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Genetic evidence for multiple paternity in the critically endangered Cuban crocodile (*Crocodylus rhombifer*)

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Abstract. Conservation strategies can be most effective when factors influencing the persistence of populations are well-understood, including aspects of reproductive biology such as mating system. Crocodylