., 2019). Nevertheless, we did not find differences in male body size between *P. salvatori* and *P. moratoi*, although calls of *P. salvatori* are 300Hz higher pitched if compared to its sister species (but see Bastos et al., 2011). Conversely, differences in dominant frequency between the calls of *P. salvatori* and *P. concavitympanum* and *P. ararype* are likely explained by male body size (Table 2). Our results provide additional diagnostic features that aid in the recognition of these morphologically similar species, reinforcing the importance of acoustic traits as an essential tool for taxonomic resolutions in *Proceratophrys* (Nascimento et al., 2019).

Despite both species being restricted to the Cerrado biome, *Proceratophrys salvatori* was only reported to occur within the Central Brazilian and Veadeiros Plateaus (both composing the highlands of Central Brazil in Goiás state, Azevedo et al., 2016; Martins-Ferreira & Campos, 2017), mostly at sites above 1000 m above sea level (Caramaschi, 1996; Brandão & Batista, 2000; Table 3). Conversely, *P. moratoi* occurs mostly below 900 m a.s.l. across the Canastra range and mountainous regions within Cerrado patches in São Paulo State (Fig. 3), with only two altitudinal records surpassing 1000 m a.s.l. in Minas Gerais State. Based on its currently known distribution, our new record in Mato Grosso State extends *P. moratoi* occurrence area in approximately 490 km north-west from the nearest locality in south-eastern

Table 3. Summary of known distribution records for *P. moratoi* and *P. salvatori.* Alt = altitude in meters above sea level. Lat = latitude; Long = longitude. * species type locality.

#	Municipality	Locality	State	Alt	Lat	Long	Source
			Proceratophrys moratoi				
1	Botucatu*	Rubião Junior	SP	865	-22.883	-48.5	Jim & Caramaschi, 1980
2	Avaré	Fazenda Recreio	SP	675	-22.887	-48.947	Maffei et al., 2011
3	Lençois Paulista	–	SP	740	-22.82	-48.88	Arruda et al., 2017
4	Bauru	Jardim Botânico Municipal de Bauru	SP	550	-22.347	-49.016	Rolim et al., 2010
5	Itirapina/Brotas	ESEC de Itirapina	SP	740	-22.215	-47.911	Brasileiro et al., 2008
6	São Carlos	–	SP	815	-22.017	-47.939	Carvalho-Jr et al., 2010
7	São Roque de Minas	Serra da Canastra	MG	1140	-20.151	-46.654	Haddad et al., 1988
8	Uberaba	–	MG	740	-19.831	-47.71	Neves et al., 2019
9	Perdizes	–	MG	1030	-19.339	-47.284	Martins & Giaretta, 2012
10	Fronteira	–	MG	460	-20.263	-49.229	Neves et al., 2019
11	Comendador Gomes	–	MG	650	-19.725	-49.196	Neves et al., 2019
12	Uberlândia	–	MG	830	-19.015	-48.266	Martins & Giaretta, 2012
13	Tupaciguara	–	MG	830	-18.662	-48.622	Neves et al., 2019
14	Monte Alegre de Minas	–	MG	745	-18.869	-48.847	Martins & Giaretta, 2012
15	Centralina	–	MG	710	-18.648	-49.155	Neves et al., 2019
16	Ituiutaba	–	MG	550	-18.949	-49.43	Martins & Giaretta, 2012
17	Paranaiguara	–	GO	410	-18.76	-50.61	Arruda et al., 2017
18	Campo Alegre de Goiás	–	GO	940	-17.553	-47.828	Passos & Paredero, 2019
19	Nova Xavantina	–	MT	300	-14.688	-52.422	**Present study**
			Proceratophrys salvatori				
20	Alto Paraíso de Goiás*	Parque Nacional Chapada dos Veadeiros	GO	1280	-14.133	-47.533	Caramaschi, 1996
21	Brasília	APA Cafuringa, Poço Azul	DF	1085	-15.582	-48.047	Brandão & Batista, 2000
22	Brasília	ESEC Águas Emendadas	DF	1060	-15.545	-47.566	Brandão & Batista, 2000
23	Brasília	ESEC Jardim Botânico de Brasília	DF	1120	-15.914	-47.885	Brandão & Batista, 2000
24	Pirenópolis	Parque Estadual dos Pirenéus	GO	1175	-15.75	-48.834	Brandão & Batista, 2000
25	Silvânia	FLONA de Silvânia	GO	865	-16.653	-48.609	Bastos et al., 2011

Goiás State, also representing the lowest altitudinal record for this species, which previously was 410 m (Arruda et al., 2017).

The genetic divergence between *Proceratophrys salvatori* and *P. moratoi* is slightly lower than the threshold of 3 % estimated for Neotropical amphibians for the same 16S segment (Fouquet et al., 2007). Moreover, new records reported have been gradually expanding the distribution of *P. moratoi* northwards from the type locality across the Cerrado Biome (Table 3), narrowing the occurrence gap between *P. salvatori* from ~980 km (when initially described; Caramaschi, 1996) to currently 130 km (Fig. 3), thus raising doubts on the specific limits of these species. Nevertheless, they seem to show a discontinuous distribution along the borders of Central Brazilian highlands in Goiás State (Fig. 3). This high altitudinal Plateau is known to harbour high levels of flora and fauna endemism (Alves et al., 2014; Colli-Silva et al., 2019), including micro-endemic species of frogs such as *Boana ericae*, *Bokermmanohyla sapiranga*, *B. pseudopseudis*, *Leptodactylus tapiti*, *P. rotundipalpebra*, and *Scinax rupestris* (Sazima & Bokermann, 1978; Brandão et al., 2012; Santoro & Brandão, 2014; Araújo-Vieira et al., 2015). Accordingly, studies focusing on endemic plants associated with montane savannas have been showing that the Central Brazilian and Veadeiros Plateaus likely acted as microrefugia during glacial cycles of the Pleistocene (Bonatelli et al., 2014; Perez et al., 2016). Therefore, it is plausible that *P. salvatori* diversified recently across these altitudinal gradients in Central Brazil, explaining the relatively lower genetic divergence compared to *P. moratoi*. In this sense, future studies should address diversification scenarios and test for their specific limits by sampling more specimens and genes under a phylogeographic framework.

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REFERENCES

Alves, R.J.V., Oliveira, J.A. & Medeiros, D. (2014). Circumscribing campo rupestre – megadiverse Brazilian rocky montane savanas. *Brazilian Journal of Biology* 74, 355–362. DOI: 10.1590/1519-6984.23212

Amaro, R.C., Pavan, D. & Rodrigues, M.T. (2009). On the generic identity of *Odontophrynus moratoi* Jim & Caramaschi, 1980 (Anura, Cycloramphidae). *Zootaxa* 2071, 61–68. DOI: 10.11646/zootaxa.2071.1.5

Araujo-Vieira, K., Brandão, R.A. & Faria, D.D.C. (2015). A new species of rock-dwelling *Scinax* Wagler (Anura: Hylidae) from Chapada dos Veadeiros, central Brazil. *Zootaxa* 10, 365–373. DOI: 10.11646/zootaxa.3915.1.2

Arruda, M.P., Costa, W.P. & Recco-Pimentel, S.M. (2017). Genetic diversity of Morato's Digger Toad, *Proceratophrys moratoi*: spatial structure, gene flow, effective size and the need for differential management strategies of populations. *Genetics and Molecular Biology* 40, 502–514. DOI: 10.1590/1678-4685-gmb-2016-0025

Azevedo, J.A.R., Valdujo, P.H. & Nogueira, C.C. (2016). Biogeography of anurans and squamates in the Cerrado hotspot: coincident endemism patterns in the richest and most impacted savanna on the globe. *Journal of Biogeography* 43, 2454–2464. DOI: 10.1111/jbi.12803

Bastos, R.P., Signorelli, L., Morais, A.R., Costa, T.B., Lima, L.P. & Pombal-Jr, J.P. (2011). Advertisement calls of three anuran species (Amphibia) from the Cerrado, central Brazil. *South American Journal of Herpetology* 6, 67–72. DOI: 10.2994/057.006.0204

Bonatelli, I.A., Perez, M.F., Peterson, A.T., Taylor, N.P., Zappi, D.C., Machado, M.C., Koch, I., Pires, A.H. & Moraes, E.M. (2014). Interglacial microrefugia and diversification of a cactus species complex: phylogeography and palaeodistributional reconstructions for *Pilosocereus aurisetus* and allies. *Molecular Ecology* 23, 3044–3063. DOI: 10.1111/mec.12780

Brandão, R.A. & Batista, C.G. (2000). Descrição do girino de *Odontophrynus salvatori* (Anura, Leptodactylidae). *Iheringia, Série Zoologia* 89, 165–170. DOI: 10.1590/S0073-47212000000200005

Brandão, R.A., Caramaschi, U., Vaz-Silva, W. & Campos, L.A. (2013). Three new species of *Proceratophrys* Miranda-Ribeiro 1920 from Brazilian Cerrado (Anura, Odontophrynidae). *Zootaxa* 3750, 321–347. DOI: 10.11646/zootaxa.3750.4.2

Brandão, R.A., Magalhães, R.F., Garda, A.A., Campos, L.A., Sebben, A. & Maciel, N.M. (2012). A new species of *Bokermannohyla* (Anura: Hylidae) from highlands of Central Brazil. *Zootaxa* 3527, 28–42. DOI: 10.11646/zootaxa.3527.1.2

Brasileiro, C.A., Martins, I.A. & Jim, J. (2008). Amphibia, Anura, Cycloramphidae, *Odontophrynus moratoi*: distribution extension and advertisement call. *Check List* 4, 382–385.

Bruford, M.W., Hanotte, O., Brookfield, J.F.Y. & Burke, T. (1992). Single and multilocus DNA fingerprinting. In *Molecular Genetic Analysis of Populations: A Practical Approach*, 225–269. Hoelzel, A.R. (ed.) I. R. L. Press: Oxford.

Caramaschi, U. (1996). Nova espécie de *Odontophrynus* Reinhardt & Lütken, 1862 do Brasil Central (Amphibia, Anura, Leptodactylidae). Boletim do Museu Nacional. *Nova Serie, Zoologia* 367, 1–8.

Carvalho-Jr, R.R., Kleinsorge, J.M.D. & Fusinatto, L.A. (2010). Amphibia, Anura, Cycloramphidae, *Odontophrynus moratoi* Jim and Caramaschi, 1980: Filling gaps. Discovery of a new population in the state of São Paulo, southeastern Brazil. *Check List* 6, 36–37.

Center for Conservation Bioacoustics. (2014). Raven Pro: Interactive Sound Analysis Software (Version 1.5) [Computer software]. Ithaca, NY: The Cornell Lab of Ornithology. Available from: http://ravensoundsoftware.com/.

Colli-Silva, M., Vasconcelos, T.N.C. & Pirani, J.R. (2019). Outstanding plant endemism levels strongly support the recognition of campo rupestre provinces in mountaintops of eastern South America. *Journal of Biogeography* 46, 1723–1733. DOI: 10.1111/jbi.13585

Cruz, C.A.G. & Napoli, M.F. (2010). A new species of smooth horned frog, genus *Proceratophrys* Miranda-Ribeiro (Amphibia: Anura: Cycloramphidae), from the Atlantic Rainforest of eastern Bahia, Brazil. *Zootaxa* 2660, 57–67. DOI: 10.11646/zootaxa.2660.1.5

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9, 772. DOI: 10.1038/nmeth.2109.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M. & Gemmell, N. (2007). Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS ONE* 2, e1109. DOI: 10.1371/journal.pone.0001109

Haddad, C.F.B., Andrade, G. & Cardoso, A.J. (1988). Anfíbios anuros do Parque Nacional da Serra da Canastra, estado de Minas Gerais. *Brasil Florestal* 64, 9–20.

Jim, J. & Caramaschi, U. (1980). Uma nova espécie de *Odontophrynus* da região de Botucatu, São Paulo, Brasil (Amphibia, Anura). *Revista Brasileira de Biologia* 40, 357–360.

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30, 3059–3066. DOI: 10.1093/nar/gkf436

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. (2012). Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. DOI: 10.1093/bioinformatics/bts199

Köhler, J., Jansen, M., Rodríguez, A., Kok, P.J.R., Toledo, L.F., Emmrich, M., Glaw, F., Haddad, C.F.B., Rödel, M. & Vences, M. (2017). The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. *Zootaxa* 4251, 1–124. DOI: 10.11646/zootaxa.4251.1.1

Kumar, S., Stecher, G. & Tamura, K. (2015). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. *Molecular Biology and Evolution* 33, 1870–1874. DOI: 10.1093/molbev/msw054

Maffei, F., Ubaid, F.K. & Jim, J. (2011). Discovery of the fifth population of a threatened and endemic toad of the Brazilian Cerrado, *Proceratophrys moratoi* (Anura, Cycloramphidae). *Herpetology Notes* 4, 95–96.

Malagoli, L.R., Mângia, S. & Haddad, C.F.B. (2016). The advertisement call of *Proceratophrys pombali* (Amphibia: Anura: Odontophrynidae) with comments on its distribution and natural history. *South American Journal of Herpetology* 11, 18–24. DOI: 10.2994/SAJH-D-15-00022.1

Mângia, S., Koroiva, R., Nunes, P.M.S., Roberto, I.J., Ávila, R.W., Sant'Anna, A.C., Santana, D.J. & Garda, A.A. (2018). A new species of *Proceratophrys* (Amphibia: Anura: Odontophrynidae) from the Araripe Plateau, Ceará state, northeastern Brazil. *Herpetologica* 74, 255–268. DOI: 10.1655/Herpetologica-D-16-00084.1

Mângia, S., Oliveira, E.F., Santana, D.J., Koroiva, R., Paiva, F. & Garda, A.A. (2020). Revising the taxonomy of *Proceratophrys* Miranda-Ribeiro, 1920 (Anura: Odontophrynidae) from the Brazilian semiarid Caatinga: morphology, calls and molecules support a single widespread species. *Journal of Zoological Systematics and Evolutionary Research* 0, 1–22. DOI: 10.1111/jzs.12365

Mângia, S., Santana, D.J. & Feio, R.N. (2010). Advertisement call of the Cycloramphid toad *Proceratophrys melanopogon* (Miranda-Ribeiro, 1926). *South American Journal of Herpetology* 5, 127–131. DOI: 10.2994/057.005.0206

Martino, A.L. & Sinsch, U. (2002). Speciation by polyploidy in *Odontophrynus americanus*. *Zoological Society of London* 257, 67–81. DOI: 10.1017/S0952836902000663

Martins, L.B. & Giaretta, A.A. (2012). Advertisement calls of two species of *Proceratophrys* (Anura: Odontophrynidae) from Minas Gerais, Brazil, with comments on their distribution, taxonomy and conservation status. *South American Journal of Herpetology* 7, 203–212. DOI: 10.2994/057.007.0302

Martins-Ferreira, M.A.C. & Campos, J.E.G. (2017). Compartimentação geomorfológica como suporte para estudos de evolução geotectônica: aplicação na região da Chapada dos Veadeiros, GO. *Revista Brasileira de Geomorfologia* 18, 501–519. DOI: 10.20502/www.ugb.org.br rbg.v18i3.1119

Nascimento, J.S., Abreu, R.O., Menezes, L., Trevizan, C.C., Solé, M., Juncá, F.A. & Napoli, M.F. (2019). The advertisement call of *Proceratophrys minuta* Napoli, Cruz, Abreu, and Del Grande, 2011 (Anura: Odontophrynidae), with comments on acoustic parameters in the genus. *South American Journal of Herpetology* 14, 24–36. DOI: 10.2994/SAJH-D-17-00021.1

Neves, M.O., Yves, A., Pereira, E.A., Alves, L., Vasques, J.B., Coelho, J.F.T. & Silva, P.S. (2019). Herpetofauna in a highly endangered area: the Triângulo Mineiro region, in Minas Gerais State, Brazil. *Herpetozoa* 32, 113–123. DOI: 10.3897/herpetozoa.32.e35641

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L. & Grabowski, G. (1991). The Simple Fool's Guide to PCR, Version 2.0. Department of Zoology, Kewalo Marine Laboratory, University of Hawaii.

Passos, M.A. & Paredero, R.C.B. (2019). New record of the endangered toad *Proceratophrys moratoi* (Jim and Caramaschi, 1980) (Amphibia, Anura, Odontophrynidae) from municipality of Campo Alegre de Goiás, state of Goiás, Brazil. *Herpetology Notes* 12, 523–525.

Perez, M.F., Bonatelli, I.A.S., Moraes, E.M. & Carstens, B.C. (2016). Model-based analysis supports interglacial refugia over long-dispersal events in the diversification of two South American cactus species. *Heredity* 116, 550–557. DOI: 10.1038/hdy.2016.17

Pyron, R.A. & Wiens, J.J. (2011). A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution* 61, 543–583. DOI: 10.1016/j.ympev.2011.06.012

Rambaut, A. (2014). FigTree: a graphical viewer of phylogenetic trees, version 1.4.2. Available from: http://tree.bio.ed.ac.uk/software/figtree/.

R Development Core Team. (2018). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available from: http://www.R-project.org.

Rolim, D.C., Martinez, R.A.M., Almeida, S.C., Ubaid, F.K., Maffei, F. & Jim, J. (2010). Amphibia, Anura, Cycloramphidae, *Proceratophrys moratoi* (Jim and Caramaschi, 1980): distribution extension and new altitudinal record in state of São Paulo, Brazil. *Check List* 6, 576–578.

Rossa-Feres, D.C. & Jim, J. (1996). Tadpole of *Odontophrynus moratoi* (Anura: Leptodactylidae). *Journal of Herpetology* 30, 536–539. DOI: 10.2307/1565699

Santana, D.J., São-Pedro, V.A., Bernarde, P.S. & Feio, R.N. (2010). Descrição do canto de anúncio e dimorfismo sexual em *Proceratophrys concavitympanum* Giaretta, Bernarde & Kokubum, 2000. *Papéis Avulsos de Zoologia* 50, 167–174. DOI: 10.1590/S0031-10492010001100001

Santoro, G.R.C.C. & Brandão, R.A. (2014). Reproductive modes, habitat use, and richness of anurans from Chapada dos Veadeiros, central Brazil. *North-Western Journal of Zoology* 10, 365–373.

Sazima, I. & Bokermann, W.C.A. (1978). Cinco novas espécies de *Leptodactylus* do centro e sudeste brasileiro (Amphibia, Anura, Leptodactylidae). *Revista Brasileira de Biologia* 38, 899–912.

Suchard, M.A., Lemey, P., Baele, G., L., A.D., Drummond, A.J. & Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution* 4, vey016. DOI: 10.1093/ve/vey016

Sueur, J., Aubin, T. & Simonis, C. (2008). Seewave: a free modular tool for sound analysis and synthesis. *Bioacoustics* 18, 213–226. DOI: 10.1080/09524622.2008.9753600

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10, 512–526. DOI:10.1093/oxfordjournals.molbev.a040023

Tonini, J.F.R., Provete, D.B., Maciel, N.M., Morais, A.R., Goutte, S., Toledo, L.F. & Pyron, R.A. (2020). Allometric escape from acoustic constraints is rare for frog calls. *Ecology and Evolution* 10, 3686–3695. DOI: 10.1002/ece3.6155

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Changes in plasma oestradiol, testosterone and progesterone concentrations during an annual reproductive cycle in wild Aldabra giant tortoises (*Aldabrachelys gigantea*)

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Aldabra giant tortoises (*Aldabrachelys gigantea*) are currently listed as Vulnerable on the IUCN Red List of Threatened Species. However, negative impacts of sea-level rise are predicted to result in an overall population decline of 40-65 % over the next 100 years, rendering the species Endangered. Captive propagation is an important tool for in- and ex-situ species conservation, but breeding success outside the tortoises' native island habitats has been very limited. Until now, the reproductive cycle of Aldabra tortoises has only been described in anatomic and behavioural studies. During a one-year period, plasma of four female and four male wild tortoises on Aldabra Atoll were examined monthly for levels of gonadal steroid hormones (oestradiol, testosterone, progesterone). Plasma oestradiol and testosterone values as well as meteorological data of the sampling period corresponded to previously published reports on seasonal changes in anatomy, behaviour and climate on the Aldabra Atoll. Seasonal changes in plasma testosterone were evident in males, with high values from January through April, reflecting previously described testicular growth and breeding season, followed by a nadir in August and September. In females, plasma oestradiol levels displayed seasonal changes, coinciding with reported ovarian growth from January to May. The obtained data provide prerequisite knowledge for endocrinological monitoring of reproductive processes and management of breeding programs, both ex-situ as in-situ, to establish reserve- and rewilded populations.

Keywords: Aldabra giant tortoise, *Aldabrachelys gigantea*, reproduction, seasonality, steroid hormone

INTRODUCTION

Early human colonisation of the Indian Ocean Islands led to the extinction of one of two lineages of endemic giant tortoises, *Cylindraspsis*, and the almost complete elimination of the other lineage, *Aldabrachelys*, by the end of the 19th century due to excessive harvesting and translocation (Gerlach et al., 2013; Hansen et al., 2010). Today, only one species, the Aldabra giant tortoise (*Aldabrachelys gigantea*), survives. In the 1970s, conservation efforts including population monitoring and research programs were initiated, resulting in a significant increase of the single remaining population on the Aldabra Atoll and introductions of populations outside the historic range (Bourn et al., 1999). Recent introductions of captive-bred *A. gigantea* to new island habitats, such as Ile aux Aigrettes and Round Island in Mauritius, have not only served the conservation of the species, but also the restoration of ecosystem function, especially in regard to plant seed dispersal (Falcón & Hansen, 2018; Falcon et al., 2018; Hansen et al., 2010). However, natural density-related population regulatory mechanisms, late onset of reproductive age, and the extremely restricted range render *A. gigantea* population dynamics vulnerable to external impacts, be they human or natural in origin (Bourn, 1977; Gerlach et al., 2013; Haverkamp et al., 2017). The species is currently listed as Vulnerable on the IUCN Red List of Threatened Species (IUCN, 2018), but an overall population decline of 40-65 % over the next 100 years is projected due to severe negative impacts of sea-level rise, which will render the species Endangered by IUCN criteria (Gerlach et al., 2013). Captive propagation is an important tool for in- and ex-situ species conservation. However, breeding success outside the Aldabra tortoises' native island habitats has been very limited. Of the 159 zoological institutions registered as holding Aldabra tortoises, only one institution (Tulsa Zoo, USA) has been producing and

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distributing offspring to a significant degree in the last 15 years (ZIMS, 2018).

Aldabra tortoises are long-lived species with late onset of sexual maturity at approximately 20 years of age. In their natural habitat on Aldabra Atoll in the southern hemisphere, they show a distinct seasonal reproductive pattern with the mating season in December to March and nesting season from June to September, resulting in prolific numbers of offspring, but with high neonatal mortality, mostly due to predation (Swingland & Coe, 1978).

Knowledge of the reproductive physiology of Aldabra giant tortoises is based on behavioural observations and morphological examinations of gonads of freshly killed, free-ranging animals on the Aldabra Atoll (Bourn, 1977; Swingland & Coe, 1978). In captive animals in one zoological institution in the Northern hemisphere, ultrasonographic evaluation of the ovarian cycle and faecal steroid metabolite measurements revealed evidence for asynchrony of the follicular development compared to the seasonal reproductive cycle observed in the native habitat, and follicular atresia (Casares, 1995).

In contrast to the other giant tortoise species, the Galápagos giant tortoise (*Chelonoidis niger*), of which the reproductive cycle has been studied more intensively both under free-ranging as well as captive conditions (Rostal et al., 1998; Schramm et al., 1999), the endocrinological pattern of the Aldabra giant tortoise has not yet been described.

Endocrinological patterns are a crucial measurement tool for the understanding of reproductive processes, and the present study served to complement the biological observations with endocrinological reproductive patterns in wild Aldabra giant tortoises during an entire year. This is a prerequisite for endocrinological monitoring of reproductive processes, developing hormonal interventions to increase reproductive output, and evaluation of husbandry and management. In particular, knowledge is required on the role of environmental factors, circadian and annual light cycles, and resource availability for the breeding success in captive populations, both ex-situ and in-situ, to establish reserve- and reintroduction populations.

METHODS

This study was part of a multidisciplinary project on Aldabra giant tortoises on Aldabra Atoll (Falcón et al., 2018; Haverkamp et al., 2017; Turnbull et al., 2015; Walton et al., 2019). The research and sampling protocol were approved by the Seychelles Bureau of Standards.

Animals

From January to December 2013, eight wild adult Aldabra tortoises (male n=4, female n=4) were tracked by GPS-tags with VHF transmitters (E-obs GmbH; Germany) and manually restrained on a monthly basis for blood sampling. All eight animals were located on Picard Island of Aldabra Atoll (-9.225925, 46.125266) and were individually identified by a branded three-digit mark on their carapace. The animals were considered sexually mature based on their carapace exceeding 55 cm straight length and/or age estimation based on the width of the third dorsal scute (Bourn, 1977; Grubb, 1971). The intermittent encounters with the animals did not allow for continuous observations of reproductive behaviour throughout this time.

Sampling

Monthly blood sampling was performed under manual immobilisation in lateral position. Manual stabilisation and blood sampling from the proximal foreleg (*vena brachialis*) with 3 ml syringes and hypodermic 19G and 23G needles did not exceed 15 minutes to avoid stress-induced changes in steroid hormone levels (Lance et al., 2004). Blood was immediately transferred into lithium-heparin containing tubes (Vacuette Tubes, Greiner Bio-One Vacuette, Switzerland) and stored in a thermos-box with ice bricks for transport to the Aldabra Atoll laboratory. Plasma was centrifuged within 2 hrs of collection and stored at -20 ° C until further analysis.

Enzyme-linked immunosorbent assay (ELISA) for steroid hormones

In males, testosterone was measured and in females, oestradiol and progesterone were measured. To minimise repeated thawing of the samples, whenever possible multiple hormones were measured within each sample on the same day. Oestradiol concentrations were quantified using a commercially available EIA kit (Arbor Assays, Ann Arbor, Michigan, USA; KB30-H1) as per manufacturer's instructions. Testosterone and progesterone concentrations were quantified using EIA methods described previously (Kummrow et al., 2011). Antisera (C. Munro, University of California, Davis, California, USA) were diluted as follows: T (polyclonal R156/7), 1:10,000 and P (monoclonal CL425, Quidel Corp., California, USA with final purification by C. Munro), 1:8,800. Horseradish peroxidase conjugates (C. Munro) were diluted as follows: T-HRP, 1:20,000 and P-HRP, 1:40,000. Standards used were T (Steraloids Inc., A6950; 48–12,500 pg/ml) and P (Sigma P0130; 15.6–4,000 pg/ml). Samples were run neat in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per litre). Controls consisted of laboratory stocks of pooled samples obtained from cycling females and males from a variety of species, and run at 30 and 70 % binding. Standards, samples and controls were run in duplicate with <10 % CV between duplicates. Intra- and inter-assay CVs were <10 % and <15 %, respectively, for all assays.

The results were plotted graphically against previously reported behavioural and anatomical observations (Bourn, 1977; Swingland & Coe, 1978).

Meteorological data

Measurements of daily temperature (mean, maximal, and minimal; °C), precipitation (mm/day), and sunshine duration (min/day) from the Aldabra Islands (LAT -9.72259, LON 47.0407) were acquired from a commercial data bank (Meteoblue AG, 4058 Basel, Switzerland), with mean values of 1985-2018 extracted and used for analysis.

RESULTS

Female steroid hormone levels

Female plasma oestradiol levels are depicted in Figure 1 and varied between 130 and 310 pg/ml. During the annual cycle, plasma oestradiol concentrations showed a seasonal pattern with high values in February and March, a decline in May and June, followed by the nadir in September and increasing values in November and December. Plasma progesterone levels were only measurable above detection limit (19 pg/ml) in April in one of the four animals (480 pg/ml).

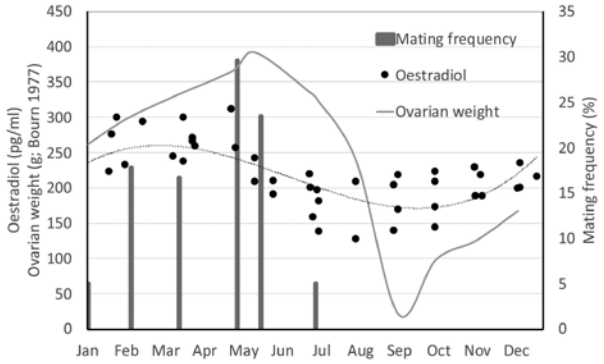

Figure 1. Plasma oestradiol (solid circles) with trendline (dotted line) of female Aldabra giant tortoises (*A. gigantea*, n=4) over an entire year in relation to published ovarian weight and mating frequency, plotted as monthly fraction of the yearly mating occurrences (from Bourn, 1977).

Male steroid hormone levels

Plasma testosterone levels in male Aldabra giant tortoises are depicted in Figure 2 and ranged from 0.14 to 3.61 ng/ml, showing a seasonal variation with highest values in January and lowest values in September.

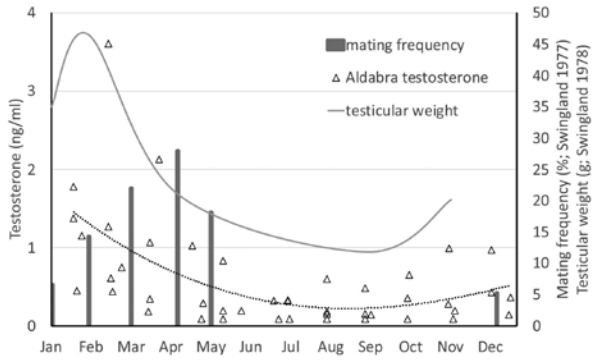

Figure 2. Plasma testosterone (triangles) with trendline (dotted line) of male Aldabra giant tortoises (*A. gigantea*, n=4) over an entire year in relation to published mating frequency, plotted as monthly fraction of the yearly mating occurrences, and testicular weight (from Swingland and Coe, 1978).

Meteorological data

In April and May, the critical time for late folliculogenesis and ovulation, precipitation in the year 2013 was below the standard deviation of the mean precipitation of the years 1985 to 2018, and sunshine duration longer, whereas mean daily temperature 2013 was only slightly higher than the mean and remained within the standard deviation (Fig. 3).

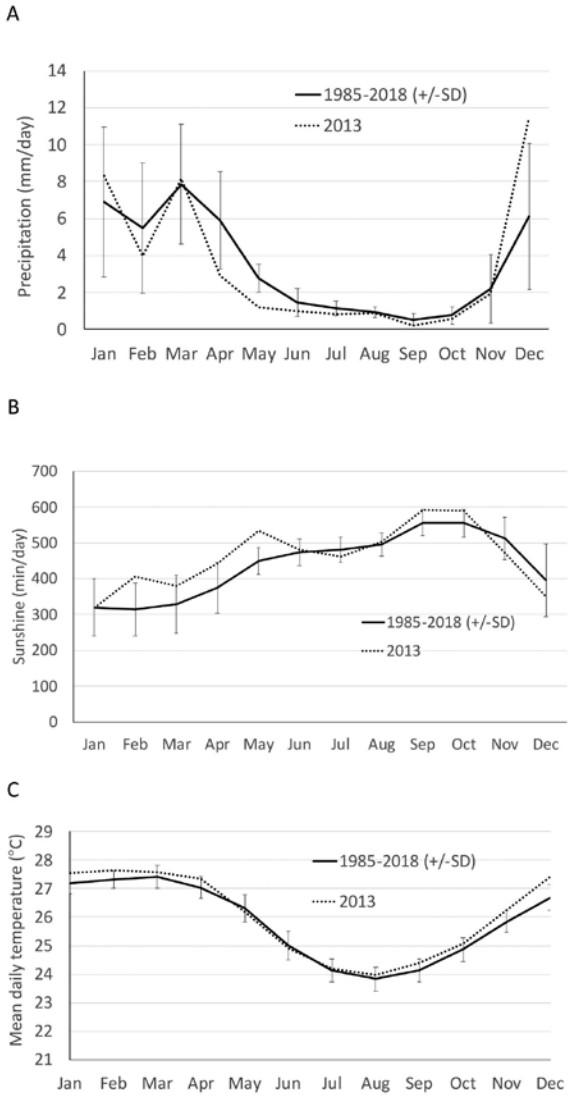

Figure 3. Meteorological data of the Aldabra Atoll, **A)** precipitation, **B)** daily sunshine duration, **C)** mean daily temperatures, mean values 1985-2018 (solid line, +/- SD) and the values of the year of sampling (2013, dotted line).

DISCUSSION

We report seasonal cyclicity in plasma oestradiol and testosterone in free-living Aldabra giant tortoise females and males, respectively, indicating a seasonal cycle of gonadal steroid hormones in their endemic range, coinciding with previously reported behavioural and anatomical observations (Bourn, 1977; Swingland & Coe, 1978).

The Aldabra giant tortoises' island habitats are located within the southern hemisphere tropical climate zone, at geographic latitude -9.23°. At such low latitudes, one would theoretically not expect a seasonal reproduction linked to a photoperiod signal, because the annual

variation in daylength is not considered sufficient for such a signal below a latitude of 11.75° (Bronson & Heideman, 1994). Yet, Aldabra giant tortoises exhibit distinct seasonality in reproduction, seemingly regulated by or adapted to climatic patterns of temperature and rainfall and available food resources (Bourn, 1977; Haverkamp et al., 2017; Schramm et al., 1999; Swingland & Coe, 1978). They show a peak in mating behaviour from February to May during the latter part of the rainy season, with the peak nesting season from June to September coinciding with the dry season, allowing the hatchlings to emerge at the beginning of the rainy season starting in October (Swingland & Coe, 1978). While for cool-climate reptile species, female reproduction is naturally restricted to the warmest times of the year, both biotic (e.g. predation on eggs, hatchlings or nesting females) and abiotic factors (e.g. precipitation, temperature) have been discussed as hypotheses to explain seasonality in tropical reptiles species (Brown & Shine, 2006).

Although not definitively investigated and proven, the timing of folliculogenesis and breeding activity during and at the end of the wet season was suggested to be associated with increased availability of food and other resources (Bourn, 1977). Seasonal changes in body condition of nesting females, and a positive association between body condition of nesting females and reproductive output, have been shown in several egg-laying reptile species (Henen, 1997; Litzgus et al., 2008; Loehr et al., 2007).

Increasing and peak plasma oestradiol levels are indicative of follicular development/ vitellogenesis, and the decrease of oestradiol levels paired with a sharp increase in progesterone are indicative of ovulation in egg laying reptile species (Callard et al., 1991). The seasonal pattern of plasma oestradiol concentrations in all four Aldabra tortoise females concurred with the observed changes in ovarian weight (Bourn, 1977), with vitellogenesis during the winter months leading up to a peak in February to April. The observed peak in mating frequency, plotted as monthly fraction of the yearly mating occurrences (Bourn, 1977), coincided with the decrease in oestradiol, facilitating ovulation, and increasing oestradiol after the end of the oviposition phase in September (Bourn, 1977) indicated the initiation a new phase of follicular development.

Interestingly, although all four *A. gigantea* females demonstrated increase of plasma oestradiol indicative of follicular development, only one female showed a single increased progesterone value during the presumed phase of ovulation in May to June. Behavioural and morphological data from field studies suggested that food availability and, in their direct influence on resource availability, population density and rainfall are primary regulative factors in the reproductive output in Aldabra tortoises (Haverkamp et al., 2017; Swingland & Coe, 1978). Although mature females, regardless of resource availability, showed the same reproductive potential, i.e. follicular development, those in high density populations regressed all or parts of their preovulatory follicles, whereas follicular atresia was not observed in the low-density populations (Swingland & Coe, 1978). In comparison to the mean values of the years 1985 to 2018, meteorological data of the year 2013 showed a distinctively lower precipitation and increased daily sunshine duration in April and May (Haverkamp et al., 2017), indicating an unusually dry season. Although only a small number of individuals were included in our study, our data may be indicative of a high rate of follicular atresia in 2013, presumably due to drought resulting in resource scarcity during the critical months in which ovulation would have occurred. Mean daily temperatures in 2013 did not differ from the mean values of 1985-2018, and are therefore less likely to qualify as a trigger factor for follicular atresia. Alternatively, methodological limitations, such as insufficient sensitivity of the ELISA to low levels of unbound circulating progesterone may have been responsible for the lack of progesterone data. Prior extraction of progesterone to expand measurement to protein-bound hormone metabolites may have increased the sensitivity of the ELISA and revealed seasonal peaks (Graham et al., 2016). The field conditions did not allow for storage at -80 °C and permanent freezing may not have always been guaranteed during transports, however, the impact of storage duration and temperature on blood steroid hormone levels have been reported to be minimal (Reyna et al., 2001; Taylor & Schuett, 2004).

Plasma testosterone levels reflected spermiogenesis in the males with high values during the wet season, corresponding with the observed peak in testicular weight and mating frequency, and decreasing levels during the dry season when mating activity ceased (Swingland & Coe, 1978).

The low number of animals and the study design based on monthly encounters with the animals for blood sampling without continuous monitoring for reproductive activity, resulting in a lack of biological validation of the hormonal data, are constraints of the study that limit the data interpretation to a descriptive study. Nevertheless, our study complements previous anatomic, behavioural and morphological studies with hormonal data and provides baseline data for endocrinological monitoring of reproductive processes and management in captive breeding programs.

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REFERENCES

Bourn, D. (1977). Reproductive study of giant tortoises on Aldabra. *Journal of Zoology* 182, 27-38.

Bourn, D., Gibson, C., Augeri, D., Wilson, C. J., Church, J. & Hay, S.I. (1999). The rise and fall of the Aldabran giant tortoise. *Proceedings of the Royal Society of London* 1091-1100.

Bronson, F. & Heideman, P. (1994). Seasonal regulation of reproduction in mammals. *Physiology of Reproduction* 2, 541-584.

Brown, G. P. & Shine, R. (2006). Why do most tropical animals reproduce seasonally? Testing hypotheses on an Australian snake. *Ecology* 87, 113-143.

Callard, I. P., Etheridge, K., Giannoukos, G., Lamb, T. & Perez, L. (1991). The role of steroids in reproduction in female elasmobranchs and reptiles. *Journal of Steroid Biochemistry and Molecular Biology* 40, 571-575.

Casares, M. (1995). Untersuchungen zum Fortpflanzungsgeschehen bei Riesenschildkröten (*Geochelone elephantopus* und *G. gianteus*) und Landschildkröten (*Testudo graeca* und *T. hermanni*) anhand von Ultraschalldiagnostik und Steroidanalyse im Kot. *Zoologischer Garten* 65, 50-76.

Falcón, W., Baxter, R. H., Furrer, S., Bauert, M., Hatt, J.-M., Schaepman-Strub, G., Ozgul, A., Bunbury, N., Clauss, M. & Hansen, D. M. (2018). Patterns of activity and body temperature of Aldabra giant tortoises in relation to environmental temperature. *Ecology and Evolution* 8, 2108-2121.

Falcón, W. & Hansen, D. M. (2018). Island rewilding with giant tortoises in an era of climate change. *Philosophical Transactions of the Royal Society B: Biological Sciences* 373, 20170442.

Falcón, W., Hansen, D. M. & Moll, D. (2018). Frugivory and seed dispersal by chelonians: A review and synthesis. *Biological Reviews* 95, 142-166.

Gerlach, J., Rocamora, G., Gane, J., Jolliffe, K. & Vanherck, L. (2013). Giant tortoise distribution and abundance in the Seychelles Islands: past, present, and future. *Chelonian Conservation and Biology* 12, 70-83.

Graham, K. M., Mylniczenko, N. D., Burns, C. M., Bettinger, T. L. & Wheaton, C. J. (2016). Examining factors that may influence accurate measurement of testosterone in sea turtles. *Journal of Veterinary Diagnostic Investigation* 28, 12-19.

Grubb, P. (1971). Growth, ecology and population structure of giant tortoises on Aldabra. *Philosophical Transactions of the Royal Society of London* 260, 327-372.

Hansen, D. M., Donlan, C. J., Griffiths, C. J. & Campbell, K. J. (2010). Ecological history and latent conservation potential: large and giant tortoises as a model for taxon substitutions. *Ecography* 33, 272-284.

Haverkamp, P. J., Shekeine, J., de Jong, R., Schaepman, M., Turnbull, L. A., Baxter, R., Hansen, D., Bunbury, N., Fleischer-Dogley, F. & Schaepman-Strub, G. (2017). Giant tortoise habitats under increasing drought conditions on Aldabra Atoll—Ecological indicators to monitor rainfall anomalies and related vegetation activity. *Ecological Indicators* 80, 354-362.

Henen, B. T. (1997). Seasonal and annual energy budgets of female desert tortoises (*Gopherus agassizii*). *Ecology* 78, 283-296.

Kummrow, M. S., Gilman, C., Mackie, P., Smith, D. A. & Mastromonaco, G. F. (2011). Noninvasive analysis of fecal reproductive hormone metabolites in female veiled chameleons (*Chamaeleo calyptratus*) by enzyme immunoassay. *Zoo Biology* 30, 95-115.

Lance, V. A., Elsey, R. M., Butterstein, G. & Trosclair Iii, P. L. (2004). Rapid suppression of testosterone secretion after capture in male American alligators (*Alligator mississippiensis*). *General and Comparative Endocrinology* 135, 217-222.

Litzgus, J. D., Bolton, F. & Schulte-Hostedde, A. I. (2008). Reproductive output depends on body condition in spotted turtles (*Clemmys guttata*). *Copeia* 2008, 86-92.

Loehr, V., Hofmeyr, M. & Henen, B. (2007). Annual variation in the body condition of a small, arid-zone tortoise, *Homopus signatus signatus*. *Journal of Arid Environments* 71, 337-349.

Reyna, R., Traynor, K. D., Hines, G., Boots, L. R. & Azziz, R. (2001). Repeated freezing and thawing does not generally alter assay results for several commonly studied reproductive hormones. *Fertility and Sterility* 76, 823-825.

Rostal, D. C., Robeck, T. R., Grumbles, J. S., Burchfield, P. M. & Owens, D. W. (1998). Seasonal reproductive cycle of the Galapagos tortoise (*Geochelone nigra*) in captivity. *Zoo Biology* 17, 505-517.

Schramm, B. G., Casares, M. & Lance, V. A. (1999). Steroid levels and reproductive cycle of the Galapagos tortoise, *Geochelone nigra*, living under seminatural conditions on Santa Cruz Island (Galapagos). *General and Comparative Endocrinology* 114, 108-120.

Swingland, I. R. & Coe, M. (1978). The natural regulation of giant tortoise populations on Aldabra atoll. Reproduction. *Journal of Zoology* 186, 285-309.

Taylor, E. N. & Schuett, G. W. (2004). Effect of temperature and storage duration on the stability of steroid hormones in blood samples from western diamond-backed rattlesnakes (*Crotalus atrox*). *Herpetological Review* 35, 14.

Turnbull, L. A., Ozgul, A., Accouche, W., Baxter, R., ChongSeng, L., Currie, J. C., Doak, N., Hansen, D. M., Pistorius, P. & Richards, H. (2015). Persistence of distinctive morphotypes in the native range of the CITES-listed Aldabra giant tortoise. *Ecology and Evolution* 5, 5499-5508.

Walton, R., Baxter, R., Bunbury, N., Hansen, D., Fleischer-Dogley, F., Greenwood, S. & Schaepman-Strub, G. (2019). In the land of giants: habitat use and selection of the Aldabra giant tortoise on Aldabra Atoll. *Biodiversity and Conservation* 28, 3183-3198.

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Mitochondrial DNA analysis reveals extremely low genetic diversity in a managed population of the Critically Endangered Gharial (*Gavialis gangeticus*, Gmelin, 1789)

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A decline in the numbers of threatened species is often reversed by reintroduction with the aim of repopulating or strengthening the population to reduce the risk of extinction. The success of reintroduction programs is associated with demographic and genetic monitoring of the reintroduced populations. We undertook a genetic assessment of the Critically Endangered gharial (*Gavialis gangeticus*) to assess the current level of genetic variation using three partial mitochondrial (mt) DNA regions: cytochrome *b*, cytochrome c oxidase subunit-I and the control region. We sequenced 103 samples collected across 14 nesting sites. A low level of mtDNA variation was observed in the sampled population (hd = 0.462 ± 0.048; Pi = 0.00029 ± 0.00004). Only five distinct haplotypes were observed in three segregating sites. This is the first assessment of the genetic variation in the wild gharial population to be made using mtDNA. Homogeneity in the 520 bp hypervariable control region of the crocodilian mtDNA is reported here for the first time. The low mitochondrial diversity and no genetic structure in the sampled population is indicative of a genetic bottleneck, the founder effect and probably associated with human-assisted augmentation of the population of the gharial. An extremely low level of genetic variation in the largest gharial population highlights the vulnerability of the gharial population in the wild and calls for immediate genetic assessment of other gharial populations so that a robust conservation plan focusing on connectivity and enhanced protection can be developed for the long-term persistence of the gharial in the wild.

Keywords: haplotype, homogeneity, low diversity, protection

INTRODUCTION

The genetic diversity of small populations is low due to various factors such as severe population declines, the founder effect and genetic bottlenecks (Banks et al., 2013). Such populations tend to lose variability rapidly as a consequence of various biotic and abiotic factors through genetic drift (Ellegren & Galtier, 2016). Drifts, regardless of any balancing force, can bring sudden and drastic changes in the allele frequency (Liao & Reed, 2009). The magnitude of such events are greater in a small population with little or no gene flow. Increased homozygosity and an increase in the frequency of recessive deleterious alleles, known as inbreeding, are immediate effects of reduced variability (Frankham et al., 1999). Long periods of isolation and inbreeding eventually lead to the decreased evolutionary adaptive potential of individuals and populations (Allendorf, 2010; Frankham et al., 1999; Galov et al., 2011; Lande et al., 1987; Liao & Reed, 2009). A decline in adaptive potential may drastically increase the extinction risk of a species locally or globally. Hence, monitoring the level of genetic variation is important for planning conservation strategies for wild and managed populations.

Species living in freshwater ecosystems are the most threatened due to natural (increasing surface temperature, non-uniform rainfall pattern) and anthropogenic (pollution, incidental capture, disturbance) factors. The gharial *Gavialis gangeticus* Gmelin (1789) is a Critically Endangered (Lang et al., 2019) freshwater crocodilian species endemic to the northern part of the Indian subcontinent (Hussain, 1999; Lang et al., 2019). The unique long-slender snout of the gharial, an adaptation for catching fish, makes it more vulnerable to accidental mortality in fishing nets (Berkovitz & Shellis, 2016; Hasan & Alam, 2016). Habitat destruction, poaching and accidental mortality in fishing gear brought the species to near-extinction. Between the 19th century and the mid-20th century, the population declined by an estimated 85 % (Hussain, 2009; Whitaker et al., 1974). Many gharial populations were extirpated in the early 1970s. By 1979, the largest known population was the one in the Chambal River, in which there were 107 individuals (all size classes) (Whitaker & Daniel, 1980).

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Table 1. List of primers used for the amplification of three partial mitochondrial DNA sequences

Gene	Primer	Sequence	Ta (˚C)	Source
coxI	FishF1	5′-TCAACCAACCACAAAGACATTGGCAC-3′	56	Ward et al. (2005)
	FishR1	5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′		
cytb	CP14715	5′-TGAGGAGCAACCGTAATTACCAACCT-3′	56	Meganathan et al. (2009)
	CP15546	5′-TCTGTCTTACAAGGCCAGTGCTTT-3′		
CR	L15637	5′-GCATAACACTGAAAATGTTAAYATGG-3′	56	Oaks (2011)
	H16258	5′-CTAAAATTACAGAAAAGCCGACCC-3′		

A conservation recovery programme was initiated in the mid-1970s to avert extinction. Captive-bred and captive-reared individuals were reintroduced in the existing range, and augmentation was carried out to re-populate a suitable area with low number of gharials. In spite of tremendous conservation efforts, throughout most of its range, the gharial either failed to recover or showed extremely slow recovery rates (Nair et al., 2012).

The gharial survives in a few small, isolated populations in India and Nepal (Lang et al., 2019). The genetic diversity of the species has been considered little or not at all despite its importance in planning the conservation of threatened wildlife (Frankel, 1974). Since the ultimate goal of any conservation program is to ensure the persistence of the population in the wild, assessment of genetic diversity is essential for planning short- and long-term conservation strategies. In this study, we used three partial mitochondrial DNA (mtDNA) regions—cytochrome ***b*** (*cytb*), cytochrome c oxidase subunit-I (*coxI*) and the control region (CR)—to assess the current level of genetic variability in the largest managed gharial population.

METHODS

Study Area

The Chambal River originates in the Vindhya Hill Range in central India. It forms a part of the greater Gangetic drainage system, flowing in a north-easterly direction through the states of Madhya Pradesh, Rajasthan and Uttar Pradesh before it meets the Yamuna River. A 600 km stretch of the Chambal River between Jawahar Sagar and Panchnada was notified as National Chambal Sanctuary (NCS) in the late 1970s under Project Crocodile, for conservation of aquatic reptiles including crocodiles, freshwater turtles and the Gangetic river dolphin. Since the inception of Project Crocodile, the population in NCS has been augmented continuously. As a result of this and protection of the habitat, NCS harbours approximately 85 % of the global gharial population (Hussain, 2009).

Sample Collection

Biological sampling was conducted as part of a long-term project of ecological monitoring of the Chambal River Basin. The sampling was conducted in 14 nesting locations along the Chambal River, within NCS (Fig. 1), in 2017. We collected biological samples in the form of tissue from dead hatchlings and eggshells with the embryonic membrane intact after hatching. The samples were stored in absolute ethanol at room temperature and later, in a −20 °C in the laboratory for long-term storage. Out of 103 biological samples used in the current study, 60 samples were from obtained unique nests, 16 samples from sibling groups, and 27 samples of unknown origin.

DNA extraction, PCR and mitochondrial DNA sequencing

We carried out total genomic DNA extraction (n=103) using the Phenol–Chloroform method (Sambrook et al., 1989) with overnight digestion of embryonic membrane in a lysis buffer with Proteinase K at 56 °C.

We selected three partial mtDNA regions (*coxI*, *cytb* and CR) to assess the genetic variation in gharials. MtDNA is useful in population genetics studies because it is inherited maternally and its nucleotide substitution rate is high (Brown et al., 1979; Castro et al., 1998). The primers used to amplify partial fragments of selected regions were described by Ward et al. (2005), Meganathan et al. (2009) and Oaks (2011) respectively (Table 1). Polymerase Chain Reaction (PCR) was carried out in 20 µL volumes containing 2 µL (10−20 ng) of the DNA template, 2 µL of 10X DreamTaq buffer, 0.2 mM of each dNTP, 3 pmol of each primer and 0.1 µL (0.5 units per reaction) of DreamTaq DNA polymerase (Thermo Fisher Scientific) to amplify the fragments. The thermal profile was 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 35 seconds, 56 °C for 40 seconds and 72 °C for 45 seconds, with a final extension at 72°C for 10 minutes. The amplified products were visualised using 2 % agarose gel, and positive amplicons were cleaned up with Exonuclease-I and Shrimp Alkaline Phosphatase (USB, Cleveland, OH) and sequenced using forward primers in an Applied Biosystems 3500xL Genetic Analyzer. Standard protocols were followed when carrying out the sequencing.

Mitochondrial DNA analyses

All the gharial mtDNA sequences generated in this study were aligned using the CLUSTAL W algorithm (Thompson et al., 1994) in BioEdit, V. 7.2.6 (Hall et al., 1999). The aligned sequences and associated electropherograms were checked manually. Variations were confirmed by re-sequencing and considered only when the base Q value was greater than 20 (QV20+). The Q values were determined using Thermo Fisher Cloud (https://apps.thermofisher.com/apps/spa/#/apps). The sequences were concatenated subsequently using MEGA, V. 10.0.5 (Kumar et al., 2018). Summary statistics, including the number of haplotypes (h), haplotype (gene) diversity (hd) and nucleotide diversity (Pi), were generated for the concatenated mtDNA fragment (1800–1806 bp) using DnaSP, V. 6.12 (Rozas et al., 2017). Genealogical relationships among haplotypes were assessed using a

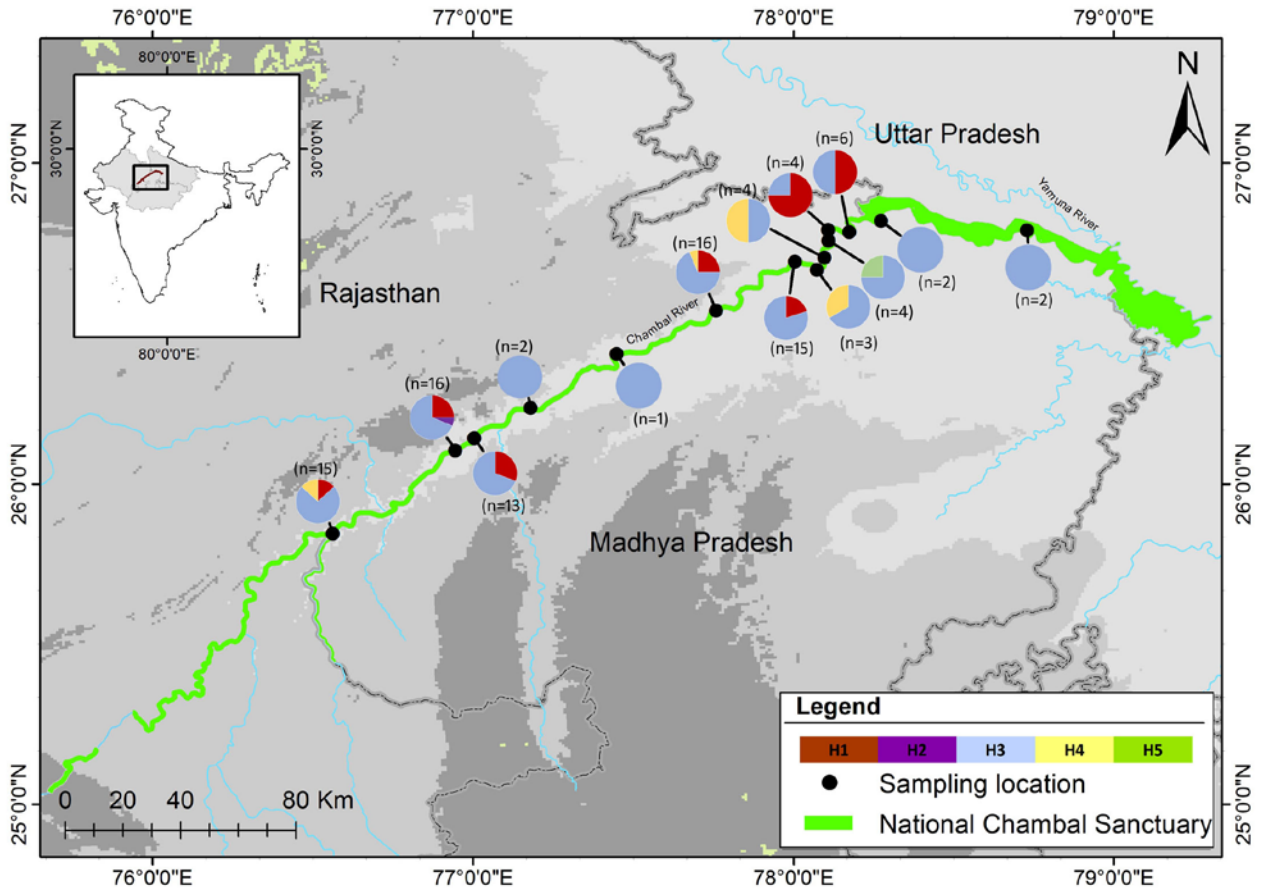

Figure 1. Map showing haplotype distribution of *G. gangeticus* in sampling locations. Pie charts represent the respective frequencies of mitochondrial DNA haplotypes in particular sampling location and n= number of samples. The colours of the pie charts represent different haplotypes.

median-joining network constructed in PopArt (Leigh & Bryant, 2015). Standard neutrality tests (*Tajima's D* and *Fu's Fs*) were performed using coalescent simulations with 10,000 permutations in Arlequin, V. 3.1 (Excoffier et al., 2005). The demographic expansion was investigated by comparison the mismatch distributions under an expected constant population and a fluctuating population with 10,000 coalescent simulations using DnaSP, V. 6.12 (Rozas et al., 2017).

RESULTS

Mitochondrial DNA diversity

1,798 – 1,806 bp of sequence data (609 bp *coxI*, 673–676 bp *cytb* and 488–521 bp CR) was obtained from 103 individuals. We observed five distinct haplotypes (H1–H5), with three segregating sites (one singleton and two parsimony informative sites) (Table 2). The sequences were submitted to GenBank (Supplementary Table S1). The haplotype (gene) diversity (hd, mean ± SD) was 0.462 ± 0.048, and the nucleotide diversity (Pi, mean ± SD) was 0.00029 ± 0.00004. Because there was no variable site in the CR, the diversity was not calculated for separate gene sequences.

The haplotype network showed no genetic cluster among the nesting localities (Figs. 1 and 2). The network was resolved with three major haplotypes. Most of the haplotypes differed from each other by one or two nucleotides. The haplotype H3 was the most common haplotype, and it was found in 72 samples (69.9 %) distributed among all the nesting locations. H1 contained 23 samples (22.3 %) from seven nesting localities, and H4 was found in six samples (5.8 %) from four nesting localities. H2 and H5, each contained one sample (1 %) each.

The mismatch distribution curve appeared to be unimodal. The standard neutrality test for population stability *Tajima's D* = -0.21 (*p*-value 0.45) and *Fu's Fs* = -1.394 (p-value 0.23) yielded a negative non-significant value.

DISCUSSION

This study represents the first genetic assessment of the wild gharial population using mtDNA. The study reveals that the variability in the 1,806 bp of mtDNA analysed here is low. The low level of variation in the mtDNA sequences is concordant with reports of low levels of mtDNA variation reported in other crocodilian species (Bloor et al., 2015; Glen et al., 2002; Luck et al., 2012; Posso-Pelaez et al., 2018; Ray et al., 2004). However, the homogeneity of the hypervariable control region is unusual. This is the first study to report homogeneity in a 520 bp control region in crocodilians. The low levels of haplotype diversity and nucleotide diversity observed in the sampling localities are possibly explained by the

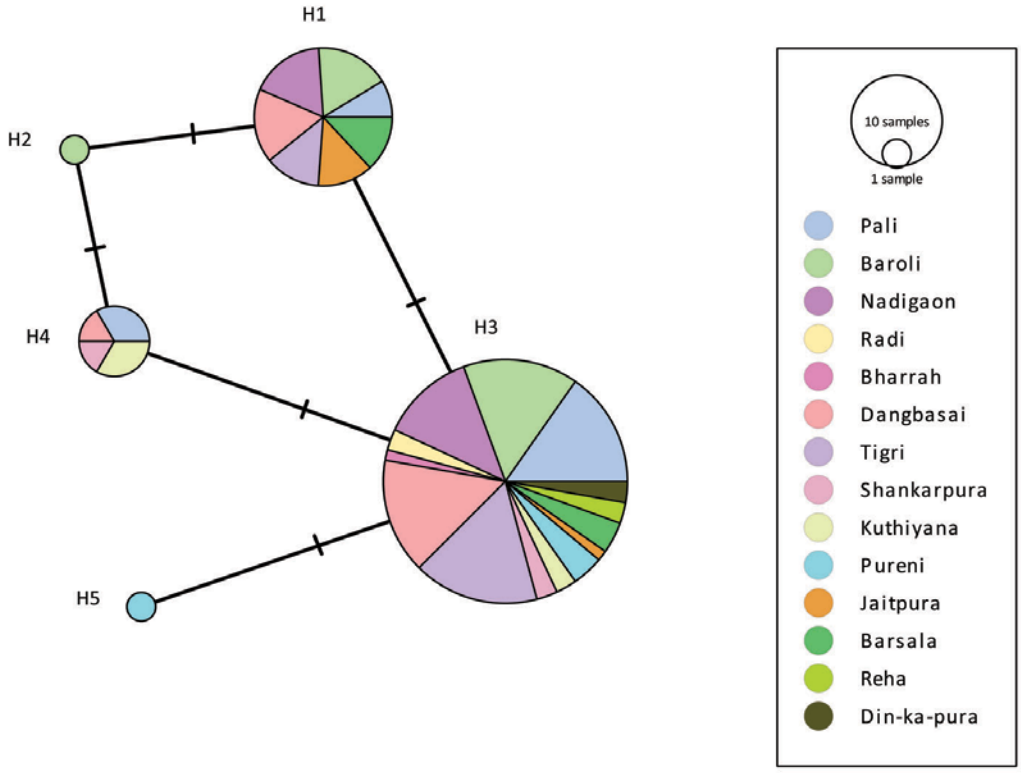

Figure 2. Median-joining haplotype network inferred from 1,806 bp of mtDNA sequence data for *G. gangeticus*. Each circle represents a different haplotype and the size is indicative of number of individuals present within the haplotype. The colours represent the proportions of the haplotypes from each nesting locality.

Table 2. Variable nucleotide position for five haplotypes observed using the concatenated approach.

Halotype number	Nucleotide position 108	Nucleotide position 703	Nucleotide position 1182
H1	T	G	C
H2	.	.	T
H3	C	.	.
H4	C	.	T
H5	C	A	T

known history of a severe population decline, genetic bottleneck and assisted population recovery with a small number of founder individuals.

The genealogical relationship determined using the median-Joining network, with no distinct clusters in the nesting localities, suggests that there is a high degree of haplotype sharing. This could be attributed to the continuous and unstructured release of young gharials that has taken place in the upstream sections of the river. The non-significant negative value obtained from the standard neutrality tests (*Tajima's D* and *Fu's Fs*) serves as a caution against drawing inferences. The unimodal mismatch distribution curve is indicative of a population expansion. However, the time of occurrence and the magnitude of the event remain unknown.

Further study needs to be carried out using nuclear microsatellite markers, single nucleotide polymorphism or other high-throughput molecular tools to substantiate the findings of our study and to gather evidence about the time and impact of demographic events. Genetic analyses of samples from other gharial populations are critical in order to establish the possible reason for the low mtDNA variation observed in this study and to develop a robust conservation strategy.

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REFERENCES

Allendorf, F.W., Hohenlohe, P.A. & Luikart, G. (2010). Genomics and the future of conservation genetics. *Nature Reviews Genetics* 11, 697 –709.

Banks, S.C., Cary, G.J., Smith, A. L., Davies, I. D., Driscoll, D. A., Gill, A. M., Lindenmayer, D. B. & Peakall, R. (2013). How

does ecological disturbance influence genetic diversity? *Trends in Ecology & Evolution* 28, 670–679.

Berkovitz, B.K. & Shellis, R.P. (2016). Reptiles 3: Crocodylia. In: *The teeth of non-mammalian vertebrates*. Academic Press, Elsevier Inc.

Bloor, P., Ibánez, C. & Viloria-Lagares, T.A. (2015). Mitochondrial DNA analysis reveals hidden genetic diversity in captive populations of the threatened American crocodile (*Crocodylus acutus*) in Colombia. *Ecology and Evolution* 5, 130–140.

Brown, W.M., George, M. & Wilson, A.C. (1979). Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences* 76, 1967–1971.

Castro, J.A., Picornell, A. & Ramon, M. (1998). Mitochondrial DNA: A tool for populational genetics studies. *International Microbiology* 1, 327–332.

Ellegren, H. & Galtier, N. (2016). Determinants of genetic diversity. *Nature Reviews Genetics* 17, 422-433.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* 1, 47-50.

Frankel, O.H. (1974). Genetic conservation: Our evolutionary responsibility. *Genetics* 78, 53–65.

Frankham, R., Lees, K., Montgomery, M.E., England, P.R., Lowe, E.H. & Briscoe, D.A. (1999). Do population size bottlenecks reduce evolutionary potential? *Animal Conservation* 2, 255-260.

Galov, A., Kocijan, I., Lauc, G., Gomerčić, M.Đ., Gomerčić, T., Arbanasić, H., Šatović, Z., Šeol, B., Vuković, S. & Gomerčić, H. (2011). High genetic diversity and possible evidence of a recent bottleneck in Adriatic bottlenose dolphins (*Tursiops truncatus*). *Mammalian Biology* 76, 339–344.

Gmelin, J.F., (1789). Caroli a Linné: Systema Naturae. In: *Beer GE*, Eds Tomus.I, Lipsiae 1057–1058.

Glenn, T.C., Staton, J.L., Vu, A.T., Davis, L.M., Bremer, J.R., Rhodes, W.E., Brisbin, Jr. I.L. & Sawyer, R.H. (2002). Low mitochondrial DNA variation among American alligators and a novel non-coding region in crocodilians. *Journal of Experimental Zoology* 294, 312–324.

Hall, T., Biosciences, I. & Carlsbad, C. (1999). BioEdit: An important software for molecular biology. *Green Earth Research Foundation Bulletin of Biosciences* 2, 60–61.

Hasan, K.M. & Alam, S.A.B.M. (2016). Gharials of Bangladesh. International Union for Conservation of Nature, Bangladesh Country Office, Dhaka, Bangladesh.

Hussain, S.A. (1999). Reproductive success, hatchling survival and rate of increase of gharial *Gavialis gangeticus* in National Chambal Sanctuary, India. *Biological Conservation* 87, 261–268.

Hussain, S.A. (2009). Basking site and water depth selection by gharial *Gavialis gangeticus* Gmelin 1789 (Crocodylia, Reptilia) in National Chambal Sanctuary, India and its implication for river conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems* 19, 127-133.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35, 1547–1549.

Lande, R., Barrowclough, G.F. & Soule, M.E. (1987). Effective Population Size, Genetic Variation and Their Use in Population Management. In: *Viable Populations for Conservation*. Cambridge University Press, Cambridge, UK.

Lang, J., Chowfin, S. & Ross, J.P. (2019). *Gavialis gangeticus* (errata version published in 2019). The IUCN Red List of Threatened Species 2019: e.T8966A149227430. Downloaded on 3 October 2019. http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T8966A149227430.en.

Leigh, J.W. & Bryant, D. (2015). Popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6, 1110–1116.

Liao, W. & Reed, D.H. (2009). Inbreeding–environment interactions increase extinction risk. *Animal Conservation* 12, 54–61.

Luck, N.L., Thomas, K.C., Morin-Adeline, V.E., Barwick, S., Chong, A.Y., Carpenter, E.L., Wan, L., Willet, C.E., Langford-Salisbury, S.M., Abdelsayd, M. & Ang, R.A. (2012). Mitochondrial DNA analyses of the saltwater crocodile (*Crocodylus porosus*) from the Northern Territory of Australia. *Australian Journal of Zoology* 60, 18–25.

Meganathan, P.R., Dubey, B. & Haque, I. (2009). Molecular identification of crocodile species using novel primers for forensic analysis. *Conservation Genetics* 10, 767–770.

Nair, T., Thorbjarnarson, J.B., Aust, P. & Krishnaswamy, J. (2012). Rigorous gharial population estimation in the Chambal: Implications for conservation and management of a globally threatened crocodilian. *Journal of Applied Ecology* 49, 1046–1054.

Oaks, J. R. (2011). A time-calibrated species tree of Crocodylia reveals a recent radiation of the true crocodiles. *Evolution: International Journal of Organic Evolution* 65, 3285–3297.

Posso-Pelaez, C., Ibanez, C. & Bloor, P. (2018). Low mitochondrial DNA variability in the captive breeding population of the critically endangered Orinoco crocodile (*Crocodylus intermedius*) from Colombia. *Herpetological Conservation and Biology* 13, 347–354.

Ray, D.A., Dever, J.A., Platt, S.G., Rainwater, T.R., Finger, A.G., McMurry, S.T., Batzer, M.A., Barr, B., Stafford, P.J., McKnight, J. & Densmore, L.D. (2004). Low levels of nucleotide diversity in *Crocodylus moreletii* and evidence of hybridization with *C. acutus*. *Conservation Genetics* 5, 449–462.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E. & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution* 34, 3299–3302.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). Preparation and Analysis of Eukaryotic Genomic DNA. In: *Molecular Cloning: A Laboratory Manual*, 2nd Eds. Cold spring Harbor Laboratory Press, New York, USA.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–468.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. & Hebert, P.D. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences* 360, 1847-1857.

Whitaker, R. & Daniel, J.C. (1980). The status of Indian crocodilians. *Journal of the Bombay Natural History Society* 75, 369.

Whitaker, R., Rajamani, V., Basu, D. & Balakrishnan, V. (1974). Preliminary survey of the Gharial, *Gavialis gangeticus*-Survey Report. Madras Snake Park Trust and conservation centre, India

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Molecular phylogeny and taxonomic evaluation of the genus *Asaccus* Dixon and Anderson, 1973 (Reptilia: Phyllodactylidae) in Iran

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The Iranian species of the phyllodactylid geckos of the genus *Asaccus* are found only in the valleys of the Zagros Mountains, a region which represents an important area of endemism in western Iran. Recently, many relict species have been described from the central and southern parts of the Zagros Mountains, which were previously known as *A. elisae*. The recent descriptions of species within this complex suggest that diversity within the genus may be higher than expected and that its taxonomy and systematics should be revised. In the present study, phylogenetic relationships within the genus *Asaccus* were evaluated using two mitochondrial and one nuclear gene. Genetically, the genus shows high levels of variability. The molecular phylogeny of the genus suggests the presence of three main clades along the Zagros Mountains with the southern population (from the Hormozgan province) and one clade (*A.* sp8 and *A.* sp9) being sister taxon to *A. montanus* from UAE. The remaining samples are separated into two reciprocally monophyletic groups: the northern (Kurdistan, Kermanshah and Ilam provinces) and the central (Lorestan, Khuzestan, Kohgilouye-Bouyer Ahmad and Fars provinces) Zagros groups. The results of the present study suggest that populations attributed to *A. elisae* in Iran correspond to distinct lineages with high genetic distances. In brief, our results suggest that the genus needs a major taxonomical revision The Arabian origin of the genus has not been confirmed, because two populations from Zagros were located within the *A. montanus*, *A. gallagheri* and *A. platyrhynchus* clade. Further morphological analyses are needed to systematically define each genetic lineage as a new taxon.

Keywords: *Asaccus*, genetic variability, Iran, Phyllodactylidae, species diversity, Zagros Mountains

INTRODUCTION

The high diversity and endemicity in Iran may be found in the Zagros Mountain region (Šmíd et al., 2014). The old geological history of this region, its geographical location between two different zoogeographical realms, the existence of many peaks and canyons distributed across more than 1,600 km in a northwest-southeast direction, and its many local microclimates are the main reasons for high diversity and endemism of the local fauna and flora (Mandaville, 1977). The uplifting of the Zagros Mountains was initiated by the northward collision of the Arabian plate with the Eurasian landmass that took place from the Oligocene to the Miocene (35 – 20 million years ago (MYA)) (Mouthereau, 2011). Major uplift of the Zagros Mountains was initiated from 12.4 MYA (Khadivi, 2010). Most species present in this region are endemic to the area and adapted to the local ecological conditions (Eskandarzadeh et al., 2018). There are many species of reptiles, with representatives from the Viperidae, Colubridae, Lacertidae, Gekkonidae and Phyllodactylidae families being the main inhabitants of the Zagros Mountains (Šmíd et al., 2014; Eskandarzadeh et al., 2018; Rajabizadeh, 2018).

The genus *Asaccus* Dixon and Anderson, 1973 corresponds to a group of geckos distributed in the Middle East (Carranza et al., 2016). Traditionally, the genus consists of only three species (two from the Zagros Mountains and one from the Hajar Mountains), but recent taxonomic revisions in Iran, UAE and Oman increased the species number to 19, ten from Iran of which nine are endemic to the country (Werner, 1895; Arnold, 1972; Dixon & Anderson, 1973; Arnold & Gardner, 1994; Gardner, 1994; Rastegar-Pouyani, 1996; Rastegar-Pouyani et al., 2006; Werner, 2006; Afrasiab & Mohamad, 2009; Torki, 2010; Torki et al., 2011;

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Carranza et al., 2016; Nasrabadi et al., 2017; Uetz, 2019). These geckos were placed previously within the family Gekkonidae, but were recently extracted from this family based on both morphological and molecular approaches and were placed within the Phyllodactylidae (Gamble et al., 2008). The Hajar Mountains in Oman and the United Arab Emirates (UAE) is one of the richest areas for the genus *Asaccus* and many local populations were elevated to new species recently thanks to the combination of molecular and morphological data (Carranza et al., 2016). Based on these results, Carranza et al. (2016) suggested that the Zagros Mountains most probably also included many undescribed species of Asaccus (Carranza et al., 2016). Simó-Riudalbas et al. (2017, 2018) provided more evidence for the high level of diversity and endemicity in the Hajar Mountains and suggested that this mountain range presented higher levels of reptile endemicity than the southernmost part of the Zagros Mountains. Rastegar-Pouyani et al. (2006) proposed that the Zagros Mountains are the center of origin of the genus *Asaccus*. However, the most recent molecular studies (Papenfuss et al., 2010; Carranza et al., 2016; Tamar et al., 2019) placed *Asaccus montanus* (one of the endemic species from the Hajar Mountains) as the sister taxon to all the other species, reinterpreting the history of this genus.

In this study, we aimed at resolving the systematics and evolution of *Asaccus* in Iran, through analyses of populations from north Zagros in Kurdestan and Kermanshah provinces to south Zagros in Khuzestan. Our specific aims were as follows: a) to comprehensively sample across the Zagros Mountains, from western to southern Iran to clarify the systematics and biogeography of *Asaccus*; and b) to test the previous hypothesis of the center of origin of the genus by adding many newly found populations of *Asaccus* from Iran.

METHODS

Sampling, DNA extraction and amplification

A total of 97 tissue samples of *Asaccus* were collected from the Iranian Plateau during field work from 2002 to 2018 (Fig. 1). We used the following GenBank sequences to complete the study: 20 sequences belonging to *A. montanus* (1 sequence), *A. nasrullahi* (1), *A. griseonotus* (1), *A. gallagheri* (1), *A. platyrhynchus* (1), *A. margaritae* (4), *A. caudivolvulus* (5), *A. gardneri* (5) and *Haemodracon riebeckii* (1) as outgroup (Simó-Riudalbas et al., 2018). Our dataset contains all described species of the genus *Asaccus* in Iran from their type localities and different unknown populations. Localities and coordinates for each sample are presented in the online supplementary materials, Table S1. All voucher specimens were deposited in the Sabzevar University Herpetological Collection (SUHC), Iran.

DNA was extracted from tissue samples using the high salt SDS method described in Kabir et al. (2006). The obtained DNA was quantified by 1 % agarose gels and Nanodrop 1000. We amplified three genes: two mitochondrial genes, 12S (12S) and Cytochrome *b* (*Cytb*), and one nuclear gene, Melanocortin 1 receptor (MC1R), because we aimed to combine our data with the previous published datasets (Carranza et al., 2016; SimÓ-Riudalbas et al., 2018; Tamar et al., 2018). These genes were amplified using four pairs of primers (primer information and PCR conditions are presented in Table S2 of the online supplementary materials).

Phylogenetic analyses and divergence time estimation

We used ClustalW within BioEdit v.7.0 (Hall, 1999) to align sequences. We used Mega v.6.0 (Tamura et al., 2013) to translate protein coding sequences (*Cytb* and *MC1R*) into amino acids and no stop codons were observed. Uncorrected genetic distances (*p*-distances) were calculated using Mega v.6.0 for the two mitochondrial genes separately.

Phylogenetic analyses were carried out to resolve the evolutionary history of the genus in Iran. For this purpose, a concatenated alignment was prepared and the phylogenetic trees were calculated under Maximum Likelihood (ML) and Bayesian Inference (BI) criteria. We used Modeltest 3.7 (Posada & Crandall, 1998) to find the best-fit models of nucleotide evolution. The evolutionary models obtained were as follows: *12S* = GTR+I+G; *Cytb* = TrN+I+G; *MC1R* = TrN+I+G. Two methods were used for phylogenetic analyses: Maximum Likelihood (ML) and Bayesian Inference (BI). For this purpose, all gene sequences were aligned and combined to reach a final concatenated alignment totaling 1,857 bp (*Cytb*: 937 bp; 12S: 392 bp; *MC1R*: 528 bp). RaxML 7.4.2 (Stamatakis, 2006), as implemented in RaxmlGUI 1.3 (Silvestro & Michalak, 2012), was used for ML analysis with the GTR+G+I model. The ML analysis was run in heuristic search method and node support was obtained using bootstrapping with 1000 replicates (Felsenstein, 1985). The BI analysis was conducted with MrBayes 3.2.1 (Ronquist et al., 2012) and the best fit evolutionary models indicated above. The analyses were run for 107 generations with a sample frequency of every 1000 generations. The adequacy of the runs was evaluated using variation in log Likelihoods (lnL) and the requirement of a split frequency lower than 0.01. The first 25 % of all trees was discarded as burn-in (Condamine et al., 2015).

Estimating divergence time

There are no internal calibration points for the Iranian Asaccus and their relatives, so we applied direct estimations as substitution rates for two mitochondrial genes. These substitution rates were calculated for three lizard families from the Canary Islands: *Tarentola* (Phyllodactylidae) (Carranza et al., 2000), *Gallotia* (Lacertidae) (Cox et al., 2010) and *Chalcides* (Scincidae) (Brown & Pestano, 1998). Many studies have used these substitution rates to estimate divergence times (Carranza & Arnold, 2012; Sindaco et al., 2012; Šmíd et al., 2013; Hosseinian Yousefkhani et al., 2019), although we are aware that this approach is far from ideal and could be an additional factor, along with use of partitions, that potentially leads to poor estimation of posteriors on divergence times (Jin & Brown, 2018). We used BEAST v. 1.8 (Heled & Drummond, 2010; Drummond et al.,

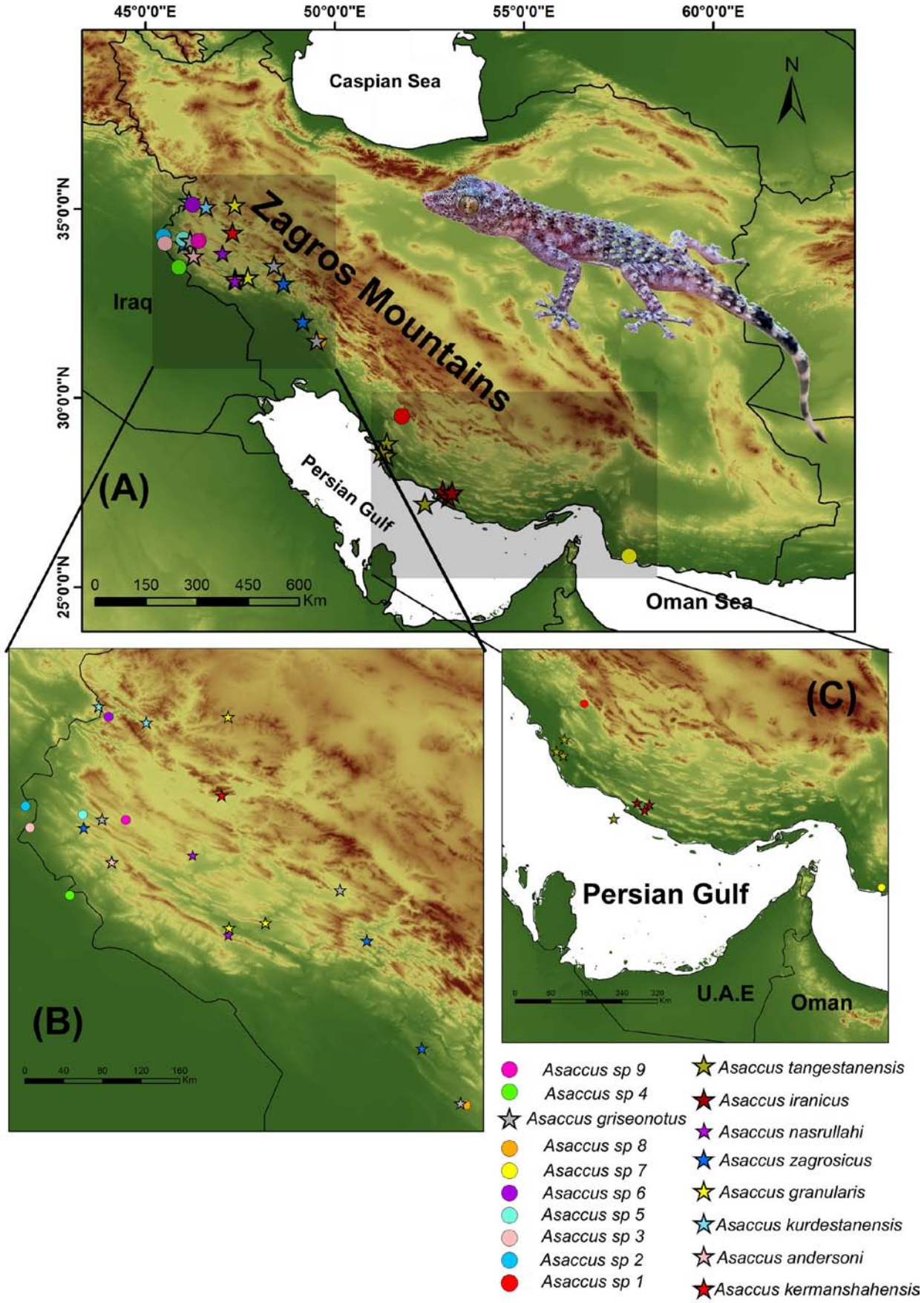

Figure 1. A) Sampling localities for all geckos used in the study. **B)** Western Iran and species in that regions. **C)** Sampling localities in south of Iran for the genus *Asaccus*.

2012) to estimate divergence times, among all species and populations of the genus *Asaccus*. The models and priors were set as follows: separate evolutionary models for each gene; random starting tree; clock models were set as lognormal relaxed clock; tree priors were set as coalescent and constant size. Ucld priors were set for 12S (mean: 0.00755, stdev: 0.00247) and *Cytb* (mean: 0.0228, stdev: 0.00806) separately (Carranza & Arnold, 2012).

RESULTS

Sequences and phylogenetic analyses

The dataset included 117 samples containing two mitochondrial fragments *12S* (366 bp; *V* = 173, *Pi* = 141) and *Cytb* (657 bp; *V* = 412, *Pi* = 339) and one nuclear gene fragment *MC1R* (629 bp; *V* = 152, *Pi* = 79) that provided 1,652 bp in total. Protein coding sequences were checked using MEGA 6.0 to ensure they did not contain stop codons. All accession numbers of new and retrieved sequences are provided in Supplementary Table S1.

Because of the similar topology of both ML and BI trees, we show only the BI tree (Fig. 2). Un-corrected genetic distances (*p*-distances) were calculated among clades based on the phylogenetic tree and are shown in Table 1. Genetic divergence for the *Cytb* gene among clades is relatively high and in most cases above 20 % (Table 1).

The results of this study uncovered high levels of hidden diversity in the Zagros Mountains and highlights the importance of this unique mountain chain as a hotspot of reptile diversity that needs priority for wildlife conservation management. The genus *Asaccus* shows high differentiation in Iran and at least 18 genetic lineages can be distinguished (Fig. 2). A clade formed by *A. gallagheri*, *A. platyrhynchus*, *A. montanus*, *A.* sp. 9 and *A.* sp. 8 originates from the earliest node within the tree and therefore forms a sister clade to the clade that includes all other *Asaccus* species included in the analyses (Fig. 2). It is perhaps surprising that the two distinct genetic lineages *A.* sp8 and *A.* sp9, are nested among the Arabian species of *Asaccus*. These two unknown lineages were collected from Izeh, Khuzestan and Islamabad Gharb, Kermanshah provinces, respectively.

Asaccus elisae is a species complex in Iran and comprises many local populations that are defined morphologically as *A. elisae*, but our study shows high divergence among them. For example, node support is high for the branch leading to the sample from Kazeroun, in Fars province indicating a new genetic

Table 1. Genetic distance between clades in Figure 1. Lower-diagonal entries are genetic distances for *Cytb* and upper-diagonal entries are *12S*. 1) *Asaccus kermanshahensis*; 2) *A.* sp2; 3) *A. griseonotus*_type; 4) *A. kurdestanensis*_type; 5) *A.* sp8; 6) *A.* sp9; 7) *A.* sp3; 8) *A.* sp5; 9)*A. zagrosicus*; 10) *A. andersoni*; 11) *A.* sp1; 12) *A. granularis*; 13) *A.* sp4; 14) *A. iranicus*; 15) *A. kurdestanensis*_A; 16) *A. nasrullahi*; 17) *A.* sp7; 18) *A. tangestanensis*; 19) *A.* sp6; 20) *A. montanus*; 21) *A. gallagheri*; 22) *A. platyrhynchus*; 23) *A. margaritae*; 24) *A. caudivolvulus*; 25) *A. gardneri* (the names of these groups correspond with those in Figure 1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1		13.2	07.5	07.5	11.2	11.2	06.8	07.8	12.1	10.0	12.5	11.4	12.5	12.0	07.5	06.9	13.2	11.9	06.4	14.9	11.2	13.2	12.2	11.5	10.1
2	23.5		13.9	12.3	15.6	15.6	11.9	12.2	11.4	14.1	10.8	09.6	09.5	11.4	12.3	14.4	16.6	11.2	12.2	18.6	14.6	17.3	15.9	17.6	16.5
3	20.8	24.2		08.0	10.8	10.8	08.7	07.9	11.7	10.7	12.2	11.0	12.5	12.6	08.1	09.8	12.3	12.4	06.7	15.1	10.2	12.8	11.2	11.1	09.8
4	16.5	27.4	25.0		09.4	09.4	08.9	04.5	12.4	12.2	12.4	11.6	12.7	12.9	00.3	09.5	15.8	12.7	03.7	15.3	11.4	13.8	11.4	12.7	10.3
5	25.0	26.8	26.9	28.7		00.0	10.2	11.5	12.2	11.0	12.5	10.6	14.9	13.1	09.4	10.9	15.6	12.9	09.8	07.1	11.9	13.6	13.9	12.2	10.4
6	23.7	29.6	26.7	25.5	23.9		10.5	11.5	12.2	11.0	12.5	10.6	14.9	13.1	09.4	10.9	15.6	12.9	09.8	07.1	11.9	13.6	13.9	12.2	10.4
7	25.4	17.6	24.0	25.2	23.2	24.1		08.1	08.8	10.0	10.2	08.2	11.5	11.7	09.2	05.0	10.8	11.6	07.8	14.9	11.9	11.9	09.8	09.8	09.1
8	-	-	-	-	-	-	-		13.3	10.0	13.6	11.9	11.2	13.1	04.8	09.4	14.9	12.9	02.4	15.9	11.2	14.6	12.2	11.9	10.4
9	26.1	17.3	23.2	26.7	24.6	25.8	04.6	-		14.4	11.0	05.9	11.4	11.0	12.6	11.9	14.0	10.8	13.2	16.0	13.0	14.2	13.9	15.0	13.5
10	25.7	25.4	24.7	26.8	28.3	28.1	22.1	-	22.6		13.4	12.1	12.7	12.6	12.5	08.0	13.4	12.2	10.0	14.1	12.4	14.7	12.0	12.0	13.7
11	25.0	23.5	21.8	24.3	29.0	27.0	20.6	-	20.7	22.4		09.0	11.9	11.8	12.6	11.9	14.2	11.6	11.5	16.9	13.2	14.9	13.9	15.6	14.5
12	24.5	17.4	21.0	26.6	23.8	24.4	16.5	-	16.2	24.8	22.5		09.9	08.8	11.6	10.4	13.3	08.6	10.7	15.0	13.6	14.7	13.7	13.1	12.3
13	26.2	14.5	21.9	29.4	27.3	26.0	18.1	-	17.0	23.7	21.8	17.3		09.7	13.0	13.8	15.3	09.4	11.5	16.3	15.3	16.3	16.9	16.6	15.3
14	24.3	19.9	20.8	27.2	29.4	27.5	18.8	-	17.9	24.7	18.4	17.4	16.0		13.2	12.2	13.1	00.5	12.0	15.5	13.4	15.8	14.5	15.5	14.8
15	26.1	16.5	23.6	25.7	23.5	24.8	01.5	-	03.4	22.4	20.2	16.2	17.0	18.4		09.6	16.0	13.0	03.8	15.2	11.4	13.9	11.6	12.8	10.4
16	25.5	27.4	21.5	24.9	28.6	30.1	22.5	-	22.9	16.4	23.9	22.2	23.8	26.4	22.5		11.9	12.1	08.5	13.7	12.2	12.5	11.2	09.9	09.9
17	31.0	33.2	28.1	28.1	31.1	29.0	27.9	-	29.1	29.4	29.7	29.2	31.0	30.3	28.3	30.4		12.9	14.6	17.6	15.3	14.9	12.5	11.5	13.5
18	24.2	20.8	21.5	26.9	27.5	26.2	20.5	-	19.5	25.0	20.8	18.9	17.2	08.6	19.9	27.5	29.4		11.9	15.1	13.3	15.6	14.3	15.3	14.6
19	18.0	25.7	21.8	13.7	27.9	23.7	23.5	-	23.8	23.5	22.1	24.5	26.6	24.4	23.5	23.9	25.7	24.3		15.6	11.2	13.9	11.5	11.2	15.2
20	27.9	31.6	26.7	30.9	27.2	29.2	27.9	-	28.9	29.8	28.3	29.8	29.2	29.1	28.7	30.4	34.1	29.3	27.9		12.9	14.9	16.3	16.3	15.2
21	26.1	25.4	24.0	29.9	27.2	22.1	24.3	-	25.3	29.0	25.0	25.9	27.3	27.3	24.6	28.6	31.3	28.2	26.1	30.5		11.9	11.9	12.9	11.8
22	22.8	29.0	26.8	24.6	24.6	02.4	24.6	-	26.2	27.6	25.7	23.8	24.8	26.9	25.4	29.9	28.6	25.8	23.2	29.0	22.4		08.8	11.9	12.1
23	24.2	26.7	24.4	25.6	27.8	25.6	26.7	26.7	-	26.9	26.9	24.9	23.3	23.3	22.4	27.1	25.3	23.6	23.1	29.0	27.1	25.6		09.8	09.8
24	23.5	27.9	24.7	24.6	27.6	24.1	25.4	25.4	-	24.9	24.3	25.7	23.9	23.9	24.0	25.7	24.6	23.8	21.7	30.1	26.1	23.2	24.5		04.7
25	21.4	23.4	23.3	23.3	26.0	24.7	21.2	21.2	-	21.4	22.8	25.5	21.0	21.0	21.6	21.5	24.5	22.2	20.5	29.9	24.2	24.9	23.1	14.1	

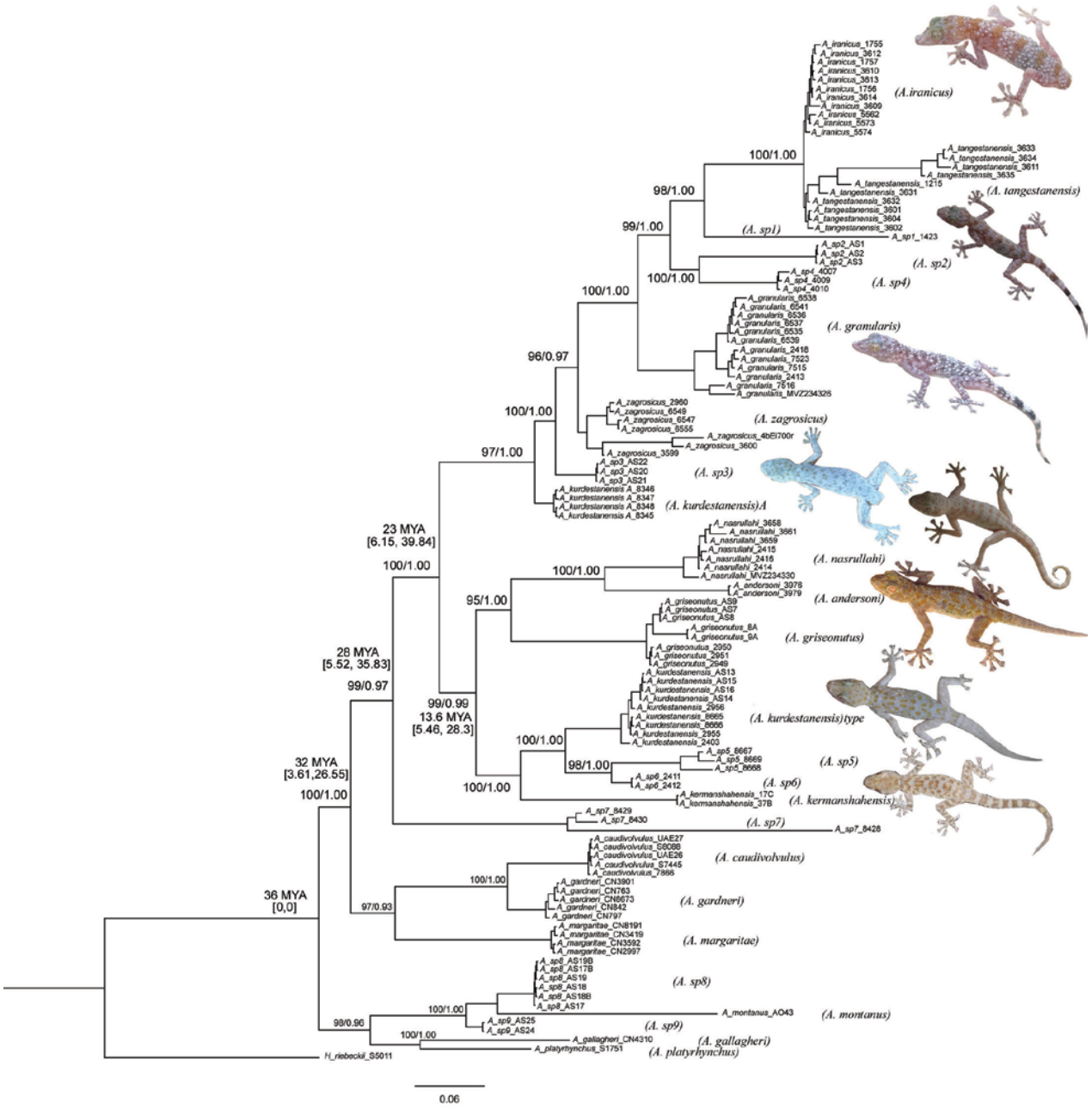

Figure 2. Bayesian Inference (BI) gene tree of spider geckos inferred from 1857 bp of mitochondrial (*12S* and *Cytb*) and nuclear (*MC_1R*) gene fragments. ML bootstrap support and posterior probability of Bayesian analyses are presented next to the nodes, respectively. Age estimates based on the substitution rates are denoted near the relevant nodes and include the mean and, in parentheses, the HPD 95 % confidence interval.

lineage (*A.* sp1). Two distinct genetic lineages from Gilan-e Gharb, Kermanshah province appear in distinct clades of our tree (*A.* sp2, *A.* sp3). One genetic lineage in the phylogenetic tree (*A.* sp4) is from Bina & Bijar, Ilam province which appears as a sister taxon to *A.* sp2. *Asaccus* sp5 and *A.* sp6 from Patagh, Sarpol-e Zahab and Nosud area respectively, in Kermanshah province form sister lineages. These two lineages form a clade that is sister to one of the clades containing *A. kurdestanensis* samples (namely, the one comprising the holotype) and these three form a sister clade to the clade that includes *A. kermanshahensis*. Surprisingly, the samples of *A. kurdestanensis* included in the present study represent two highly divergent lineages, the one described above containing the holotype of the species and another which includes all four samples from Sarv abad- Qaleji village. In addition a geographically distant population of *Asaccus*, found near Jask in southern Iran, originates from quite an old node within the tree (*A.* sp7).

Asaccus iranicus and *A. tangestanensis* are two species that were recently described from south Iran (Torki et al., 2011), although they show only 8.6 % divergence (*p*-distance for *Cytb*) and are the most closely related species (Fig. 2). *Asaccus granularis* was known only from the type locality (near Pol Dokhtar, Lorestan province) and samples from Darreshahr in Ilam province

clustered with type samples of *A. granularis*. One sample from the Museum of Vertebrate Zoology (MVZ 234326) that was deposited in GenBank as *A. griseonotus* from 99 km SW Khorram Abad clustered with our *A. granularis* clade, which suggests a possible error in this record.

Asaccus zagrosicus, represents a distinct genetic lineage that is sister to the clade containing *A. granularis*, *A. iranicus*, *A. tangestanensis*, *A.* sp2 and *A.* sp4. *Asaccus andersoni* and *A. nasrullahi* are two closely related species that clustered with *A. griseonotus* (Fig. 2). The genetic distance between *A. andersoni* and *A. nasrullahi* is very high (19.4 % for *Cytb*) and indicates their deep history of divergence. *Asaccus kermanshahensis* from the type locality, 32 km north-east of Kermanshah city, is another species that is clearly distinct from other groups. This taxon has high genetic distance from other clades (more than 16 % for C*ytb*), with *A. kurdestanensis* showing the lowest distance from it, 16 % in the *Cytb* gene fragment.

Divergence time estimation

Ages obtained from the phylogeny are shown in Figure 2 and show that diversification within the genus *Asaccus* started about 36 MYA. Based on our estimation time of divergence, the majority of the Iranian populations of the genus *Asaccus* began diversification about 28 MYA (95 % HPD = 5.5-35.8), when they divided into southern and western clades.

DISCUSSION

Using a molecular phylogenetic approach, we have presented the diversification patterns for a clade of phyllodactylid geckos from the Zagros Mountains, which is a major geographical system in western Iran that separates the Central Iranian Plateau in the east from the Mesopotamian plain in the west. Zagros runs in a north-west to south-east direction from West Azarbaijan province to Hormozgan province in south Iran in a 1600 km long line (Falcon, 1974). It is part of the Alpine-Himalayan mountain system that borders the Arabian shield and is very interesting for herpetologists because of the large numbers of deep valleys in the foothills (Falcon, 1974). In addition to the tectonic events in the Zagros formation, several paleoclimatic fluctuations played important role in the Middle East biodiversity (Zachos et al., 2001; Fathinia et al., 2018). For example, climatic conditions in the Zagros Mountains, led to the ancient immigration of mesic adapted species from the Mediterranean basin (García-Antón et al., 2002).

The genus *Asaccus* currently has ten described species in Iran (Šmíd et al., 2014), but specimens from several taxa, e.g., *A. elisae* and *A. kurdestanesis*, appear in distinct lineages along with several unnamed ones. On the other hand, distinct species appear genetically much more closely-related than previously considered (e.g., *A. iranicus* with *A. tangestanensis*). Our study highlights the high level of variation and local isolation of populations within the genus in the Zagros Mountains (Fig. 1). The results of our study support the importance of the Zagros Mountains due to the high level of endemicity in reptile and amphibian species found there (Hosseinzadeh et al., 2014). Based on the literature, there are many endemic species and isolated populations of reptiles in Iran (about 21.09 % of all Iranian reptiles are endemic) (Hosseinzadeh et al., 2014), most of which are situated in the east of Iran and the Zagros Mountains. These results emphasise the need for more investigation in the area to understand its biodiversity.

The central part of the Zagros Mountains in Charmahal Bakhtiari and Lorestan provinces have many deep and hardly accessible valleys that are suitable as microendemic areas. Among several local species of the genus *Asaccus*, the status of *A. elisae*, with a wide distribution range, is controversial and needs serious revision. The molecular phylogenetic tree, surprisingly demonstrates the huge genetic diversity among local populations within this species.

Our analysis reveals 9 distinct unnamed lineages of *Asaccus* in Iran that each of which has the potential to be considered as full species (based on the molecular data). Our study focused on the unknown populations from the northern to the southern parts of the Zagros Mountain region, so our findings illustrate hidden genetic diversity within the populations traditionally attributed to *Asaccus elisae*. Many isolated populations of the genus *Asaccus* were added into the analyses and phylogenetically differentiated from other species and populations. Combining these findings with further morphological and ecological information will help to provide more clarification. Carranza et al. (2016) referred to Arabia as the origin of the genus *Asaccus*. In our study, *A.* sp9, *A.* sp. 8, *A. montanus*, *A. platyrhynchus* and *A. gallagheri* originate from one of the earliest lineages within the tree, which challenges the view provided by Carranza et al. (2016).

Our molecular phylogenetic tree was produced based on all available populations of the species from Iran and GenBank samples from Arabia. We have demonstrated at least nine new genetic lineages with divergences comparable to the divergences found between well-recognised species. Further investigations including morphological and ecological studies are needed to describe these new species properly, and such a study has already been already started. The present results highlight the need for future conservation programs for the Zagros Mountains as one of the most important hotspots of endemism in Iran.

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REFERENCES

Afrasiab, S. R. & Mohamad, S. I. (2009). A study on cave-dwelling geckos in Iraq, with the description of a new species from Saffine mountain: (Reptilia: Gekkonidae). *Zoology in the Middle East* 47, 49-56.

Arnold, E. N. (1972). Lizards with northern affinities from the mountains of Oman. *Zoologische Mededelingen* 47, 111-128.

Arnold, E. N., & Gardner, A. S. (1994). A review of the Middle Eastern leaf-toed geckoes (Gekkonidae: *Asaccus*) with descriptions of two new species from Oman. *Fauna of Saudi Arabia* 14, 424-441.

Brown, R. P. & Pestano, J. (1998). Phylogeography of skinks (Chalcides) in the Canary Islands inferred from mitochondrial DNA sequences. *Molecular Ecology* 7, 1183-1191.

Carranza, S. & Arnold, E. N. (2012). A review of the geckos of the genus *Hemidactylus* (Squamata: Gekkonidae) from Oman based on morphology, mitochondrial and nuclear data, with descriptions of eight new species. *Zootaxa* 3378, 95.

Carranza, S., Arnold, E. N., Mateo, J. A. & López-Jurado, L. F. (2000). Long-distance colonization and radiation in gekkonid lizards, *Tarentola* (Reptilia: Gekkonidae), revealed by mitochondrial DNA sequences. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 267, 637-649.

Carranza, S., Simó-Riudalbas, M., Jayasinghe, S., Wilms, T. & Els, J. (2016). Microendemicity in the northern Hajar Mountains of Oman and the United Arab Emirates with the description of two new species of geckos of the genus *Asaccus* (Squamata: Phyllodactylidae). *PeerJ* 4, e2371.

Condamine, F. L., Nagalingum, N. S., Marshall, C. R. & Morlon, H. (2015). Origin and diversification of living cycads: a cautionary tale on the impact of the branching process prior in Bayesian molecular dating. *BMC Evolutionary Biology* 15, 65.

Cox, S. C., Carranza, S. & Brown, R. P. (2010). Divergence times and colonization of the Canary Islands by Gallotia lizards. *Molecular Phylogenetics and Evolution* 56, 747-757.

Dixon, J. R. & Anderson, S. C. (1973). A new genus and species of gecko (Sauria: Gekkonidae) from Iran and Iraq. *Bulletin of the southern California Academy of Sciences* 72, 155-160.

Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29, 1969-1973.

Eskandarzadeh, N., Rastegar-Pouyani, N., Rastegar-Pouyani, E., Fathinia, B., Bahmani, Z., Hamidi, K. & Gholamifard, A. (2018). Annotated checklist of the endemic *Tetrapoda* species of Iran. *Zoosystema* 40, 507-537.

Falcon, N. L. (1974). Southern Iran: Zagros Mountains. *Geological Society, London, Special Publications 4*, 199-211.

Fathinia, B., Rastegar-Pouyani, N. & Rastegar-Pouyani, E. (2018). Molecular phylogeny and historical biogeography of genera *Eristicophis* and *Pseudocerastes* (Ophidia, Viperidae). *Zoologica Scripta* 47, 673-685.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791.

Gamble, T., Bauer, A. M., Greenbaum, E. & Jackman, T. R. (2008). Out of the blue: a novel, trans-Atlantic clade of geckos (Gekkota, Squamata). *Zoologica Scripta* 37, 355-366.

García-Antón, M., Maldonado-Ruiz, J., Morla-Juaristi, C., & Sainz- Ollero, H. (2002). Fitogeografía histórica de la Península Ibérica. In F. D. Pineda, J. M. de Miguel, M. A. Casado, & J. Montalvo (Eds.), La Diversidad Biológica de España (pp. 45–63). Madrid, Spain: Prentice Hall.

Gardner, A. S. (1994). A new species of *Asaccus* (Gekkonidae) from the mountains of northern Oman. *Journal of herpetology* 28, 141-145.

Hall, T. A. (1999, January). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series (Vol. 41, No. 41, pp. 95-98). [London]: Information Retrieval Ltd., c1979-c2000.

Heled, J. & Drummond, A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution* 27, 570-580.

Hosseinian Yousefkhani, S. S., Aliabadian, M., Rastegar-Pouyani, E. & Darvish, J. (2019). Taxonomic revision of the spider geckos of the genus *Agamura* senso lato Blanford, 1874 (Sauria: Gekkonidae) in the Iranian Plateau. *Herpetological Journal* 29, 1-12.

Hosseinzadeh, M. S., Aliabadian, M., Rastegar-Pouyani, E. & Rastegar-Pouyani, N. (2014). The roles of environmental factors on reptile richness in Iran. *Amphibia-Reptilia* 35, 215-225.

Jin, Y., & Brown, R. P. (2018). Partition number, rate priors and unreliable divergence times in Bayesian phylogenetic dating. *Cladistics* 34, 568-573.

Kabir, S., Shahriar, M., Kabir, A. H., & Uddin, M. G. (2006). High salt SDS-based method for the direct extraction of genomic DNA from three different gram-negative organisms. *The CDR Journal* 1, 57-64.

Khadivi, S., Mouthereau, F., Larrasoaña, J. C., Vergés, J., Lacombe, O., Khademi, E. & Suc, J. P. (2010). Magnetochronology of synorogenic Miocene foreland sediments in the Fars arc of the Zagros Folded Belt (SW Iran). *Basin Research* 22, 918-932.

Mandaville J. (1977). The scientific results of the Oman flora and fauna survey 1975. *Plants Journal of Oman Studies Special Report* 1, 229-267.

Mouthereau, F. (2011). Timing of uplift in the Zagros belt/Iranian plateau and accommodation of late Cenozoic Arabia–Eurasia convergence. *Geological Magazine* 148, 726-738.

Nasrabadi, R., Rastegar-Pouyani, N., Rastegar-Pouyani, E. & Gharzi, A. (2017). A revised key to the lizards of Iran (Reptilia: Squamata: Lacertilia). *Zootaxa* 4227, 431-443.

Papenfuss, T. J., Jackman, T. R., Bauer, A. M., Stuart, B. L., Robinson, M. D. & Parham, J. F. (2010). Phylogenetic relationships among species of Southwest Asian leaf-toed geckos (*Asaccus*). *Proceedings of the California Academy of Sciences* 61, 587-596.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* (Oxford, England) 14, 817-818.

Rajabizadeh, M. (2018). *The snakes of Iran*. Iranshenasi press. Pp 468.

Rastegar-Pouyani, N. (1996). A new species of *Asaccus* (Sauria: Gekkonidae) from the Zagros Mountains, Kermanshahan

province, western Iran. *Russian Journal of Herpetology* 3, 11-17.

Rastegar-Pouyani, N., Nilson, G. & Faizi, H. (2006). A new species of *Asaccus* (Sauria: Gekkonidae) from Kurdistan province, western Iran. *Hamadryad* 30, 141-150.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S. & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61, 539-542.

Silvestro, D. & Michalak, I. (2012). raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity & Evolution* 12, 335-337.

Simó-Riudalbas, M., Metallinou, M., de Pous, P., Els, J., Jayasinghe, S., Péntek-Zakar, E. & Carranza, S. (2017). Cryptic diversity in *Ptyodactylus* (Reptilia: Gekkonidae) from the northern Hajar Mountains of Oman and the United Arab Emirates uncovered by an integrative taxonomic approach. *PloS one* 12, e0180397.

Simó-Riudalbas M., Tarroso, P., Papenfuss T., Al-Sariri T. & Carranza S. (2018). Systematics, biogeography and evolution of *Asaccus gallagheri* (Squamata, Phyllodactylidae) with the description of a new endemic species from Oman. *Systematics and Biodiversity* 16, 323-339.

Sindaco, R., Metallinou, M., Pupin, F., Fasola, M. & Carranza, S. (2012). Forgotten in the ocean: systematics, biogeography and evolution of the *Trachylepis* skinks of the Socotra Archipelago. *Zoologica Scripta* 41, 346-362.

Šmíd, J., Moravec, J., Kodym, P., Kratochvíl, L., Hosseinian Yousefkhani, S. S., Rastegar-Pouyani, E. & Frynta, D. (2014). Annotated checklist and distribution of the lizards of Iran. *Zootaxa* 3855, 1-97.

Šmíd, J., Moravec, J., Kratochvíl, L., Gvoždík, V., Nasher, A. K., Busais, S. M. & Carranza, S. (2013). Two newly recognized species of *Hemidactylus* (Squamata, Gekkonidae) from the Arabian Peninsula and Sinai, Egypt. *ZooKeys* 355, 79-107.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688-2690.

Tamar, K., Mitsi, P., & Carranza, S. (2019). Cryptic diversity revealed in the leaf-toed gecko *Asaccus montanus* (Phyllodactylidae) from the Hajar Mountains of Arabia. *Journal of Zoological Systematics and Evolutionary Research* 57, 369-382.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30, 2725-2729.

Torki, F. (2010). Beschreibung eines neuen *Asaccus* (Reptilia: Phyllodactylidae) aus der Provinz Lorestan, Iran. *Sauria* 32, 3-16.

Torki, F., Fathinia, B., Rostami, H. A., Gharzi, A. & Nazari-Serenjeh, F. (2011). Beschreibung eines neuen *Asaccus* (Sauria: Phyllodactylidae) aus dem Iran. *Sauria* 33, 51-62.

Uetz, P., Freed, P. & Hošek, J. (eds.) (2019) The Reptile Database, http://www.reptile-database.org, accessed [December 2019].

Werner, F. (1895). Über eine sammlung von Reptilien aus Persien, Mesopotamien und Arabien. *Verhandlungen der zoologisch-botanischen Gesellschaft in Wien* 45, 13-22.

Werner, Y. L. (2006). Retraction of *Ptyodactylus* Goldfuss from the fauna of Iran and its replacement by a new species of *Asaccus* Dixon and Anderson (Reptilia: Sauria: Gekkonidae). *Hamadryad* 30, 135-140.

Zachos, J. C., Pagani, M., Sloan, L., Ellen, T., & Billups, K. (2001). Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* 292, 686-693.

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Responses of crocodilians to construction of a hydro-electric dam on the Madeira River in the Brazilian Amazon

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The spillways of the Santo Antônio Hydro-electric Dam on the Madeira River in Brazilian Amazonia were closed in November 2011, inundating more than 100 km of river and reducing the annual fluctuations in water level. We surveyed the crocodilians in the affected area for two years before and for eight years after dam filling in order to evaluate the effects of the dam on the size structure of the population, the distribution of each species, and the detectability of individuals to interpret changes in apparent density. Our methodology was probably not appropriate to evaluate trends in population characteristics of *Paleosuchus palpebrosus* or *P. trigonatus*, but there was little evidence of an effect of the dam on the numbers of *Caiman crocodilus* and *Melanosuchus niger* in the area, and the distributions of all caiman species along the river changed only slightly after the dam was constructed. However, the proportions of small *C. crocodilus* and large *M. niger* detected in surveys increased eight years after dam filling. Despite having detectable effects on some population characteristics, the dam does not appear to represent a threat to the persistence of the species in the area if deforestation along the banks of the reservoir can be avoided.

Keywords: environmental impact, dam, crocodilians, Amazonia, conservation

INTRODUCTION

Large dams modify the aquatic environment (Agostinho et al., 2005; Zeilhofer & Moura, 2009; Finer & Jenkins, 2012) and can have negative effects on the aquatic fauna (Mérona & Tejeriba, 2005; Sá-Oliveira et al., 2015), including crocodilians (Mourão & Campos, 1995; Botha et al., 2011). The Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis (IBAMA) requires studies of crocodilians as part of the monitoring activities in newly formed reservoirs in Brazil. Nevertheless, the changed abiotic conditions also affect the detectability of crocodilians, so simple counts may give misleading indications of species responses.

Surveys of crocodilians are strongly affected by water level (Da Silveira et al., 2008) and the principle effect of dams is to increase water levels, even in so-called run-of-the-river dams, which do not form the large lentic reservoirs of conventional dams (Benchimol & Peres, 2015). Increased water levels and reduction in seasonal fluctuations in water level are likely to strongly impact crocodilian population dynamics due to changes in the availability of nesting areas (Mourão & Campos, 1995; Campos, 2019), foraging areas and facilitation of access by hunters (Campos, 2015). In some areas, the dams also affect pollution levels (Botha et al., 2011).

We studied the effects of the Santo Antônio Hydroelectric dam on the Rio Madeira in south-eastern Amazonia, Brazil, on four species of crocodilians (*Caiman crocodilus*, *Melanosuchus niger*, *Paleosuchus palpebrosus* and *P. trigonatus*). This and other dams in the Brazilian Amazon have been strongly criticised for presumed negative environmental impacts (Lees et al., 2016). We surveyed the crocodilians in the affected area for two years before and for eight years after dam filling, in order to evaluate the impacts of the dam on the size structure of the population, the distribution of each species and the detectability of individuals to interpret changes in apparent density.

MATERIALS AND METHODS

Ethics Statement

The research project was approved by the Brazilian Environment Agency (IBAMA permit No. 017/02) and by the Chico Mendes Institute for Biodiversity Conservation (ICMBio permanent license No. 13048-1) for capture and marking caimans (Normative regulation N° .154/2007). All procedures followed ethical practices for animals approved by the Committee on the Ethics of Animal Research of the Brazilian Agricultural Research Organization (Embrapa No 009/2016). No biological material, such as blood or tissue, was collected in this study. These species are classified by the International

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Union for Conservation of Nature (IUCN) as Lower Risk, least concern for conservation.

Nocturnal Surveys

Surveys were carried out at night, generally between 19:30h and 23:30h, in the Madeira River and its main tributaries using a 7 m aluminum canoe with 15 hp outboard motor. Each survey period lasted 7 nights. Surveys extended from shortly above the Santo Antônio dam wall to just below the wall of the Jirau Dam (built after the Santo Antônio dam) (Fig. 1). Three surveys were undertaken before dam filling in July-August 2010 and 2011 (low water), and January-February 2011 (high water). Post-filling surveys were undertaken in July-August between 2012 and 2019 (7 surveys), and January-February in 2012 and 2013 (2 surveys). Water levels were as high in all post-filling surveys as they were during the highwater season before the dam was built. However, there was still variation in water level during this period and water levels were slightly lower during surveys in July and August than in January and February. Caiman were identified as *Caiman crocodilus yacare* (Ccy), *Melanosuchus niger* (Mn), *Paleosuchus palpebrosus* (Pp) or *P. trigonatus* (Pt). We use the subspecies definition for Ccy because its taxonomic status is unclear. There is a genetic and morphological cline between the Pantanal caiman (usually referred to as *C. yacare*) and the Amazonian spectacled caiman (usually referred to as *C. crocodilus*) and these two taxa cannot be reliably distinguished in a > 2000 km intergrade zone along the Madeira River (Farias et al., 2013a; Farias et al., 2013b). Individuals in the upper Madeira River are morphologically more similar to the Pantanal caiman than they are to the Amazonian spectacled caiman on the lower reaches of the Madeira River. As the definition of the species is arbitrary, we used the subspecific name to indicate probable relationships. Individuals that could not be approached sufficiently close for confident identification (eyes only – 16.7 % of individuals) were not included in analyses.

The location of each individual was recorded with a GPS (Garmin) and between 25 and 52 individuals per species were captured and their the snout-vent length (SVLM – cm) measured after visual estimation of the length (SVLE – cm). The relationship between SVLM and SVLE of each species was as follows: for Ccy SVLM = 4.03 + 0.956* SVLE (N = 30; r^2 = 0.951, P < 0.001), for Mn SVL_M = 9.358 + 0.819* SVL_E (N = 25; r^2 = 0.951, P < 0.001), for Pp SVL_M = 6.368 + 0.907* SVL_E (N = 52, r^2 = 0.931, P < 0.001), and for Pt SVL_M = 4.518 + 0.939* SVL_E (N = 64, r^2 = 0.93, P < 0.001).

Only one individual from each sib-group of recently-hatched caimans that we located (SVL < 24 cm) was included in analyses. Hatchling groups are difficult to locate and are comprised of individuals that cannot be considered independent samples, so including all group members would artificially inflate sample sizes for this size class. All statistical analyses were undertaken in SYSTAT® and the maps were created in QGIS 2.18.

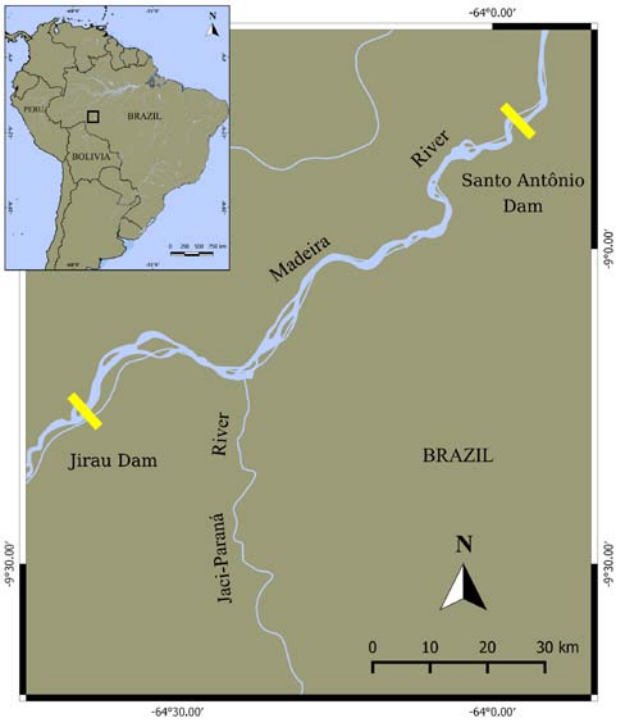

Figure 1. Study area encompassing the reservoir of the Santo Antônio Hydro-electric Dam to the wall of the Jirau Hydro-electric Dam (UHE Jirau) on the Madeira River, Brazilian Amazonia.

RESULTS

Analysis of Variance (ANOVA) indicated that the number of individuals seen in surveys of *Melanosuchus niger* ($F_{1,10}$ = 12.48, P = 0.005) and *Paleosuchus trigonatus* ($F_{1,10}$ = 5.45, P = 0.042) decreased after dam filling, though the numbers were similar before and after filling for *P. palpebrosus* ($F_{1,10}$ = 0.54, P = 0.478) and *Caiman crocodilus* ($F_{1,10}$ = 1.89, P = 0.200). However, when water level (WL), which is a surrogate for detectability, was included as a covariate, Analysis of Covariance (ANCOVA) indicated no significant difference in the number of caiman seen before and after reservoir filling (BA) for *M. niger* (WL: $F_{1,9}$ = 13.268, P = 0.005; BA: $F_{1,9}$ = 0.821, P = 0.389), *P. trigonatus* (WL: $F_{1,9}$ = 5.485, P = 0.044; BA: $F_{1,9}$ = 0.707, P = 0.422), and *C. crocodilus* (WL: $F_{1,9}$ = 5.526, P = 0.043; BA: $F_{1,9}$ = 1.861, P = 0.206). Only for *P. palpebrosus* was there indication of a difference in number seen independent of water level (WL: $F_{1,9}$ = 6.676, P = 0.030; BA: $F_{1,9}$ = 7.418, P = 0.023). Fewer *P. palpebrosus* were seen than expected, even after taking into account the effect of water level on detectability. With the possible exception of *M. niger*, the numbers of caimans seen after reservoir filling were similar to those seen at comparable water levels before filling (Fig. 2).

A Kolmogorov-Smirnov test indicated that the size structure of individuals seen differed between high- and low-water seasons before dam filling for *P. trigonatus* (P = 0.011) and *P. palpebrosus* (P = 0.040), but not for *M. niger* (P = 0.986) or *C. crocodilus* (P = 0.455). Therefore, in comparisons between before and after filling, when water levels were high, we used only the data for the pre-

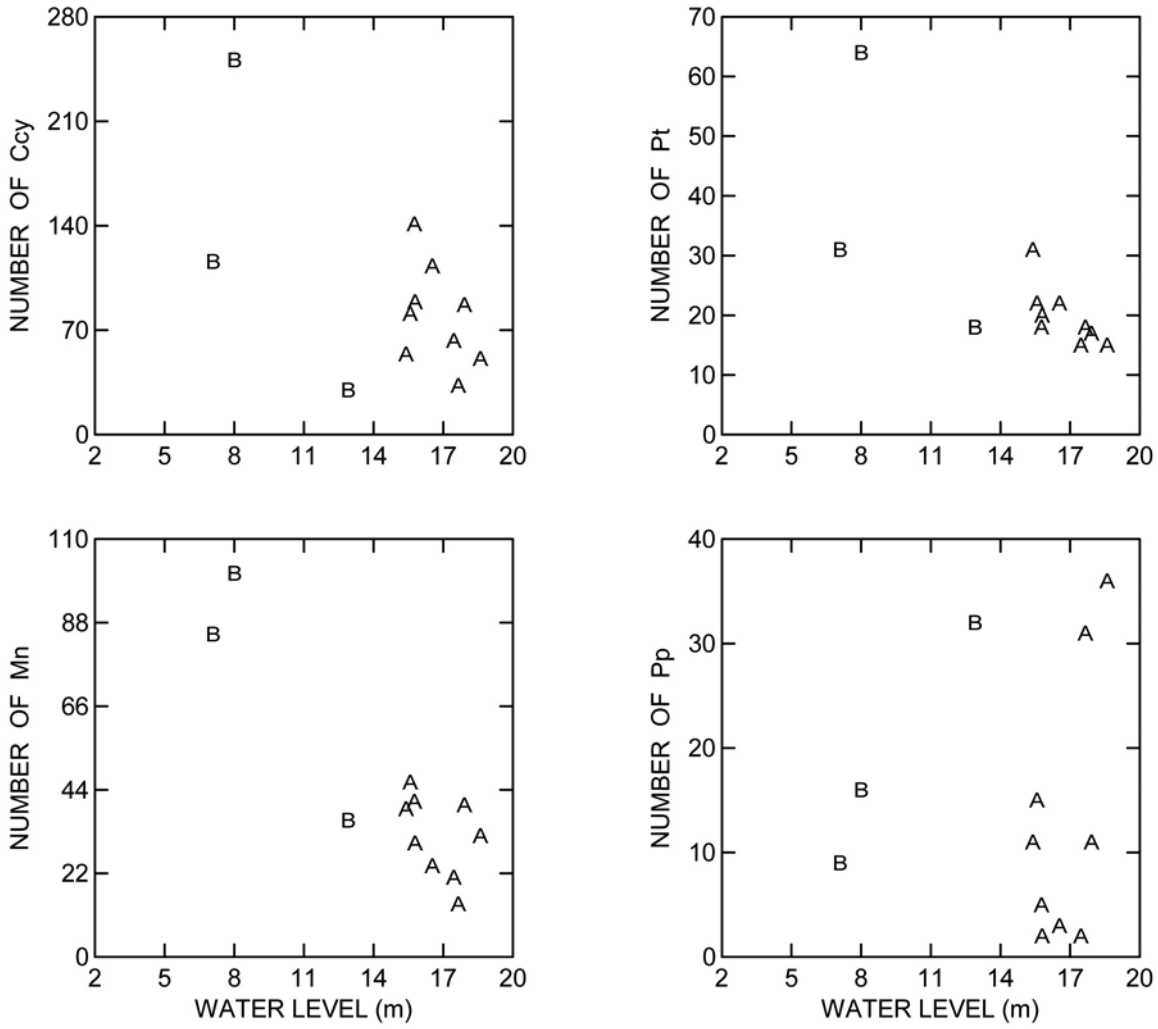

Figure 2. Number of caimans (Ccy = *C. crocodilus yacare*; Mn = *M. niger*; Pt = *P. trigonatus*; Pp = *P. palpebrosus*) in relation to water level (m), before (B) and after (A) filling of the Santo Antônio reservoir.

filling high-water season for the species of *Paleosuchus* and all data for the other two species. Given the large number of caimans seen, it is unlikely that the size of any one individual was estimated in more than one survey, so for the tests we combining years, assuming that all individuals were independent data points.

For *P. trigonatus*, Kolmogorov-Smirnov tests showed no significant differences in size structure between the pre-filling surveys and post-filling surveys until 2017 ($P \geq 0.331$ in all cases). There was evidence for a change in size structure in 2018 and 2019 ($P = 0.016$ and 0.068, respectively). Sufficient individuals of *P. palpebrosus* for yearly analyses ($N \geq 42$) were only seen until 2013. In 2012 and 2013 there was equivocal evidence of change in size structure ($P = 0.153$ and 0.063, respectively). Combining all post-filling surveys indicated a significant change in size structure for this species ($P = 0.006$).

Caiman crocodilus showed consistent differences between before and after reservoir filling ($P \geq 0.045$ in all cases), except for 2012 ($P = 0.175$). Combining all post-filling years indicated a significant overall change in size structure ($P < 0.001$). In contrast, the Kolmogorov-Smirnov tests for differences in size structure of *M. niger* were inconsistent, showing no significant differences in most years ($P \geq 0.192$), but significant differences in 2012 and 2018 ($P \leq 0.001$). However, the pooled post-filling surveys ($P = 0.019$) indicated size-structure change.

The proportions of large individuals of *P. trigonatus* (SVL > 60 cm) and *P. palpebrosus* (SVL > 75 cm) declined (Fig. 3). Although statistically significant, the changes in size structure of individuals seen of the other species tended to be subtle. The proportion of individuals of *C. crocodilus* between 40 and 55 cm SVL tended to increase and the proportion between 55 and 70 cm SVL tended to decline. More large (SVL > 135 cm) *M. niger* were seen after reservoir filling. The proportions of other size classes were similar before and after filling.

The caimans were not uniformly distributed along the river before reservoir filling (Figs. 4 & 5). Most individuals of all species were in the upstream reaches and not near the proposed site of the dam wall, which was situated on a long series of rapids. However, the dam apparently did not have much effect on the distributions of *P. trigonatus* and *M. niger*. The correlation between densities in river segments before reservoir filling and 4-8 years after was high ($r = 0.93$, $P < 0.001$ for both species). The strength

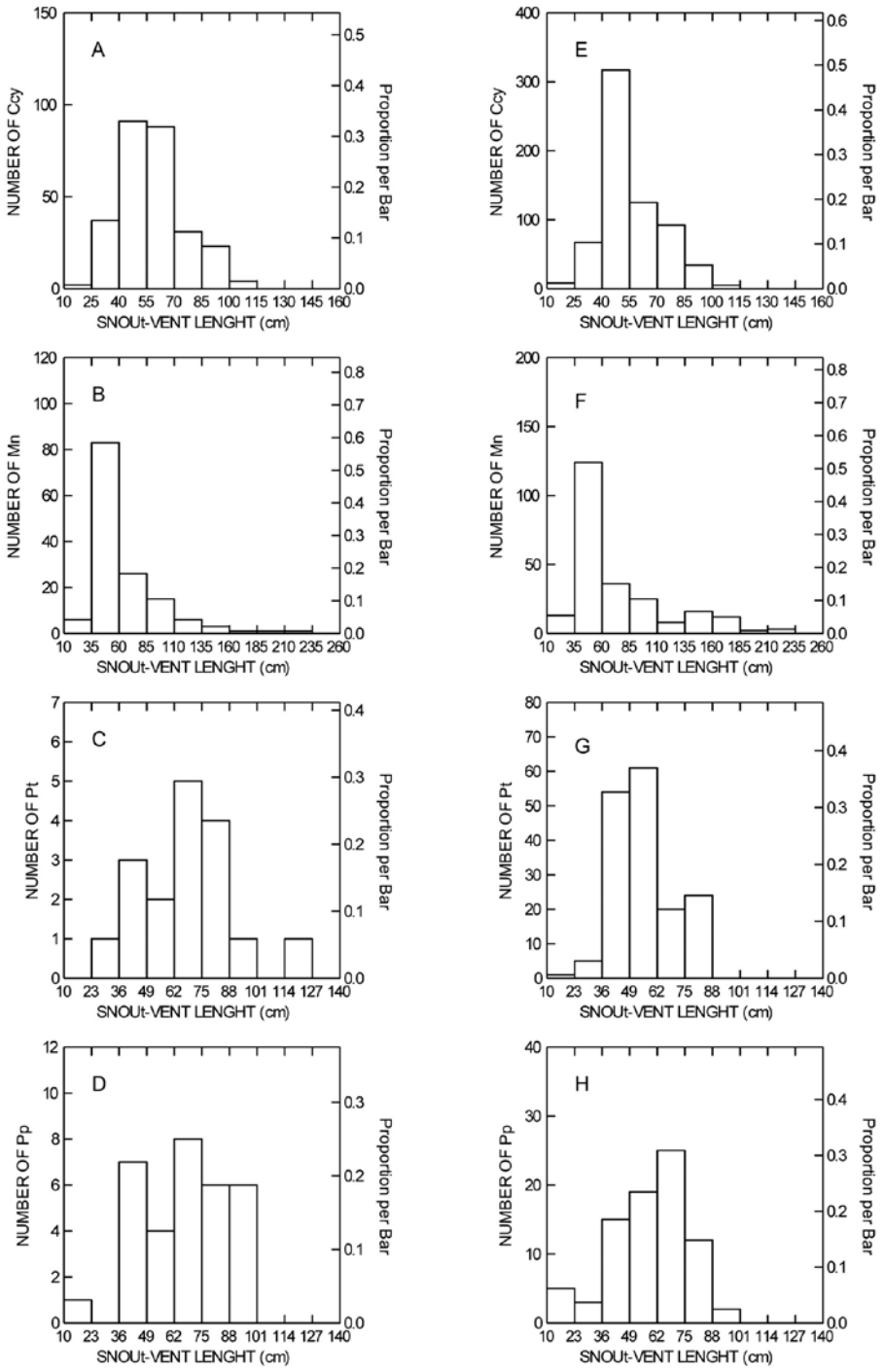

Figure 3. Size structure of caimans before (A, B, C, D) and after filling (E,F, G H) of the Santo Antônio reservoir. Ccy = *C. crocodilus yacare*, Mn = *M. niger*, Pt = *P. trigonatus*, Pp = *P. palpebrosus*.

of the correlation was less for *C. crocodilus* (r = 0.75, P = 0.001), indicating that the reservoir had a greater effect on the distribution of this species. Even so, after filling it was found in most areas that it had occupied before and the change was probably mainly due to the expansion of its distribution (Fig. 4). There was little relationship between the densities of *P. palpebrosus* (Fig. 5) in segments before and after dam filling (r = 0.31, P = 0.185).

DISCUSSION

The number of caimans of all species seen in surveys after reservoir filling tended to decline, but for three of the four species the decrease was not greater than expected given the higher water levels. For those species, the numbers seen after dam filling were similar to those recorded during high-water surveys before the dam was completed. This is consistent with numerous studies that have shown that water level is the principle determinant of the number of caimans seen in boat surveys (e.g.

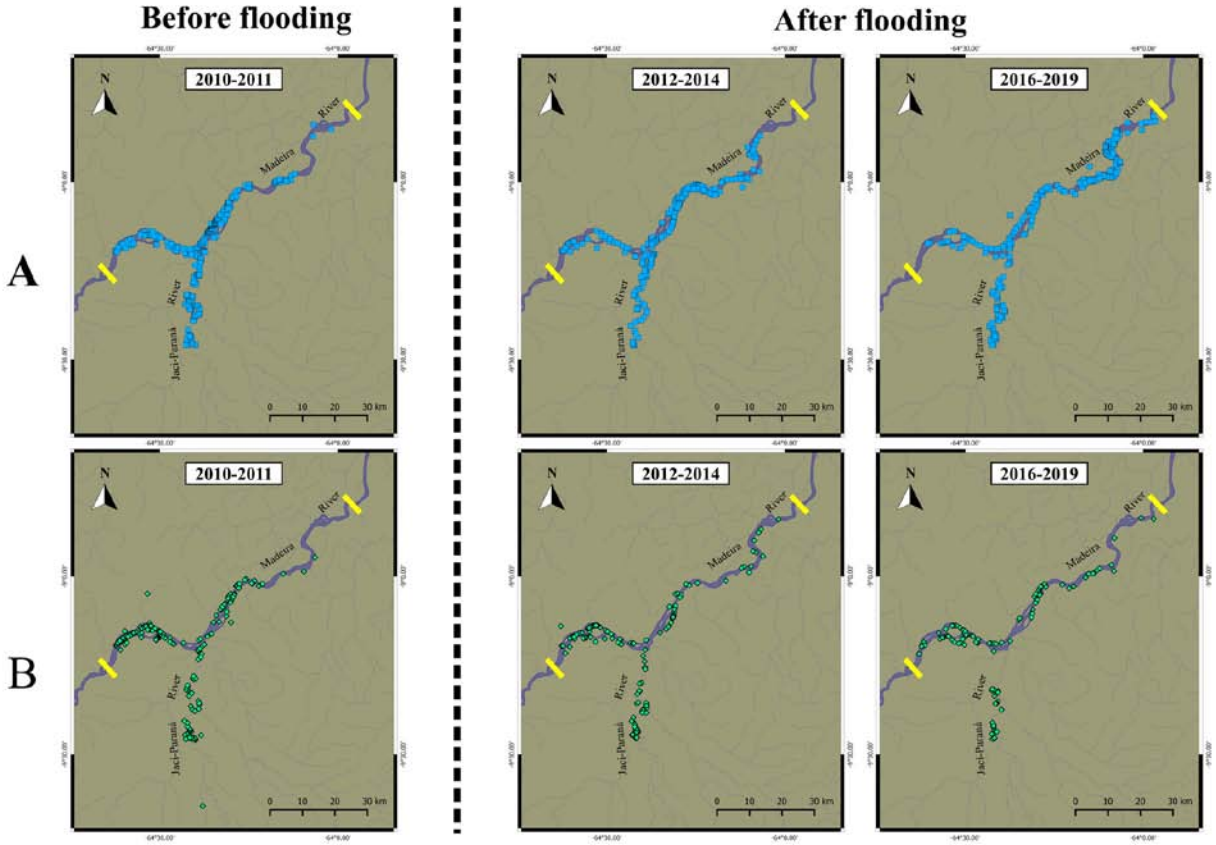

Figure 4. Spatial distribution of caiman before and after filling of the Santo Antônio reservoir. **A.** *C. crocodilus yacare*; **B.** *M. niger*. Data are for the pooled results of three pre-filling surveys (2010-2011), five early post-filling surveys (2012-2014) and four late post-filling surveys (2016-2019).

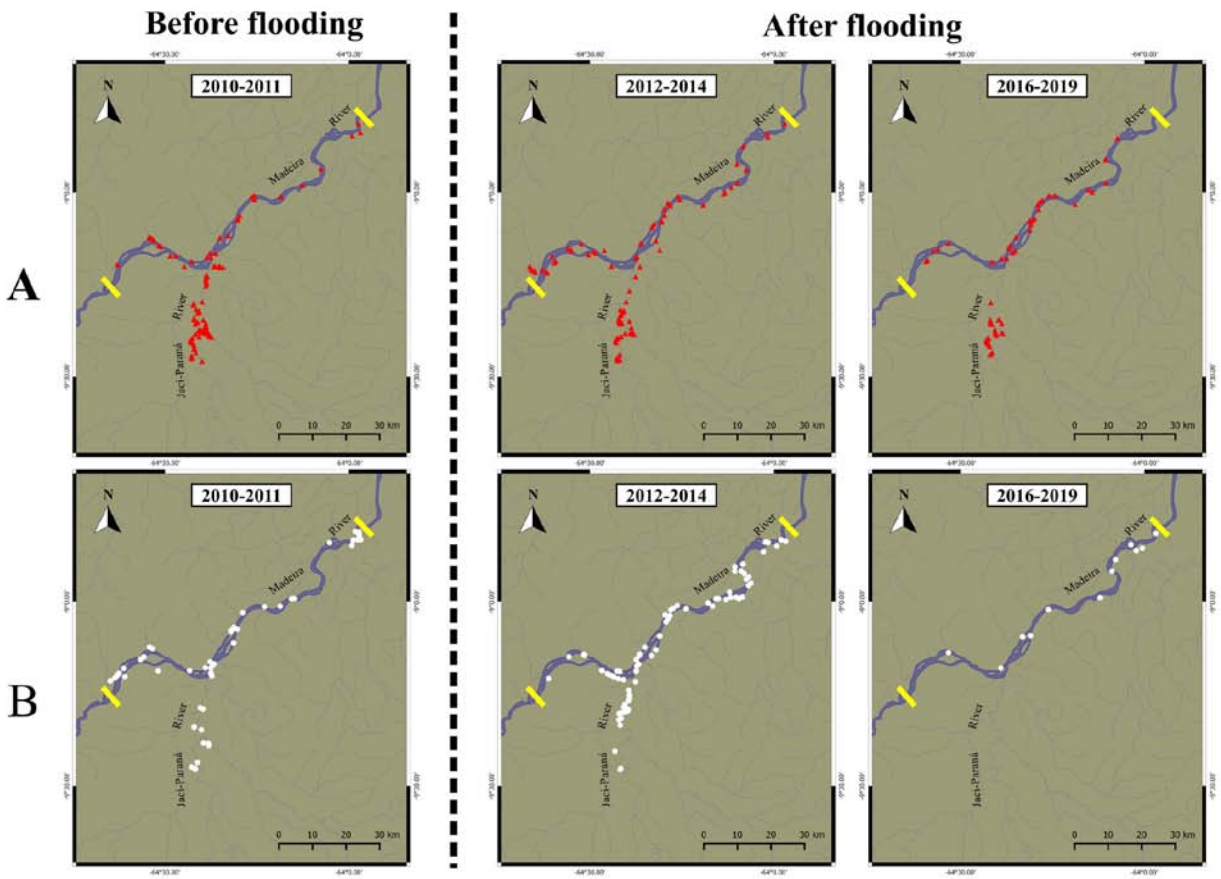

Figure 5. Spatial distribution of caimans before and after filling of the Santo Antonio reservoir. **A.** *P. trigonatus*; **B.** *P. palpebrosus*. Data are for the pooled results of three pre-filling surveys (2010-2011), five early post-filling surveys (2012-2014) and four late post-filling surveys (2016-2019).

Da Silveira et al., 2008; Fujisaki et al., 2011). Although the number of *Paleosuchus palpebrosus* seen declined more than expected due to increased water levels, we are hesitant to ascribe any biological significance to this because most individuals of *P. palpebrosus* live in habitats that are not appropriate for boat surveys (Campos et al., 2010; Campos & Magnusson, 2016; Campos et al., 2017). Most *P. trigonatus* individuals live in small streams, often far from large rivers, and individuals found around large rivers are probably vagrants (Magnusson & Lima, 1991). Although we did not register a decline for this species, the number of individuals near the banks of large rivers may not be a useful index of the abundance of the species in the region. In contrast, the margins of large water bodies are considered to constitute the principle habitat of *Melanosuchus niger* and *Caiman crocodilus*, so boat surveys probably provide reasonable indices of abundance for these species.

Hunting and other human activities affect the size structure of crocodilian populations (Mourão et al., 1996), so the distribution of sizes may be a more sensitive index of perturbation than attempts to estimate densities. For the reasons given above, we do not believe that boat surveys are appropriate to estimate densities of either species of *Paleosuchus*, and the reduction in the proportion of large individuals of these species seen may just be related to breeding adults moving further into the forest. However, the consistent finding of small individuals four to seven years after dam filling indicates that recruitment, and hence breeding, is occurring in the area.

The proportion of large individuals of *C. crocodilus* diminished after dam filling, with a concomitant increase in individuals of intermediate sizes. However, large individuals continued to be seen and the difference may simply have been a result of increased breeding after dam filling. *Caiman crocodilus* is the most widespread species of caiman and occupies a wide range of habitats (Velasco et al., 2010) including those that have been highly modified by humans. Studies should be continued, but there is presently little evidence that the change in size structure will negatively affect the species in the area in the long term.

The proportion of large individuals of *M. niger* increased after dam filling, with no concomitant reduction in the number of small individuals seven years after dam filling. It is possible that the increase in number of large individuals seen is a result of negative effects forcing the larger individuals into more exposed conditions, but it seems more likely that the dam increased the favorability of the river for this species, which is known to occur principally in lentic conditions (Marioni et al., 2013), with the change in size distribution indicating successful breeding and high survival of larger individuals.

The distributions of all species of caimans were originally higher in the upstream reaches away from the rapids where the dam wall was built. None of the species normally occur at high densities in highly turbulent water (Medem, 1981; Magnusson & Campos, 2010). This situation continued for some species after reservoir filling and the relative densities of *P. trigonatus* and *M. niger* in segments along the river were similar to those before dam construction. The relative densities of *C. crocodilus* in different segments of river were less correlated with the densities before dam construction, but the pattern was similar and differences apparently arose from expansion rather than contraction of areas with higher relative densities. We attribute no biological significance to the lack of consistency of pre- and post-filling densities of *P. palpebrosus* because the major habitat of this species cannot be surveyed by boat.

Dam construction may cause hardship to some species (Sá-Oliveira et al, 2015; Campos, 2015). However, construction of the Santo Antônio dam does not appear to have eliminated any of the crocodilian species or reduced them to such low numbers that they are in imminent danger of extinction. However, there are still forested areas abutting much of the reservoir and increased deforestation rates associated with dam infrastructure could change that situation in the future. It is unfortunate that environmental legislation in Brazil only requires monitoring of the effects of dams for a few years after construction. There are many hydro-electric dams that were constructed in the last century that could be used to evaluate the long-term effects of dams on crocodilians if the funds were available to resurvey them.

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Author Contributions

ZC and FM collected the data; FM prepared maps; GM and WM made the statistical analyses; ZC and WM wrote the manuscript text.

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REFERENCES

Agostinho, A.A., Thomaz, S.M. & Gomes, L.C. (2005). Conservação da biodiversidade em águas continentais do Brasil. *Megadiversidade* 1, 70–78.

Benchimol, M. & Peres, C.A. (2015). Predicting local extinctions of Amazonian vertebrates in forest islands created by a mega dam. *Biological Conservation* 187, 61–72.

Botha, H., Van Hoven, W. & Jr Guilhette, L.J. (2011). The decline of the Nile Crocodile population in Loskop Dam, Olifantes Rivers, South Africa. *Water AS* 37, 103–108.

Campos, Z., Llober, A.Q., Pina, C.I. & Magnusson, W.E. (2010). Yacare caiman *Caiman yacare*. In Manolis SC, Stevenson C,

editors. Crocodiles. Status Survey and Conservation Action Plan. Third Edition. Crocodile Specialist Group: Darwin, Australia. 23–-28.

Campos, Z. (2015). Size of caimans killed by humans at a hidyroelectric dam in the Madeira River, Brazilian Amazon. *Herpetozoa* 28, 101–104.

Campos, Z. & Magnusson, W.E. (2016). Density and biomass estimates by removal for an Amazonian crocodilian, *Paleosuchus palpebrosus*. *PloS ONE* 11, e0156406.

Campos, Z., Mourão, G. & Magnusson, W. E. (2017). The effect of dam construction on the movement of dwarf caimans, *Paleosuchus trigonatus* and *Paleosuchus palpebrosus*, in Brazilian Amazonia. *Plos one* 12 (11), e0188508.

Campos, Z. (2019). Disruption of reproductive behavior of black caiman, *Melanosuchus niger* in the Santo Antônio hydroeletric dam, Madeira River, Brazilian Amazon. *The Herpetological Bulletin* 148, 26–18.

Da Silveira, R., Magnusson, W.E. & Thorbjarnarson, J.B. (2008). Factors affecting the number of caimans seen during spotlight surveys in the Mamirauá Reserve, Brazilian Amazonia. *Copeia* 2008, 425–430.

Farias, I.P., Marioni, B., Verdade, L.M., Bassetti, L., Coutinho, M.E, Mendonça, S. H.S.T., Vieira, T.Q., Magnusson, W.E. & Campos, Z. (2013a). Avaliação do risco de extinção do jacaré-do-pantanal *Caiman yacare* (Daudin, 1802) no Brasil. *Biodiversidade Brasileira* 3, 21–30.

Farias, I.P., Marioni, B., Verdade, L.M., Bassetti. L., Coutinho, M.E., Mendonça, S.H.S.T., Vieira, T.Q., Magnusson, W.E. & Campos, Z. (2013b). Avaliação do risco de extinção do jacaré-tinga *Caiman crocodilus* (Linnaeus, 1758) no Brasil. *Biodiversidade Brasileira* 3, 4–12.

Finer, M. & Jenkins, C.N. (2012). Proliferation of hydroelectric dams in the Andean Amazon and implications for Andes-Amazon connectivity. *PloS ONE* 7, e35126.

Fujisaki, I., Mazzotti, F.J., Dorazio, R.M., Rice, K.G., Cherkiss, M. & Jeffery, B. (2011). Estimating trends in alligator populations from nightlight survey data. *Wetlands* 31, 147–155.

Lees, A.C., Peres, C.A, Fearnside, P.M., Schneider, M. & Zuanon, J.A. (2016). Hydropower and the future of Amazonian biodiversity. *Biodiversity and Conservation* 25, 451–466.

Magnusson, W.E. & Lima, A. (1991). The ecology of a cryptic predator, *Paleosuchus trigonatus*, in a tropical rainforest. *Journal of Herpetology* 25, 41–48.

Magnusson, W. & Campos, Z. (2010). Cuvier`s Smooth-fronted Caiman *Paleosuchus palpebrosus*. In Manolis S.C., Stevenson C., editors. Crocodiles. Status Survey and Conservation Action Plan. Third Edition, Crocodile Specialist Group: Darwin, Australia; 40–42.

Marioni, B., Farias, I.P., Verdade, L.M., Bassetti. L., Coutinho, M.E., Mendonça, S. H.S.T., Vieira, T.Q., Magnusson, W.E. & Campos, Z. (2013). Avaliação do risco de extinção do jacaré-açu *Melanosuchus niger* (Spix, 1825) no Brasil. *Biodiversidade Brasileira* 3, 31–39.

Medem, F. (1981). Los Crocodylia de Sur America Vol. 1. Los Crocodylia de Colombia. Colciencias, Bogota. Ed. Carrera 7ª Ltda. Colombia.

Mérona, B., Vigouroux, R. & Tejeriba-Garr, F.I. (2005). Alteration of fish diversity downstream from petit-Saut dam in French Guiana. Implication of ecological strategies of fish species. *Hydrobiology* 551, 33–47.

Mourão, G. & Campos, Z. (1995). Survey of broad-snouted caiman *Caiman latirostris*, marsh deer *Blastocerus dichotomus* and capybara *Hydrochaeris hydrochaeris* in the area to be inundated by Porto Primavera dam, Brazil. *Biological Conservation* 73, 27–31.

Mourão, G., Campos, Z., Coutinho, M. & Abercrombie, C. (1996). Size structure of illegally harvested and surviving caiman *Caiman crocodilus* yacare in Pantanal, Brazil. *Biological Conservation* 75, 261–265.

Sá-Oliveira, J.C., Hawes, J.E., Isaac-Nahum, V.J. & Peres, C.A. (2015). Upstream and downstream responses of fish assemblages to an eastern Amazonian hydroelectric dam. *Freshwater Biology* 60, 2037–2050.

Velasco, A., Ayarzagüena, J., Manolis, S. & Stevenson, C. (2010). Spectacled caiman *Caiman crocodilus*. *Crocodiles, Status Survey and Conservation Action Plan* 10–15.

Zeilhofer, P. & Moura, R.M. (2009). Hydrological changes in the northern Pantanal caused by the Manso dam: impact analyses and suggestions for mitigation. *Ecological Engineering* 35, 105-117.

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SHORT NOTE

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New insights about ovarian pigmentation in Anura

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Amphibians have pigmented cells in organs beyond just skin. Their functions involve free radical neutralisation, early innate response, and a relationship with environmental temperature and UV light. In gonads, pigment containing-cells seem to be restricted mainly to the testes and related to sperm production. However, we report for the first time ovarian melanisation in *Pseudis minuta* and its ontogenetic changes in larval and postmetamorphic stages. Melanin containing-cells on the ovarian surface initially appear at early premetamorphic stages whereas in the cortex they occur later. In consecutive stages, melanin containing-cells were more evident among oocytes but without a clear pattern, being located randomly within the germinal epithelium or in the stroma. Although their function is unclear, a relationship with the fast acquisition of sexual maturity must be further explored.

Keywords: Anuran, germ cells, melanin, ovary, *Pseudis minuta*

Melanic pigment cells in internal organs have intrigued researchers for decades. In amphibians and fishes, pigment cells containing melanin occur in various tissues and organs such as skin, but also in liver, spleen, kidney, peritoneum, lung, heart, blood vessels, thymus, gonads, and meninges (Agius & Agbede, 1984; Zuasti et al., 1998; Gallone et al., 2002; Zieri et al., 2007; Franco-Belussi et al., 2011, 2012). All, but those in the skin, constitute the so-called extracutaneous pigmentary system (Breathnach, 1988).

The functions of the extracutaneous pigmentary system involve cytoprotection against free radicals and oxidative stress (Zuasti et al., 1998; McGraw, 2005), detoxification from pollutants (Fenoglio et al., 2005), and protection against bacteria (Franco-Belussi et al., 2013). Visceral melanin pigmentation in anurans seems to be related to other environmental factors too, since the amount of melanocytes responds to changes in temperature and UV light exposure (Franco-Belussi et al., 2016). However, coloration in all organs seems to be also correlated with the phylogeny (Franco-Belussi et al., 2009; Provete et al., 2012), but also responds differently to climatic variables depending on the lineage and locality in which species occur (Franco-Belussi et al., 2017).

Delmore et al. (2018), in a study of bat testicular pigmentation, called the melanin containing-cells in the tissues surrounding the gonads 'reproductive melanin', and the state of possessing this trait 'reproductive melanisation'. They suggested an association with sperm production or protection. In anurans, as in most tetrapods, reproductive melanisation seems to be restricted mainly to the testes (e.g., Guillette et al., 1983; Faivovich, 2002; De Oliveira et al., 2002; De Oliveira et al., 2003; De Oliveira & Zieri, 2005; Zieri et al., 2007; Franco-Belussi et al., 2009; Provete et al., 2012; Goldberg et al., 2020). When present in adults, testicular pigmentation begin to accumulate during the differentiation of the testes and it seems to be correlated with germinal cell differentiation (Goldberg et al., 2020). In those species with a fast rate of testicular differentiation, melanisation began at very early premetamorphic stages and increased at a continuous rate up to the juvenile period, when it acquired its maximum density (Goldberg et al., 2020). However, there is a mention of ovarian pigmentation in the ovarian surface of some specimens of *Dendropsophus labialis* (Pinto-Erazo et al., 2016), and a "black pigment" has been described inside degenerative germinal cells in adult anuran ovaries (Ogielska et al., 2010).

The comparative ontogeny of gonadal pigmentation is largely unstudied (but see Guillette et al., 1983; Goldberg et al., 2020). This is even more notable in ovaries. This lack of knowledge might be due to the paucity of studies that have focused on describing the ontogeny of melanin pigmentation. As a part of an ongoing research project describing and comparing gonadogenesis in different anuran species, we observed that ovaries of the lesser swimming frog *Pseudis minuta* presented a degree of melanisation during their development.

Pseudis minuta (Hylidae) is an aquatic species that inhabits temporary ponds in north-eastern Argentina, Uruguay and extreme southern Brazil (Frost, 2020). Highly pigmented testes that begin to accumulate melanocytes at premetamorphic stages have been described (Goldberg et al., 2020). Together with other species of the genus (*P. paradoxa* and *P. platensis*), *P. minuta* exhibits a highly accelerated rate of germ cell development and it has been hypothesised that the juvenile stage is included within their prolonged delayed

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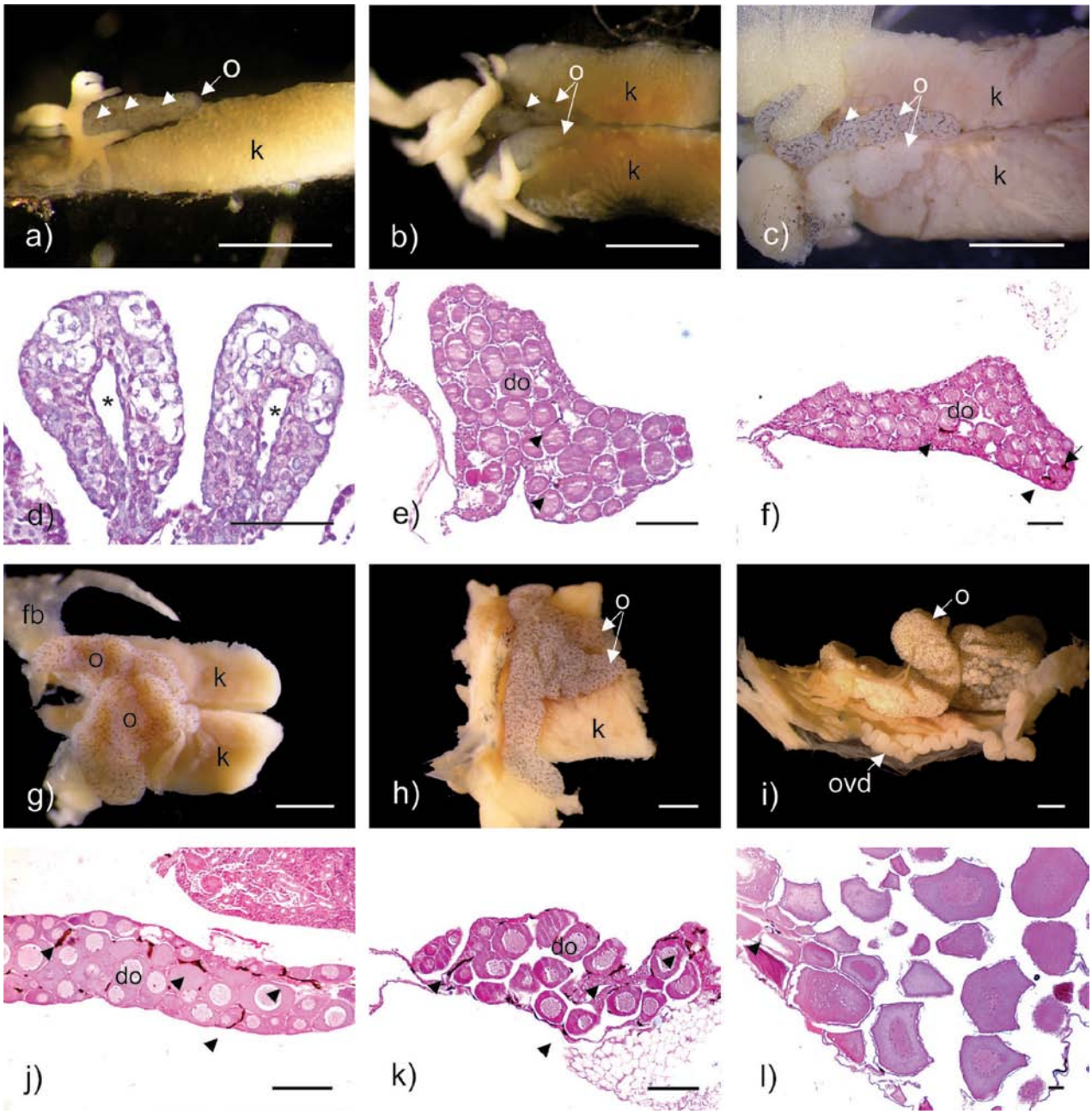

Figure 1. Ovarian differentiation in *P. minuta*. a, d. Stage 29 (-Stage I). (a) external morphology with pigmented cells on the ovarian surface (white arrowheads); (d) differentiated ovary with a distinct central lumen (asterisk), and primordial germ cells in mitotic division placed in the cortex. b, e. Stage 32 (-Stage II). (b) external lobulation evident; (d) the ovarian cavity enlarged with the cortex formed by several layers of oocytes. A few melanocytes are evident among oocytes (black arrows). c, f. Stage 36 (-Stage III). Ovaries were externally pigmented (white arrowheads) whereas, histologically (f), few scattered melanocytes (black arrows) were located between the oocytes. g, j. Stage 42 (-Stage V). Ovarian size increase was attributable to an increase in the number and size of diplotene oocytes. More melanocytes can be distinguished among the oocytes. h, k. Stage 45 (-Stage X). By the end of metamorphosis external pigmentation increased. Melanocytes appeared located without a clear pattern. i, l. One-year-old postmetamorphic specimen. Externally, both ovaries appear highly pigmented whereas a few melanocytes can be found between maturing oocytes. do = diplotene oocytes. fb = fat bodies. k = kidney. ovd = oviduct. Scale bar equal to 1 mm in a, b, c, g, h, i; 50 µm in d; and 100 µm in e, f, j, k, l.

metamorphosis (Downie et al., 2009; Fabrezi et al., 2010; Goldberg et al., 2016). Sexual maturity is achieved in males as soon as the metamorphic period ends (Fabrezi et al., 2010; Goldberg et al., 2016).

Ontogenetic changes in ovarian pigmentation is a feature that has never been described before in any other anuran species. In this context, here we report for the first time the ontogenetic changes related to this feature in *P. minuta* and in pinpointing it, represent an important step in the comprehension of the ontogeny of gonadal pigmentation.

We studied larval (N = 29) and postmetamorphic (N = 8) specimens of *P. minuta*. Larval development was staged according to Gosner (1960). However, the approximate Fabrezi et al. (2009) Stage is also given for comparative purposes (~followed by the stage numbers

in Roman numerals). We used this table because it recognises, in *P. platensis*, several more metamorphic stages than those described in the commonly used table of Gosner (1960). Specimens were also classified as premetamorphic (up to Gosner stage 36; N = 9), prometamorphic (between stages 37 and 41; N = 10), metamorphic (between stages 42 and 45; N = 10), juvenile (without mature secondary sexual characters; N = 2), or adult (with mature gonads and secondary sexual characters; N = 5) following Etkin (1936) and Quinzio et al. (2015). Specimens were euthanised in an aqueous solution of chloretone, and fixed in 10 % formalin. Adults were then preserved in 70 % alcohol. All specimens were collected in temporary and semitemporary ponds near the interception of El Pescado River and Street 31 (35°1'19.81''S, 57°51'10.11''W), La Plata department, and in Punta Indio (35°35'39.3''S, 57°29'07.41''W), Punta Indio department, both in province of Buenos Aires, Argentina. Larval specimens, accessioned as lots, and adult specimens, accessioned with individual numbers, are deposited in the Herpetological Collection of the Instituto de Bio y Geociencias (IBIGEO)-CCT–CONICET Salta with the following catalogue numbers and collecting dates: IBIGEO-A 1581 (08/12/2011), 1582 (15/12/2011), 1578 (17/06/2006), 1593 (16/10/2014), 1594 (16/10/2014), 1997 (16/10/2014), 1998 (16/10/2014), and 1599 (22/10/2014). This research adheres to The British Herpetological Society's Ethical Policy and Guidelines (British Herpetological Society, 2017). Specimen collection permits were issued by the Secretaría deFauna y Flora, Gobierno de la Provincia de Buenos Aires, Argentina (Res. 319/10 and 42/11).

Data were obtained from the following sources: (i) Manual dissection of larval and postmetamorphic specimens to describe changes in gonads. (ii) Histological sections of ovaries. To do this, ovaries were separated from preserved specimens, dehydrated, embedded in paraffin, and sectioned at 6 µm. Sections were stained with hematoxylin and eosin. The age of postmetamorphic specimens were estimated by two independent observers by counting the number of lines of arrested growth (LAGs) in transverse section of phalangeal bones of toe IV, following Hemelaar (1986).

Early during premetamorphosis, at Gosner stage 29 (-Stage I), the first sign of ovarian differentiation is discernible with the incipient lobulation of the cords (Fig. 1a). Scattered pigmented cells appeared distributed on the midline surface of one or both ovaries (Fig. 1a). Histologically, a cortico-medullary structure is clearly, with a cortex composed of primordial germ cells and darkly stained somatic cells (Fig. 1d).

By stages 32-33 (-Stage II), the ovaries begin to show their distinctive morphology as they become divided into lobules (Fig. 1b). Melanocytes still occupy the midline of the ovarian surface (Fig. 1b). Histologically, the cortex is mainly composed of previtellogenetic primary oocytes (Fig. 1e). Each oocyte appear individualised and surrounded by proliferating prefollicular cells (Fig. 1e). A few melanocytes appear between the oocytes without a clear pattern (Fig. 1e).

By stage 36-37 (-Stage III), gonad size increases with an increasing number of germ cells (Fig. 1c, f). The degree of lobulation, is more defined than in previous stages. Pigmentation, when present, appears as scattered brownish cells giving the organ a faint surface coloration (Fig. 1c). In some cases, the pigmentation is asymmetrical, with one ovary more pigmented than the other (Fig. 1c). In light microscopy, each oocyte appears individualised and surrounded by proliferating prefollicular cells (Fig. 1f). By these stages, a few spots of melanocytes accumulation are also discernible in the spaces between oocytes (Fig. 1f).

At the beginning of metamorphosis (Gosner stage 42; -Stage V), the ovaries continue to grow in size (Fig. 1g). Melanocytes are located all around the ovaries surface (Fig. 1g). Oocytes increase in size and present a highly basophilic cytoplasm and a larger nucleus with numerous nucleoli (Fig. 1j). Melanin containing-cells are more evident than in previous stages among oocytes but without a clear pattern, being located randomly within the germinal epithelium or in the stroma (Fig. 1j).

By the end of metamorphosis (Gosner stages 44-45; -Stages IX-X), lobules appear much wider than in previous stages (Fig. 1h). The presence of several pigmented cells gives a darker, like dotted brown, colour to the structure (Fig. 1h). Histologically, pigmentation remains as dark accumulations of melanocytes unevenly distributed around among oocytes (Fig. 1k). No pigmentation is observed in the oocytes in this phase. The external appearance of scattered melanocytes remains in juvenile and adult ovaries (Fig. 1i). Individual lobules contained developing oocytes of variable size in stages I to IV (sensu Dumont, 1972), encircled by follicular cells and a theca (internal and external) (Fig. 1l).

Ogielska & Kotusz (2004) distinguished three types of developmental rates of anuran ovaries and germ cells relative to somatic development: basic, retarded, and accelerated. In *P. minuta*, the ovarian cavity and diplotene oocytes are evident at premetamorphic stages; therefore the rate of ovarian differentiation of this species is accelerated. The same condition was described in *Euphlyctis cyanophlyctis* (Phuge & Gramapurohit, 2013), *Pseudis paradoxa* (Downie et al., 2009), *Scinax fuscovarius* (Goldberg, 2015), *Microhyla ornata* (Mali & Gramapurohit, 2015), *Dendropsophus labialis* (Pinto-Erazo et al., 2016) and in several ranid species (Ogielska & Kotusz, 2004; Gramapurohit et al., 2000). However, none of these species, with the exception of *D. labialis*, showed evidence of melanocytes on the surface or between oocytes.

Pigmented cells in anuran gonads have only been reported in testis of several species (De Oliveira et al., 2002, 2003; De Oliveira & Zieri, 2005; Zieri et al., 2007; Franco-Belussi et al., 2009), whereas in two bufonid species, *Rhinella diptycha* and *R. icterica*, pigmented cells were observed in the medullar region of the Bidder's organ (Farias, Carvalho-e-Silva & Brito-Gitirana, 2002; Silberschmidt Freitas et al., 2015). The only previous report in anuran ovaries only refer to those cases in *Dendropsophus labialis*, with no melanocytes among oocytes (Pinto-Erazo et al., 2016). In other vertebrates, ovarian pigmentation is also a rare trait and a review of the literature resulted in a single report in the Chinese silky fowl (*Gallus gallus domesticus* Brisson), which has

hyperpigmentation in several organs (Muroya et al., 2000).

In *P. minuta*, melanocytes begin to accumulate in testes and ovaries during premetamorphosis (Goldberg et al., 2020; this study). All prometamorphic specimens had pigmented testes whereas ovaries showed a variable pattern with both or one ovary pigmented. At metamorphic climax, the testes had acquired their final ovoid shape and all specimens had highly pigmented testes whereas ovaries, although less pigmented than testes, present a large number of melanocytes (Goldberg et al., 2020). However, not all species with highly pigmented testis presents pigmented ovaries as we have seen in different species such as *Physalaemus biligonigerus* and *Lysapsus limellum* (Goldberg pers. obs.). In fact, *Pseudis minuta*, and those cases in *D. labialis*, are the only anuran species with this characteristic described so far. Interestingly, both species present an accelerated rate of ovarian differentiation (Pinto-Erazo et al., 2016).

Many anuran species produce pigmented eggs during vitellogenesis (Altig & McDiarmid, 2007), including *P. minuta*. In these mature oocytes, the animal hemisphere becomes dark brown with the deposit of melanin and the vegetal hemisphere remains an opaque, light yellow (Uribe Aranzábal, 2011). In some anurans species, the presence of dark pigment occurs in oocytes during degeneration (Ogielska et al., 2010). Melanin present in ovarian tissue could have functions related to protection of tissue against free radicals or potentially toxic agents produced during the process of cell degeneration (Zuasti et al., 1998; McGraw, 2005). However, ovarian and oocyte pigmentation seem to be two independent events, being the former possibly stimulated by steroids and the latter by gonadotropins (Jørgensen, 1992; Uribe Aranzábal, 2011). It is well known that melanocytes are capable of transferring melanin granules to epidermal cells (Nordlund et al., 1989), and therefore the source of oocyte could in theory follow that process. However, oocyte pigmentation is widespread among anuran species, but ovarian melanocytes are rare, indicating a different process.

The description of the ovarian differentiation in *P. minuta* presented here allowed the recognition for the first time in this species of reproductive melanisation in anuran ovaries. It is interesting to note that, regardless of sex, melanocytes can accumulate in gonads in a process regulated by an endocrine pathway (Zieri et al., 2015). Even when its function is unclear, and a relationship with the fast acquisition of sexual maturity must be further explored, it is evident that the present report represents a useful starting point to study a trait that in the past might have been unnoticed.

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REFERENCES

Altig, R. & McDiarmid, R.W. (2007). Morphological diversity and evolution of egg and clutch structure in amphibians. *Herpetological Monographs* 21, 1–32.

Agius, C. & Agbede, S.A. (1984). An electron microscopical study on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. *Journal of Fish Biology* 24, 471–488.

Breathnach, A.S. (1988). Extra-cutaneous melanin. *Pigment Cell Research* 1, 234–237.

British Herpetological Society. (2017). Ethical Policy and Guidelines. *The Herpetological Bulletin* 141, 46–48.

De Oliveira, C., Santana, A.C., Omena, P.M., Santos, L.R.S. & Zieri, R. (2003). Morphological considerations on the seminiferous structures and testes of anuran amphibians: *Bufo crucifer*, *Physalaemus cuvieri* and *Scinax fuscovarius*. *Biociencias* 11, 39–46.

De Oliveira, C., Zanetoni, C. & Zieri, R. (2002). Morphological observations on the testes of *Physalaemus cuvieri* (Amphibia, Anura). *Revista Chilena de Anatomía* 20, 263–268.

De Oliveira, C. & Zieri, R. (2005). Pigmentação testicular em *Physalaemus nattereri* (Steindachner) (Amphibia, Anura) com observacões anatomicas sobre o sistema pigmentar extracutâneo. *Revista Brasileira de Zoologia* 22, 454–460.

Delmore, J.L., Brennan, P.L.R. & Orr, T.J. (2018). Reproductive melanization may protect sperm from harmful solar radiation. *Evolutionary Ecology* 32, 127–139.

Downie, J.R., Sams, K. & Walsh, P.T. (2009). The paradoxical frog *Pseudis paradoxa*: larval anatomical characteristics, including gonadal maturation. *Herpetological Journal* 19, 1–10.

Dumont, J.N. (1972). Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *Journal of Morphology* 136, 153–179.

Etkin, W. (1936). The phenomena of the anuran metamorphosis. III. The development of the thyroid gland. *Journal of Morphology* 59, 68–89.

Fabrezi, M., Quinzio, S.I. & Goldberg, J. (2009). Giant tadpole and delayed metamorphosis of *Pseudis platensis* Gallardo, 1961 (Anura, Hylidae). *Journal of Herpetology* 43, 228–243.

Fabrezi, M., Quinzio, S.I. & Goldberg, J. (2010). The ontogeny of *Pseudis platensis* (Anura, Hylidae): Heterochrony and the effects of larval development on postmetamorphic life. *Journal of Morphology* 271, 496–510.

Faivovich, J. (2002). A cladistic analysis of Scinax (Anura: Hylidae). *Cladistics* 18, 367–393.

Farias, C.F., Carvalho-e-Silva, S.P. & Brito-Gitirana, L. (2002). Bidder´s organ of *Bufo ictericus*: A light and electron microscopy analysis. *Micron* 33, 673–679.

Fenoglio, C., Boncompagni, E., Fasola, M., Gandini, C., Comizzoli, S., Milanesi, G. & Barni, S. (2005). Effects of environmental pollution on the liver parenchymal cells and Kupffer-melanomacrophagic cells of the frog *Rana esculenta*. *Ecotoxicology and Environmental Safety* 60, 259–268.

Franco-Belussi, L., Castrucci, A.M. de L. & De Oliveira, C. (2013). Responses of melanocytes and melanomacrophages of *Eupemphix nattereri* (Anura: Leiuperidae) to Nle4, D-Phe7-α-melanocyte stimulating hormone and lipopolysaccharides. *Zoology* 116, 316–324.

Franco-Belussi, L., De Souza Santos, L.R., Zieri, R. & De Oliveira, C. (2012). Visceral pigmentation in three species of the genus *Scinax* (Anura: Hylidae): Distinct Morphological Pattern. *Anatomical Record* 295, 298–306.

Franco-Belussi, L., Santos, L.R. de S., Zieri, R. & De Oliveira, C. (2011). Visceral pigmentation in four *Dendropsophus* species (Anura: Hylidae): Occurrence and comparison. *Zoologischer Anzeiger* 250, 102–110.

Franco-Belussi, L., Nilsson Sköld, H. & De Oliveira, C. (2016). Internal pigment cells respond to external UV radiation in frogs. *Journal of Experimental Biology* 219, 1378–1383.

Franco-Belussi, L., Provete D. & De Oliveira C. (2017). Environmental correlates of internal coloration in anurans vary throughout space and lineages. *Ecology and Evolution* 7, 9222–9233.

Franco-Belussi, L., Zieri, R., Santos, L.R. de S., Moresco, R.M. & De Oliveira, C. (2009). Pigmentation in anuran testes: Anatomical pattern and variation. *Anatomical Record* 292, 178–182.

Frost, D.R. (2020). Amphibian Species of the World: an Online Reference. Version 6.0 (Date of access: 23rd, January, 2020). Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA. doi.org/10.5531/db.vz.0001.

Gallone, A., Guida, G., Maida, I. & Cicero, R. (2002). Spleen and liver pigmented macrophages of *Rana esculenta L*. A new melanogenic system? *Pigment Cell Research* 15, 32–40.

Goldberg, J. (2015). Gonadal differentiation and development in the snouted treefrog, *Scinax fuscovarius* (Amphibia, Anura, Hylidae). *Journal of Herpetology* 49, 468–478.

Goldberg, J., Barrasso, D.A., Agostini, M.G. & Quinzio, S. (2016). Vocal sac development and accelerated sexual maturity in the lesser swimming frog, *Pseudis minuta* (Anura, Hylidae). *Zoology* 119, 489–499.

Goldberg, J., Valverde, B.S.L. & Franco-Belussi, L. (2020). Testicular melanization in anuran species: ontogeny and sexual maturity. *Amphibia-Reptilia* 41, 75–86.

Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183–190.

Gramapurohit, N.P., Shanbhag, B.A. & Saidapur, S.K. (2000). Pattern of gonadal sex differentiation, development, and onset of steroidogenesis in the frog, *Rana curtipes*. *General and Comparative Endocrinology* 119, 256–264.

Guillette, L.J. Jr., Weigel, J. & Flater, G. (1983). Unilateral testicular pigmentation in the Mexican lizard *Sceloporus variabilis*. *Copeia* 1, 155–161.

Hemelaar, A. (1986). Demographic study of *Bufo bufo L.* (Anura, Amphibia) from different climates, by means of skeletochronology (Ph.D. Thesis). University of Nijmegen, Nijmegen, Netherlands.

Jørgensen, C.B. (1992). Growth and reproduction. Pp. 439–466, in: Feder, M.E. & Burggren, W.W. (eds). Environmental Physiology of the Amphibians. USA: The University of Chicago Press.

Mali, P.V., Gramapurohit, N.P. (2015): Pattern of gonadal differentiation and development up to sexual maturity in the frogs, *Microhyla ornata* and *Hylarana malabarica*: a comparative study. *Journal of Experimental Zoology Part A* 323, 666–678.

McGraw, K.J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Animal Behaviour* 69, 757–764.

Muroya, S., Tanabe, R., Nakajima, I. & Chikuni, K. (2000). Molecular characteristics and site specific distribution of the pigment of the Silky Fowl. *Journal of Veterinary Medical Science* 62, 391–395.

Nordlund, J.J., Zalfa, M.D., Abdel-Malek, A., Boissy, R.E. & Rheins, L.A. (1989). Pigment Cell Biology: An Historical Review. *The Journal of Investigative Biology* 92, 53S–63S

Ogielska, M. & Kotusz, A. (2004). Pattern of ovary differentiation with reference to somatic development in anuran amphibians. *Journal of Morphology* 259, 41–54.

Ogielska, M., Rozenblut, B., Augustyńska, R., & Kotusz, A. (2010). Degeneration of germ line cells in amphibian ovary. *Acta Zoologica* 91, 319–327.

Phuge, S.K. & Gramapurohit, N.P. (2013). Gonadal sex differentiation, development up to sexual maturity and steroidogenesis in the skipper frog, *Euphlyctis cyanophlyctis*. *General and Comparative Endocrinology* 181, 65–71.

Pinto-Erazo, M.A., Goldberg, J. & Jerez, A. (2016). Gonadal development in the Neotropical high Andean frog *Dendropsophus labialis* (Amphibia: Hylidae). *Cuadernos de Herpetología* 30, 57–68.

Provete, D.B., Franco-Belussi, L., De Souza Santos, L.R., Zieri, R., Moresco, R.M., Martins, I.A., De Almeida, S.C. & De Oliveira, C. (2012). Phylogenetic signal and variation of visceral pigmentation in eight anuran families. *Zoologica Scripta* 41, 547–556.

Quinzio, S., Goldberg, J., Quinzio, S.I., Goldberg, J., Cruz, J.C., Pereyra, M.C. & Fabrezi, M. (2015). La morfología de los Anuros: pasado, presente y futuro de nuestras investigaciones. *Cuadernos de Herpetología* 29, 51–67.

Silberschmidt Freitas; J., Franco-Belussi, L. & de Oliveira, C. (2015). Morphological and histochemical studies of Bidder's organ in *Rhinella schneideri* (Amphibia: Anura) males. *Italian Journal of Zoology* 82, 479–488.

Uribe Aranzabal, M. C. (2011). Hormones and the female reproductive system of amphibians. Pp. 55–81, in: Norris, D.O. & Lopez, K.H. (eds.). Hormones and Reproduction of Vertebrates. Amphibians. Vol. 2. USA: Academic Press.

Zieri, R., Taboga, S.R. & De Oliveira, C. (2007). Melanocytes in the testes of *Eupemphix nattereri* (Anura, Leiuperidae): Histological, stereological, and ultrastructural aspects. *Anatomical Record* 290, 795–800.

Zuasti, A., Jiménez-Cervantes, C., García-Borrón, J.C. & Ferrer, C. (1998). The melanogenic system of Xenopus laevis. *Archives of Histology and Cytology* 61, 305–316.

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SHORT NOTE

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Taxonomic status of the Guyanese endemic caecilian *Caecilia pressula* Taylor, 1968 (Amphibia: Gymnophiona: Caeciliidae)

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The taxonomic status of the poorly known Neotropical caecilian species *Caecilia pressula* Taylor, 1968 is reconsidered based on examination of the type series. The single reported diagnostic feature, a laterally compressed body, that purportedly distinguishes *C. pressula* from *Caecilia tentaculata* Linnaeus, 1758 is not consistent across the seven specimens that constitute the type series and the only reported specimens, is variable in the Holotype depending on how it is held, and is considered to be artefactual. *Caecilia pressula* is considered to be a junior synonym of *Caecilia tentaculata*. Dentitional features of the smallest and presumed youngest specimens in the type series provide evidence that *C. tentaculata* practices maternal dermatophagy.

Keywords: Guyana, neotropics, reproduction, systematics

Compared to many other herpetological taxa, caecilian taxonomy can be challenging. Lack of limbs, and of well-developed eyes and tails limits the number of taxonomic characters, and the relative rarity of many caecilian species in scientific collections limits understanding of variation. Consequently, species limits are sometimes poorly understood. Taxonomic uncertainty is a major reason given for the data deficient conservation status of many caecilian species in the IUCN red list.

The caecilian species *Caecilia pressula* Taylor, 1968 is one of 13 species of the Neotropical *Caecilia* Linnaeus, 1758 described by Taylor (1968) in his revision of caecilian taxonomy. The description of *C. pressula* was based on seven specimens in the collections of the American Museum of Natural History (AMNH 49470-49076) that were collected from the Marudi Mountains of Guyana by R. Snedigar in 1938 during the Terry-Holden Expedition (https://siarchives.si.edu/collections/auth_exp_fbr_eace0098). A single adult male, AMNH A-49475, was designated the holotype and six much smaller and no doubt much younger specimens were designated as paratypes. These specimens were previously considered by Dunn (1942) and Parker & Dunn (1964) to be specimens of the type species of its genus, *Caecilia tentaculata* Linnaeus, 1758, which has a broad distribution in the Guianas and Amazon basin. Coloma et al. (2004) listed Venezuela, Surinam, French Guiana, Brazil, Peru, Ecuador and Colombia as countries of occurrence noting that, despite an absence of records, it presumably also occurs in Guyana. Cole et al. (2013) confirmed this presumption.

Taylor (1968:431) commenced his diagnosis of *Caecilia pressula* thus: "A species somewhat resembling *Caecilia tentaculata*, but with the body strongly compressed for most of its length (width 12.5, height 17.2, reaching a known length of 437 mm)." Although several other features are also mentioned in Taylor's diagnosis, including eye visibility, tentacle position, annulation pattern and squamation, none of these serve to further distinguish *C. pressula* from Linnaeus's long-standing species *C. tentaculata*, and I had long held doubts about the separation of these two species and hence the reality and taxonomic status of the former. Other than the description of its scales (Taylor, 1972), and its inclusion in faunal lists (as a Guyanese endemic, Cole et al., 2013), taxonomic summaries (e.g. Wilkinson & Nussbaum, 2006; Wilkinson et al., 2011), and conservation assessments (as data deficient, Reynolds et al., 2004) there have been no additional reports of *C. pressula* in the literature and no additional specimens have been newly collected or identified in historical collections.

I recently examined the type series of *Caecilia pressula* at the AMNH (Fig. 1). The holotype, in my considered opinion, is a specimen of *C. tentaculata*. In terms of features that are most often relied upon for caecilian taxonomy (including colour, shape, size, positions of sensory organs, annulation, dentition, squamation) I find no compelling evidence to support the suggestion that AMNH 49475 is a member of a taxon that is distinct from *C. tentaculata*. Counts of meristic features (annulation, teeth) all fall within the known ranges for *C. tentaculata* (e.g. Taylor, 1968; Maciel & Hoogmoed, 2011). While it is true that the type specimen of *C. pressula* is somewhat laterally compressed, the extent of this varies along the body and depends on how the specimen is held. Taylor (1968) reports midbody widths of 12.5 or 13 mm and a depth of 17.2 mm. My measures are similar (width 12.9, depth 17 mm) or not (width 16, depth 13) depending on how the specimen is held. In regions where the lateral

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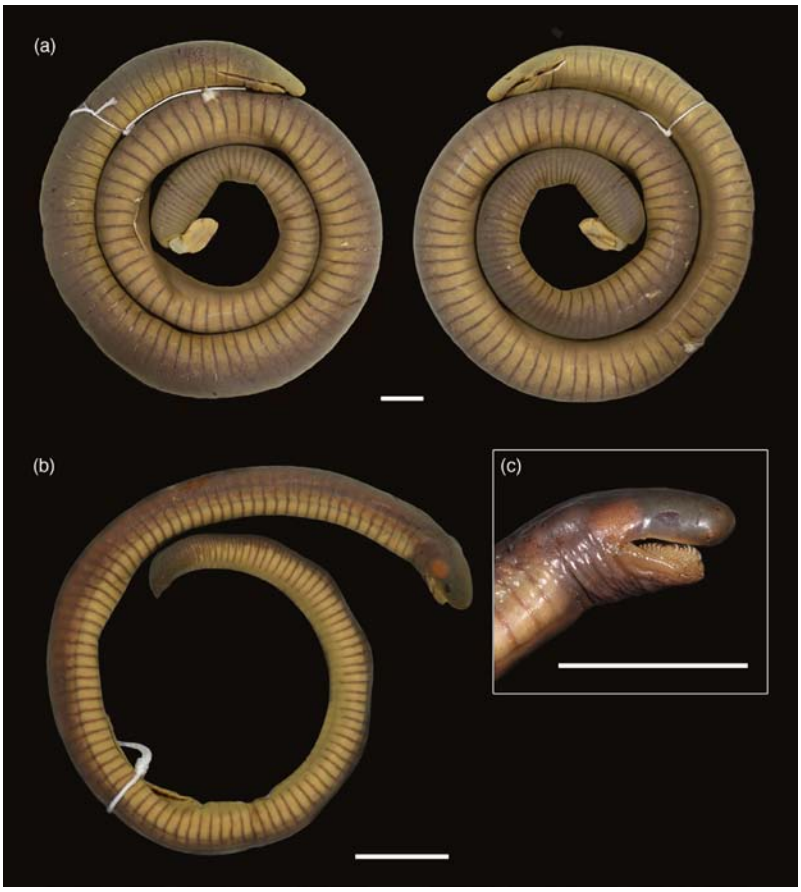

Figure 1. *Caecilia pressula* Taylor, 1968. (a) Holotype (AMNH A-19475), whole body. (b) 146 mm paratype (AMNH A-49471), whole body. (c) Close up of AMNH A-49471 showing vernal dentition. Scale bars are 10 mm.

body wall is less flaccid, anteriorly (width 11, depth 10 mm), at the level of the heart (width 14, depth 11 mm) and a little anterior to the vent (width 13.5, depth 9.8 mm), any compression is slight and dorsoventral rather than lateral. The midbody region of most specimens of *Caecilia* is slightly dorsoventrally compressed but lateral compression contingent upon how a specimen is held is not rare. The slightly flaccid body of the type specimen of *C. pressula* may result from inadequate filling of the coelom during initial fixation and it is certainly, in my opinion, no basis for inferring a different species. Lateral compression is not apparent in any of the paratype specimens of *C. pressula* which are all subcircular or slightly dorsoventrally compressed at midbody. In view of these observations and considerations, I place *Caecilia pressula* Taylor 1968 in the synonymy of *Caecilia tentaculata* Linnaeus, 1758.

The smallest specimens of the type series of *Caecilia pressula* (130 to 146 mm, total length) were of interest to Parker & Dunn (1964) because they have a non-adult dentition on their lower jaws (Fig. 1c). Their multiple rows of small spatulate teeth with tiny distal spicules are similar in crown form and arrangement to the teeth that had been reported in viviparous typhlonectid caecilian foetuses. Presumably based on Parker & Dunn's (1964) description, Wake (1977) listed "foetal" teeth as evidence of viviparity in *C. tentaculata*. Current understanding is that similar teeth can be found in the hatchlings of some maternal dermatophagous (skin feeding) oviparous caecilian species (e.g. Kupfer et al., 2006; Wilkinson et al., 2008). Thus such teeth, renamed "vernal" by San Mauro et al. (2014); provide no compelling evidence of viviparity but do provide evidence of the nutrition of young (hatchlings or foetuses) through hypertrophied and lipidified skin or oviduct epithelia. Thus, in contrast to Wake (1977), San Mauro et al. (2014) interpreted Parker & Dunn (1964) as providing evidence of maternal dermatophagy in *Caecilia tentaculata*, but both interpretations overlooked that the relevant specimens had been transferred to a different species. *Caecilia tentaculata* is a well-known species in the sense that there are many specimens in collections but despite the abundance of specimens, foetuses have never been found. Combined with this absence, the smallest specimens in the type series of *C. pressula* provide strong evidence that *C. tentaculata* is oviparous and practices maternal dermatophagy.

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REFERENCES

Cole, C.J., Townsend, C.R., Reynolds, R.P., MacCulloch, R.D. & Lathrop, A. (2013). Amphibians and reptiles of Guyana, South America: illustrated keys, annotated species accounts, and a biogeographic synopsis. *Proceedings of the Biological Society of Washington* 125, 317–578.

Coloma, L.A., Ron, S., La Marca, E., Hoogmoed, M., Castro, F., Lynch, J. & Wilkinson, M. (2004). *Caecilia tentaculata*. The IUCN Red List of Threatened Species 2004: e.T59530A11958955. Downloaded of 24 June 2020. https://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T59530A11958955.en.

Dunn, E.R. (1942). The American caecilians. *Bulletin of The Museum of Comparative Zoology, Harvard* 91, 339–540.

Kupfer, A., Müller, H., Antoniazzi, M.M., Jared, C., Greven, H., Nussbaum, R.A. & Wilkinson, M. (2006). Parental investment by skin feeding in a caecilian amphibian. *Nature* 440, 926–929.

Maciel, A.O. & Hoogmoed, M.S. (2011). Taxonomy and distribution of caecilian amphibians (Gymnophiona) of Brazilian Amazonia, with a key to their identification. *Zootaxa* 2984, 1–53.

Parker, H.W. & Dunn, E.R. (1964). Dentitional metamorphosis in the Amphibia. *Copeia* 1964, 75–86.

Reynolds, R., Hoogmoed, M., MacCulloch, R., Gaucher, P. & Wilkinson, M. (2004). *Caecilia pressula*. The IUCN Red List of Threatened Species 2004: e.T59526A11957102. Downloaded on 24 Jun 2020. https://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T59526A11957102.en.

San Mauro, D., Gower, D.J., Müller, H., Loader, S.P., Zardoya, R., Nussbaum, R.A. & Wilkinson, M. (2014). Life-history evolution and mitogenomic phylogeny of caecilian amphibians. *Molecular Phylogenetics and Evolution* 73, 177–189.

Taylor, E.H. (1968). *Caecilians of the World*. Lawrence: USA. 848 p.

Taylor, E.H. (1972). Squamation in caecilians, with an atlas of scales. *University of Kansas Science Bulletin* 49, 989–1164.

Wake, M.H. (1977). The reproductive biology of caecilians: an evolutionary perspective. In: *The Reproductive Biology of Amphibians*, 73–101. Taylor, D.H. & Guttman, S.I. (Eds.).

Wilkinson, M., Kupfer, A., Marques-Porto, R., Jeffkins, H., Antoniazzi, M.M. & Jared, C. (2008). One hundred million years of skin feeding? Extended parental care in a Neotropical caecilian (Amphibia: Gymnophiona). *Biology Letters* 4, 358–361.

Wilkinson, M. & Nussbaum, R.A. (2006). Caecilian phylogeny and classification. In: *Reproductive Biology and Phylogeny of Amphibia, Volume 3 Gymnophiona*, 53–92. J.-M. Exbrayat (Ed.), Enfield, USA.

Wilkinson, M., San Mauro, D., Sherratt, E. & Gower, D.J. (2011). A nine-family classification of caecilians (Amphibia: Gymnophiona). *Zootaxa* 2874, 41–64.

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Evidence of the peptide identity of the epidermal alarm cue in tadpoles of the toad *Rhinella arenarum*

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Chemical cues associated with predation attempts allow prey to trigger defensive behaviours. Accordingly, tadpoles of several species of anurans display strong behavioural responses to chemical cues of injured conspecifics. As part of the antipredator response, tadpoles show rapid and sustained inhibition of activity when exposed to chemical cues of predation. Although the ability to respond to cues of conspecifics has been confirmed in a wide variety of anuran species, studies about the tissue source and the chemical aspects of the molecules involved are scarce and contradictory. In the present work, we analysed the chemical characteristics, tissue source and release mechanism of the chemical alarm cue in *Rhinella arenarum* tadpoles. Our results support the hypothesis that a peptide of epidermal origin in mediates amphibian tadpole communication.

Keywords: Amphibia, Anura, tadpoles, anti-predator behaviours, conspecific cues

In anuran tadpoles, chemical cues released during predation can be detected by other tadpoles as a sign of potential predation risk. This information causes changes in the behaviour, morphology, and/or development of the animal receiver (Relyea, 2001; Laurila et al., 2002; Crossland et al., 2019). It has been reported that tadpoles of several species of anurans show a strong reduction in activity in response to chemical cues from injured conspecifics (Marquis et al., 2004; Fraker et al., 2009; Hettyey et al., 2015) or from the predator that had fed on conspecific tadpoles (Hettyey et al., 2015).

Although the ability to respond to cues from conspecifics with anti-predator behaviours has been confirmed in a wide variety of anuran species, little is known about the tissue source and chemical nature of the molecules involved. In *Lithobates sylvaticus* (*Rana sylvatica*), the tadpoles' skin cells produce a peptide alarm pheromone released through an active process of secretion after the predator attack (Fraker et al., 2009). Authors also made a biochemical characterisation of the alarm cue, confirming that it is composed of at least two small peptides (Fraker et al., 2009). On the other hand, *R. aurora* tadpoles increase ammonium secretions during predation attempt, eliciting anti-predator behaviours in conspecifics (Kiesceker et al., 1999). By contrast, in larvae of two frog species, *Lithobates pipiens* and *L. clamitans*, are thought to release a sulfated steroid as a component of the alarm cue (Austin et al., 2018), a result which supports the classic studies of Hrbáček (1950), who postulated that the alarm cue of bufonids is composed of steroids related to the bufotoxins (Hrbáček, 1950). Beyond these studies, there are no other records of the chemical characterisation of the cues that trigger alarm behaviours in tadpoles.

Given the lack of information regarding the tissue source and the chemical nature of conspecific cues in amphibian tadpoles, we sought to analyse these properties in *Rhinella arenarum*. Previously, we confirmed that these tadpoles responded to conspecific homogenates reducing the time they spent swimming (Raices, 2018), which allows us to use them in behavioural assays. In the present work, we provide evidence that these tadpoles use a peptide of epidermal origin similar to that proposed for *L. sylvaticus* (Fraker et al., 2009).

Growth and maintenance of tadpoles: *Rhinella arenarum* embryos were obtained by in vitro fertilisation, according to standard methods (Casco et al., 1992). Tadpoles were staged according to Gosner (1960) and maintained in conditions already standardised in our laboratory: five larvae per litre of dechlorinated tap water are maintained in a 12-hour light/dark cycle, at 22 ° C, and fed ad libitum with boiled chard (Distler et al., 2016). Every other day, food and waste residues were removed and water volume restored. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, Protocol #22/13, and the principles of NIH (publication 8523, revised 1985).

Behaviour essays: *Rhinella arenarum* tadpoles (stage G36-37) were exposed to control and different treatments in a circular glass aquarium (15 cm diameter) filled with 500 ml of dechlorinated tap water at 22 °C. After 15 minutes of acclimatisation, 500 µl of each stimulus (see below for details) was added homogeneously with

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a micropipette at a rate of 100 µl /sec. To avoid having the micropipette produce shadows that could disturb the tadpoles, we determined that the pipet needed to touch the surface of the water at a 45 ° angle. Five seconds after addition of the stimulus, tadpoles were recorded for 6 minutes. Videos were then analysed for the amount of time during which the tadpoles were active (Time spent moving). It was considered as "activity" every time the tadpoles flapped their tails, even if they relocated or not.

Tissue source of the alarm cue: For the preparation of stimulus, *R. arenarum* tadpoles (stage G36-37, n=8) were deeply anaesthetised by immersion in dechlorinated tap water at 0 °C and quickly decapitated to avoid suffering. Tadpoles were dissected into tails (composed by muscle and skin), skin (body skin), and carcass (composed by viscera and muscles). The tissues were then homogenised in 1:1 (weight:volume) of 1 g tissue / 1 ml of dechlorinated tap water. After homogenisation, water was added until a final concentration of 0.1 g/ml. The homogenate was centrifuged at 5000 x g for 5 min and the supernatant was collected (stock solution 1:1). Simultaneously, we prepared aliquots of dechlorinated tap water, without chemical cues, in identical 2 ml vials. We coded all vials to keep the experimenter blind to the treatment. All vials were frozen for no more than 2 weeks at -20 °C prior to use.

Evaluation of potential active release mechanisms: Previous results in *Lithobates clamitans* (*Rana clamitans*) tadpoles suggested that the chemical alarm cue might be released via a voltage coupled stimulus-secretion pathway (Fraker et al., 2009). To test if this paradigm applies to *Rhinella arenarum*, tadpoles were briefly exposed to 5 mM KCl to depolarise cell membranes and thereby activating the release of secretory vesicles via a calcium/potassium-dependent mechanism. Four sets of 5 tadpoles were sequentially immersed in 20 ml 5 mM KCl or 20 ml dechlorinated tap water (as a control group) for 15 min. and then removed (according to Fraker et al., 2009). This generated conditioned media (tadpoles-KCl and tadpoles-water, n=6 and 8, respectively) that were then used as stimuli for behavioural assays. Two additional controls included: 20 ml of 5 mM KCl (n=6) or dechlorinated tap water alone (without tadpoles, n=7) as stimuli for behavioural assays. Behavioural observations were made as described above.

Chemical identity of the alarm cue: To identify the chemical cues in the skin of the tadpole, we used only dissected tails which reduce manipulation time and facilitate the extraction protocol. Homogenates were made as described above and were subjected to different treatments prior to use in the behavioural trials: 1) heat treatment (n=4): the sample was incubated in a water bath at 100 °C for 10 min; 2) Chloroform extraction to remove lipid components (n=4): an equal volume of chloroform was added to the homogenate in a 10 ml conical polypropylene tube, vortexed vigorously, and centrifuged at 2000 x g for 10 min to separate and recover the aqueous phase from the organic phase; 3) Protein digestion (n=3): 400 µl of homogenate sample was incubated with 200 µl Proteinase K solution (Ready to use, Dako Cat. number: S3020) at 50 °C for 15 min. and then boiled for 10 min to inactivate the proteinase activity. A control with Proteinase K solution but without homogenate (n=4) was also included. See Supplementary Figure for protocol details.

Statistical analyses: Statistics were performed using the R software version 3.3.1 (R Development Core Team, 2014) applying a significance level of $\alpha = 0.05$. The results were visualised as median ± quartiles. The mean of "Total activity (min)" was analysed between each group. One-way Analysis Of Variance (ANOVA) was used, with the aov( ) function, to analyse the differences between each group. In every test, Shapiro-Wilk and Levene tests were used to check for normality and homoscedasticity assumptions, respectively. Even when the Levene test result did not show heteroscedasticity, we decided to use the argument "weights" within the function gls( ), and the function varExp( ) to specify an exponential function of the variance. The GLS models were fitted by restricted maximum likelihood (REML). For each experiment, differences in the time spent swimming depending on the stimulus applied were tested with Tukey's Honestly Significant Difference (HSD) tests by glht( ) function. R scripts and datasets are deposited at https://zenodo.org/record/3963025#.Xx9EyvhKhwp.

As proof of the existence of an alarm cue in *R. arenarum*, we found that tadpoles significantly reduced their time spent moving when they were exposed to body skin and tail homogenates compared with water (Fig. 1a: skin=1.77 ± 0.99 min, tail= 0.99 ± 0.52 min, water= 4.66 ± 0.26 min, $p<0.005$ in both cases against water). The responses triggered by cues from the body skin and the tail homogenates did not differ ($p=0.154$). On the other hand, tadpoles exposed to the carcass homogenate did not reduce the time spent moving compared to those exposed to water (carcass=4.67 ± 0.89 min vs. water= 4.66 ± 0.26 min, $p=0.999$). While the tail homogenate was mainly composed of skin and muscles, the latter tissue type can be excluded as a potential source of alarm cues, since the carcass also contained muscles but did not trigger anti-predator behaviours. In *Rana aurora* tadpoles, Kiesecker et al. (1999) proposed ammonia as an alarm cue. However, our results would rule out this possibility since urine levels should be highest in the carcass homogenate. All these results suggest that chemical alarm cues that trigger anti-predator behaviours are stored in the skin of *R. arenarum* tadpoles. Similar results have been observed in other species of *Anura* (Pfeiffer, 1966; Fraker et al., 2009). In tadpoles of the Bufonidae family, including *Rhinella arenarum* (Regueira et al., 2016), a type of epidermal cells called "giant cells" were associated with the production of chemical alarm cues (Pfeiffer, 1966). It is possible that the anti-predator responses observed here, in tadpoles exposed to skin preparations, are related to this epidermal cell type.

We next wanted to address whether the alarm cue from the tadpole is released from the skin by a stimulus-coupled secretion pathway. Therefore, were created conditioned media from tadpoles exposed to 5mM KCl and observed the behaviour of naïve *R.*

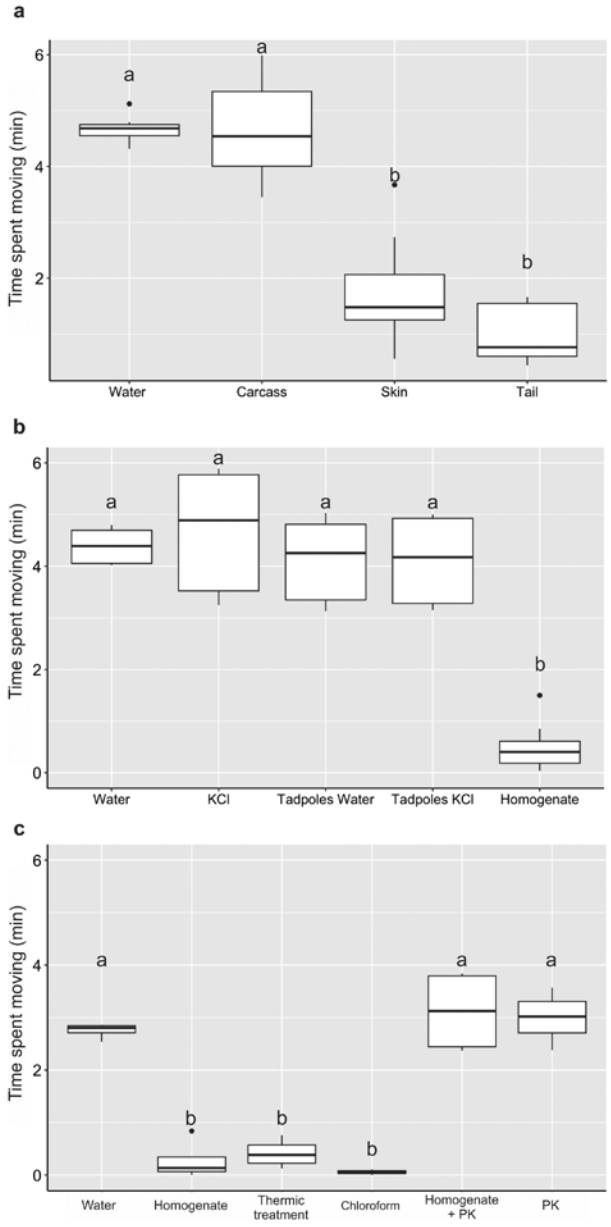

Figure 1. Boxplot representing the time spent moving (min) for larvae of *R. arenarum* exposed to different stimuli. For homogenate preparation and treatment, details see text.
a) The chemical cue of predation is derived from toad tadpole skin. Stimuli: Water (control), Carcass (viscera and tissue), Skin (body skin), Tail (skin and muscle) ($F=56.58$, $p<0.005$, $df=3$).
b) The alarm signal in *R. arenarum* is not released by an active mechanism that involves the opening of ion channels. Stimuli: Water (control), KCl (KCl 5mM), Tadpoles Water (water conditioned by tadpoles that had been immersed in tap water) or Tadpoles KCl (water conditioned by tadpoles that had been immersed in 5mM KCl) and Homogenate (tail homogenate) ($F=37.05$, $p<0.005$, $df=4$).
c) The epidermal chemical cue in *R. arenarum* is a thermostable, water-soluble and peptide-like molecule. Stimuli: Water (control), Homogenate (tail homogenate), Thermic treatment (homogenate preheat at 100 ˚C), Chloroform extraction (homogenate pre-treated with chloroform), Homogenate + PK (homogenate pre-treated with Proteinase K), PK (Proteinase K) ($F=41.09$, $p<0.005$, $df=5$). In a, b and c, thick horizontal lines and boxes represent the medians and interquartile ranges, respectively; whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; circles represent extreme data points. Different letters indicate significant differences with $p<0.05$.

arenarum tadpoles exposed to this conditioned water. Conditioned-water did not result in a reduction of time spent moving compared with water controls (Fig. 1b, tadpoles-KCl=4.11 ±0.9 min vs. water=4.39 ± 0.35 min, p=0.976). The conditioned-water obtained from controls (tadpole-water) did not trigger anti-predator behaviours (tadpoles-water= 4.13±0.79 min vs. water=4.39 ± 0.35 min, p=0.977). The total time spent moving was not influenced by the addition of 5 mM KCl either (KCl= 4.68 ±1.24 min vs. water=4.39 ± 0.35 min, p= 0.976), suggesting that the KCl itself does not affect the normal exploratory activity of the tadpoles. These results indicate that the alarm cue release does not seem to occur via a coupled stimulus-secretion pathway as opposed to what was observed by Fraker (2009) in *L. sylvaticus*, where the cue release occurs by an active mechanism that involves cell membrane depolarisation. Instead, the chemical alarm cue in *R. arenarum* seems to be released by a passive mechanism, surely involving tissue damage.

Since the controversy regarding the chemical nature of alarm cues in amphibian tadpoles, we investigated this feature in *R. arenarum* by treating the removing protein and lipid components of the homogenates (see above). The activity of tadpoles was not affected when they were exposed to homogenates that were heat-treated (100 ˚C) or chloroform extracted (untreated homogenate=0.28 ± 0.38 min vs thermic treatment=0.42 ± 0.28 min p=0.982, untreated homogenate vs chloroform extraction=0.06 ± 0.06 min, p=0.886). Therefore, we speculate that the alarm cue is a thermostable, non-lipid molecule. In contrast, the anti-predator response was significantly reduced when homogenates were pre-treated with Proteinase K (homogenate + PK = 3.12 ± 0.8 min, untreated homogenate = 0.28 ± 0.38 min, p <0.001) supporting the idea of a peptide identity. Since the alarm cue was thermostable, we favour that the cue is a small peptide, because large proteins with complex structures are usually susceptible to heat denaturation.

Information on the chemical nature of alarm cues is scarce and contradictory. Recent work on mass spectrometry has confirmed that an anion characterised by an m/z value of 501 is present in homogenates of larvae of the genus *Lithobates* the exact structure of the molecule is unknown, it was suggested that it is a sulfated steroid of approximately 26 carbon atoms (Austin et al., 2018). Those results coincide with the "steroidal" origin for the tadpoles' alarm cues postulated by Hrbáček (1950). However, other reports describe an anti-predator behaviour in tadpoles exposed to conspecific homogenates prepared in aqueous solutions (Marquis et al., 2004; Fraker et al., 2009; Hettyey et al., 2015; Crane et al., 2017). Additionally, our results in *R. arenarum* also argue against a lipid-based alarm cue because tadpoles exposed to homogenates previously subjected to chloroform extraction showed an anti-predator response as strong as that of tadpoles exposed to untreated homogenates, demonstrating that the alarm cue remained in the aqueous phase. Considering that volatility is a key feature of the molecules involved in chemical communication in the air, some authors have proposed that solubility plays a similar role in water. Supporting this assumption, it is very unlikely that the cues used for chemical communication in larvae of

anurans are large non-polar compounds such as steroids with low solubility in water. Based on this, other authors have postulated peptides as the main candidates in chemical communication in aquatic environments due to their high solubility in water (Wyatt, 2005) in agreement with our results.

In amphibians, there are a variety of bioactive peptides found in adult anuran skin cells, as well as in tadpoles. These peptides are stored in high concentrations and have various functions related to defence against parasites, immunity, and ectohormones (Giuliani et al., 2008). Fraker et al. (2009) characterised an alarm cue coming from the skin of larva*l L. clamitans* that consists of a mix of two components with different chemical properties, the combination of which triggers the anti-predator behavioural response. The components had a molecular weight less than 10 kDa and were not affected by thermal treatments or freezing, but were not extracted with chloroform. The LC-MS/MS analysis identified two small peptides as potential candidates for the alarm cues in *L. clamitans* (Fraker et al., 2009). In our case, preliminary chromatographic studies obtained from skin homogenates of *R. arenarum* tadpoles confirmed that it would be one or more peptides with a molecular weight close to 5 kDa (data not shown). In this regard, Crossland et al. (2019) identified in *Rhinella marina* tadpoles at least four compounds that caused strong avoidance responses and concluded that the alarm cue may involve a mixture of substances, but the identity of that chemical cue (or array of chemical cues) remains unknown.

In summary, the results in our work agree with the assumption that peptides are a solid candidate to participate in tadpole´s chemical communication, partially coinciding with Fraker et al.'s (2009) observations in tadpoles of other anurans. However, unlike what is proposed by Fraker et al. (2009), in *R. arenarum* the release of the alarm cues is not mediated by active secretion mechanisms but would be released by mechanical damage to the skin cells.

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REFERENCES

Austin, C.E., March, R.E., Stock, N.L. & Murray D.L. (2018). The origin and ecological function of an ion inducing anti-predator behavior in Lithobates Tadpoles. *Journal of Chemical Ecology* 44, 178–188.

Casco, V.H., Paz, D., Ruiz, G., Maldonado, C., Pisano, A., & Aoki, A. (1992). Differentiation of endocrine myocardiocytes in the developing heart of the toad (*Bufo arenarum* Hensel). *International Journal of Developmental Biology* 36(4), 537-542.

Crane, A.L., & Ferrari, M.C. (2017). Evidence for risk extrapolation in decision making by tadpoles. *Scientific Reports* 7, 43255.

Crossland, M.R., Salim, A.A., Capon, R.J. & Shine, R. (2019). The effects of conspecific alarm cues on larval cane toads (*Rhinella marina*). *Journal of Chemical Ecology* 45, 838–848.

Distler, M.J., Jungblut, L.D., Ceballos, N.R. & Pozzi, A.G. (2016). Overcrowding-mediated stress alters cell proliferation in key neuroendocrine areas during larval development in *Rhinella arenarum*. *Journal of Experimental Zoology A Ecology Genetic and Physiology* 325, 149–157.

Fraker, M.E., Hu, F., Cuddapah, V., Mc Collum S.A., Relyea R.A., Hempel, J. & Denver, R.J. (2009). Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. *Hormone & Behavior* 55, 520–529.

Giuliani, A., Pirri, G., Bozzi, A., Di Giulio, A., Aschi, M. & Rinaldi, A.C. (2008). Antimicrobial peptides: natural templates for synthetic membrane-active compounds. *Cellular and Molecular Life Sciences* 65, 2450–2460.

Gosner, K.L. (1960). A simplified table for staging anurans embryos and larvae with notes on identification. *Herpetology* 16, 183-190.

Hettyey, A., Tóth, Z., Thonhauser, K.E., Frommen, J.G., Penn, D.J. & Van Buskirk, J. (2015). The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles. *Oecologia* 179(3), 699-710.

Hrbáček, J. (1950). On the flight reaction of tadpoles of the common toad caused by chemical substances. *Experientia* 6, 100-102.

Kiesecker, J.M., Chivers, D.P., Marco, A., Quilchano, C., Anderson, M.T. & Blaustein, A.R. (1999). Identification of a disturbance signal in larval red-legged frogs, *Rana aurora*. *Animal Behaviour* 57(6), 1295-1300.

Laurila, A., Pakkasmaa, S., Crochet, P.A. & Merilä, J. (2002). Predator-induced plasticity in early life history and morphology in two anuran amphibians. *Oecologia* 132(4), 524-530.

Marquis, O., Saglio, P. & Neveu, A. (2004). Effects of predators and conspecific chemical cues on the swimming activity of *Rana temporaria* and *Bufo bufo* tadpoles. *Arch für Hydrobiologie* 160, 153-170.

Pfeiffer, W. (1966). Die Verbreitung der Schreckreaktion bei Kaulquappen und die Herkunft des Schreckstoffes. *Zeitschrift für vergleichende Physiologie* 52, 79–98.

Raices, M. (2018). Comunicación química en larvas de *Rhinella arenarum*. Caracterización del comportamiento antipredatorio y de las señales de alarma. Thesis. Universidad de Buenos Aires

Regueira, E., Dávila, C. & Hermida, G.N. (2016). Morphological changes in skin glands during development in *Rhinella arenarum* (Anura: Bufonidae). *Anatomical Records* 299, 141-156.

Relyea, R.A. (2001). The relationship between predation risk and antipredator responses in larval anurans. *Ecology* 82(2), 541-554.

Wyatt, T.D. (2005). Pheromones: Convergence and Contrasts in Insects and Vertebrates. In: *Chemical Signals in Vertebrates* 10. Springer, 7–19 pp.

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SHORT NOTE

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Contributions to *Lycodon zawi*, a little-known colubrid snake (Reptilia: Serpentes: Colubridae)

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Since the original description of *Lycodon zawi* almost two decades ago from Myanmar and Northeast India, little is known on the systematics, distributional range as well as the natural history of the species. Hence, this paper attempts to contribute updated information to enhance the genetic data, morphology, distributional records, and natural history on its feeding and the hitherto unknown breeding habit of this species from Mizoram State, Northeast India.

Keywords: Diet, eggs, morphology, phylogeny, Zaw's wolf snake

The colubrid snake *Lycodon zawi* Slowinski, Pawar, Win, Thin, Gyi, Oo & Tun, 2001 commonly known as Zaw's wolf snake was described from Myanmar and north-east India on the basis of 11 individuals (Slowinski et al., 2001). A combination of characters such as the presence of preocular scale, loreal scale not in contact with internasal, anal scale divided, poorly developed white cross-bands on a brownish-black dorsum, and without a well-developed band on the neck (nape) differentiate *L. zawi* (Fig. 1A) from its congeners (Slowinski et al., 2001). It is a nocturnal, oviparous species known to feed mainly on skinks and geckos (Slowinski et al., 2001; Whitaker & Captain, 2004). The known distributional records in India include the States of Assam (Garbhanga Reserved Forest and Tinkopani Reserve Forest), Mizoram (Ngengpui Wildlife Sanctuary, Keifang and Pachhunga University College Campus), Meghalaya (Nongkhyllem Wildlife Sanctuary, Balpakram Tiger Reserve) (Slowinski et al., 2001; Dutta et al., 2013), Tripura (Vanghmun, Jampui hills) (Majumder, 2018), and West Bengal (Ghosh et al., 2017). Outside India, it has been recorded from northern Myanmar (Sagaing Division and Rakhine State) (Slowinski et al., 2001) and Bangladesh (Lawachara National Park) (Reza, 2010). It is currently listed as Least Concern in IUCN Red List of threatened species (Wogan & Vogel, 2012). Given the paucity of data on the morphology, genetic characters, natural history and distributional range after the original description of the species, herein we provide additional data to fill the gaps in the aforementioned status.

During this study (2007–2019), we collected 17 specimens comprised mainly of dead on road (DOR) and individuals killed by local people in Mizoram State. We document *L. zawi* from 24 localities covering eight Districts of Mizoram State. Those are represented by 11 localities in Aizawl District, four in Lunglei District, three in Mamit District, two in Serchhip District, and one locality each in Lawngtai, Kolasib, Hnahthial and Champhai Districts (200–1216 m a.s.l). Details of collection data are presented in Supplementary Materials. Two live specimens we collected (MZMU 1049 and MZMU 1061) and these were anaesthetised using 250mg/kg of 0.7 % sodium bicarbonate buffered MS-222 (Tricaine Methanesulfonate) solution by intracoelomic injection, and then euthanised using a second intracoelomic injection of 0.1 ml unbuffered 50 % (v/v) MS-222 solution (see Conroy et al., 2009). Then, catalogued, fixed in 10 % buffered formalin and later transferred to 70 % ethanol in the Reptile Section, Departmental Museum of Zoology, Mizoram University (MZMU). The scalation terminology of Campbell & Lamar (2004) was used. The number of ventral scale (Ve) was counted according to Dowling (1951). Measurements were taken with a slide-caliper (Mitutoyo, 505–671) to the nearest 0.1 mm except snout-vent length (SVL) and tail length (TaL), which were measured with a ruler to the nearest 1 mm. The terminology for hemipenis by Dowling & Savage (1960) was followed. The relative tail length (RTaL) was calculated as tail length/total length (TaL/TL). Tissue samples collected from dead specimens were stored in –20 ˚C at 90 % ethanol in the facility of Developmental Biology and Herpetology Laboratory, Mizoram University, India.

We extracted DNA for a single individual of the specimen (MZMU 1379) using a DNeasy Blood and Tissue Kit (Qiagen™, Valencia, California, USA) following the standard protocol provided within the kit. PCR amplification on fragments of the mitochondrial cytochrome c oxidase I (*cox1*) gene was performed using

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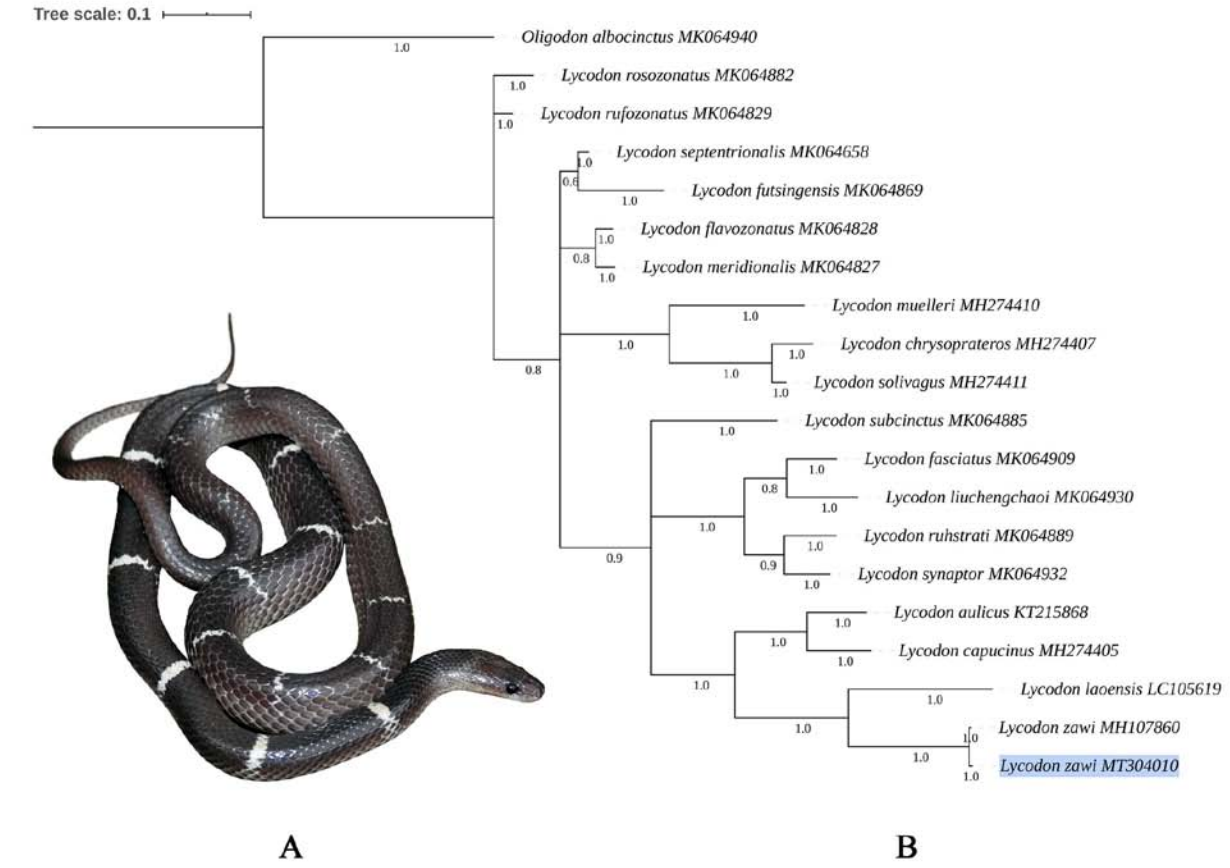

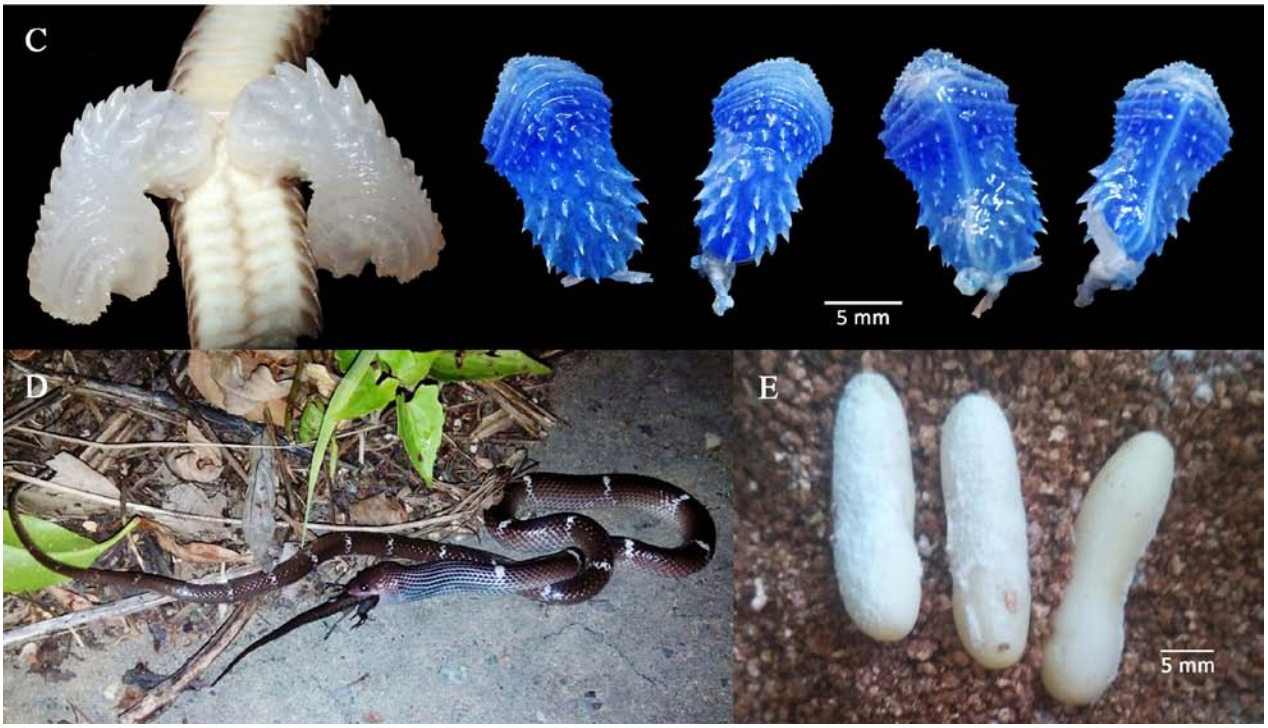

Figure 1. **(A)** Specimen of adult female *L. zawi* (MZMU 1061) in life, captured from Mission Veng, Mizoram, India. **(B)** Bayesian phylogeny based on partial *cox1* for Lycodon with other congeners. The sample generated in this study is highlighted in blue. **(C)** Everted hemipenis of *L. zawi* (MZMU 1049) (left), processed hemipenis (MZMU 1058) in asalcal view (middle two), and salcus view (right two). **(D)** *Lycodon zawi* swallowing *Eutropis macularia* in Mizoram, NE India. **(E)** Eggs of *L. zawi* just after dusting with antifungal powder, and later deposited to Departmental Museum of Zoology, Mizoram University (voucher number MZMU E1).

LCO1490 and HCO2198 primers (Folmer et al., 1994). The generated partial *cox1* sequence was deposited in the GenBank repository (accession number MT304010). In our phylogenetic analyses, we included 18 publicly available sequences of *Lycodon* species recovered from GenBank including one *Oligodon albocinctus* sequence (MK064940), which was used as an outgroup. We aligned our sequential data and GenBank database sequences together by using ClustalW (Thompson et al., 1994), and the Kimura 2 (K2P) genetic distances (Kimura, 1980) were calculated using MEGA X (Kumar et al., 2018). MrModeltest 2.3 (Nylander, 2004) was used to calculate the best fit model for Bayesian inference (BI) phylogeny. The BI phylogeny was constructed in MrBayes 3.2.5 by selecting nst=6 and rates=invgamma for the GTR+I+G model. The MCMC (one cold and three hot chains) was run for 20,000,000 generations sampling every 1000 generations, and burn-in was set to 25 %. The analysis was terminated when the standard deviation of split frequencies was less than 0.01 (Ronquist & Huelsenbeck, 2003).

We confirmed the identification of the species based on morphology (Slowinski et al., 2001; Purkayastha, 2013) and the *cox1* data (Fig. 1B). The morphometrics and pholidosis of 17 specimens we collected in this study are summarised: In males (n=8), SVL 260–514 mm, TaL 57–110 mm, RTaL 0.149–0.207 (mean=0.191), Ve 183–205, Subcaudals (Sc) 59–68. In females (n=9), SVL 169–517 mm, TaL 41–140 mm, RTaL 0.142–0.215 (mean=0.189), Ve 192–213, Sc 43–70. In sex pooled, dorsal scale rows 17:17:15, supralabials 8–9, suplalabials touching eye 3rd–5th (rarely 4th–5th), infralabials 8–10, temporals 2+3, loreal single, anal shield divided, 12–20 white transverse band on body and 3–6 poorly developed bands on the tail in five specimens. Hemipenes reaching up to 8–10 Sc, unforked, proximal half of organs with large spines followed by spinules. Ridges found at distal end of the asulcal surface extending around the sides toward the sulcus. A single sulcus spermaticus terminated in an expanded trough at the tip which agree well with the original hemipenis description by Slowinski et al. (2001) (Fig. 1C). Moreover, the specimens collected from Mission Veng and Sawleng, Mizoram, India represent the largest known female (MZMU 1061, total length 650 mm) and second largest male (MZMU 1,049, total length 599 mm) specimen for the species (see Slowinski et al., 2001; Whitaker & Captain, 2004; Dutta et al., 2013; Purkayastha, 2013), respectively.

The BLAST of the generated partial *cox1* gene matched *L. zawi* collected from Aizawl, Mizoram, India (MH107860), with a 99.76 % identity, 0 % gaps and an e-value of 0.0. From the studied mitochondrial *cox1* dataset (422 bp) of the genus *Lycodon*, a total of 143 variable sites were diagnosed with 15.5 % of mean K2P genetic distance with each other, ranging from 0.2 % to 20.8 %. The BI analysis of *L. zawi* of our specimen showed a well-supported monophyletic clade with the conspecific sequence obtained from the GenBank database (MH107860) with 0.2 % intraspecific genetic distance. Minimum and maximum interspecific genetic distances were diagnosed with *L. laoensis* (14.6 %) and *L. subcinctus* (20.8 %), respectively. Genetic distances of 16.5 %, 18.01 % and 18.07 % were also diagnosed with its congeneric species sympatric in the region *L. aulicus*, *L. septentrionalis* and *L. fasciatus*, respectively. The genetic relationship of *L. zawi* and *L. laoensis* in the present single locus tree (*cox1*) contradicts with the earlier work that used multiple genes (*c-mos*, *cytb* and *nd4*) to derive well-supported trees (Lei et al., 2014). However, we suggested the discordance between our single locus tree and the multilocus trees of *L. zawi* (Lei et al., 2014) may be due to any of the conditions such as model misspecification, due to the result of different evolutionary processes, inherent to the finite amount of data or error in sampling process (see Goodman et al., 1979; Pamilo & Nei, 1988; Takahata, 1989; Maddison, 1997; Page & Charleston, 1997; Mallo & Posada, 2016).

During a night survey on 11 August 2016, at ca. 22:30, a single *L. zawi* was observed preying on a skink, *Eutropis macularia* in the wild at Mizoram University Campus, India (23°44'15.69" N, 92°39'44.15" E, 780 m/asl., Fig. 1D) which was not mentioned specifically by earlier workers where Slowinski et al. (2001) reported that the food consists of small skinks: *Sphenomorphus maculatus* and geckos (*Hemidactylus frenatus* and *H. garnoti*). On 30 June 2019, at 15:00, a gravid female was captured from a bunch of grasses in a flower garden near the Guest House of Mizoram University, India. The female laid three eggs on 23 July 2019 (Fig. 1E), and the female was released back to the wild after taking the necessary morphological data. Eggs were soft, whitish, with a leathery texture, elongated ellipsoid, and measured 30.3 x 8.6 mm, 30.7 x 8.9 mm, and 30.9 x 8.6 mm. The eggs were incubated in a plastic container with vermiculite bedding and the eggs were occasionally dusted with clotrimazole, an antifungal powder (1 % w/w). None of the eggs hatched because of fungal infection or due to error in the optimal incubation conditions; or the eggs were not fertilised at all, hence data on the incubation period, temperature and humidity requirements, neonate size and other reproductive habits are not available at this time. All the specimens were encountered during monsoon season mostly at night (April to late September during 2013–2019). From our present work, we show that the species is not uncommon in the State and that is a species that has an apparent preference for riparian forests, where most specimens were found. Individuals were mostly active at night, and this nocturnal activity is congruent with the observations of Slowinski et al. (2001). *Lycodon zawi* were often encountered crossing tarmac roads as well as near and inside human habitations, possibly in search of prey.

Our work confirms the species identity and contributes further molecular data to a global database (GenBank Accession number: MT304010; specimen voucher MZMU 1379). It also bestowed new ranges of Ve 183–205 vs. 179 –197 in male (Slowinski et al., 2001; Reza, 2010) and 192–213 vs. 204–207 in female (Slowinski et al., 2001; Dutta et al., 2013), and new range of Sc in female i.e 43–70 vs. 49 (Dutta et al., 2013). Also, expands the known elevational range from 100–750 m/asl. to the zone of 200–1216 m/asl. (see Whitaker & Captain, 2004; Reza, 2010; Das, 2012; Dutta et al., 2013; Majumder, 2018). In addition, the species is also likely to occur in Bhutan (Jigme Tshelthrim Wangyal, pers. comm.; Vishal Santra pers. comm.), which

would extend its distributional range further towards the north-west of the known range.

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REFERENCES

Campbell, J. A. & Lamar, W. W. (2004). *The Venomous Reptiles of the Western Hemisphere. Vol. 2*. Ithaca: Comstock Pub. Associates.

Conroy, C. J., Papenfuss, T., Parker, J. & Hahn, N. E. (2009). Use of tricaine methanesulfonate (MS222) for euthanasia of reptiles. *Journal of the American Association for Laboratory Animal Science* 48, 28–32.

Das, I. (2012). *A Naturalist's Guide to the Snakes of South-east Asia* (Malaysia, Singapore, Thailand, Myanmar, Borneo, Sumatra, Java and Bali). Oxford: John Beaufoy Publishing.

Dowling, H. G. (1951). A proposed standard system of counting ventrals in snakes. *British Journal of Herpetology* 1, 97–99.

Dowling, H. G. & Savage, J. M. (1960). A guide to the snake hemipenis: a survey of basic structure and systematic characteristics. *Zoologica* 45, 17.

Dutta, D., Sengupta, S., Das, A. K. & Das, A. (2013). New distribution of records of *Lycodon zawi* (Serpentes: Colubridae) from Northeast India. *Herpetology Notes* 6, 263–265.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplifcation of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3, 294–299.

Ghosh, A., Mukherjee, N. & Rahut, B. (2017). Geographic Distribution: *Lycodon zawi* (Zaw's Wolf Snake). *Herpetological Review* 48, 129.

Goodman, M., Czelusniak, J., Moore, G. W., Romero-Herrera, A. E. & Matsuda, G. (1979). Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms constructed from globin sequences. *Systematic Biology* 28, 132–163.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111–120.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35, 1547–1549.

Lei, J., Sun, X., Jiang, K., Vogel, G., Booth, D. T. & Ding, L. (2014). Multilocus phylogeny of *Lycodon* and the taxonomic revision of *Oligodon multizonatum*. *Asian Herpetological Research* 5, 26–37.

Letunic, I. & Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research* 47, W256–W259.

Maddison, W. P. (1997). Gene trees in species trees. *Systematic Biology* 46, 523–536.

Majumder, J. (2018). Record of *Lycodon zawi* (Squamata: Colubridae) in Tripura and its range extension in northeast India: an Indo-Burma biodiversity hotspots of the world. *Biodiversity International Journal* 2, 303–304.

Mallo, D. & Posada, D. (2016). Multilocus inference of species trees and DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences* 371, 20150335.

Nylander, J. (2004). Mr.Modeltest v2, program distributed by the author. Uppsala: Evolutionary Biology Centre, Uppsala University, Uppsala.

Page, R. D. & Charleston, M. A. (1997). From gene to organismal phylogeny: reconciled trees and the gene tree/species tree problem. *Molecular Phylogenetics and Evolution* 7, 231–240.

Pamilo, P. & Nei, M. (1988). Relationships between gene trees and species trees. *Molecular Biology and Evolution* 5, 568–583.

Purkayastha, J. (2013). *An Amateur's Guide to Reptiles of Assam*. Assam: EBH Publishers (India).

Reza, A. H. M. A. (2010). Colubrid snake *Lycodon zawi* (Serpentes: Colubridae) from Lawachara national park in Bangladesh. *Russian Journal of Herpetology* 17, 75–77.

Ronquist, F. & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.

Slowinski, J. B., Pawar, S. S., Win, H., Thin, T., Gyi, S. W., Oo, S.L. & Tun, H. (2001). A new Lycodon (Serpentes: Colubridae) from northeast India and Myanmar (Burma). P*roceedings of the California Academy of Sciences* 52, 397–405.

Takahata, N. (1989). Gene genealogy in three related populations: consistency probability between gene and population trees. *Genetics* 122, 957–966.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.

Whitaker, R. & Captain A. (2004). *Snakes of India: The Field Guide*. Chennai: Draco Books.

Wogan, G. & Vogel, G. (2012). *Lycodon zawi*. The IUCN Red List of Threatened Species 2012:e.T176846A1449629. https://dx.doi.org/10.2305/IUCN.UK.20121.RLTS.T176846A1449629.en [accessed 23 April 2020].

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Websites:

Lang, J., Chowfin, S. & Ross, J.P. (2019). *Gavialis gangeticus*. The IUCN Red List of Threatened Species 2019: e.T8966A149227430. Downloaded on 3 October 2019. http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T8966A149227430.en.

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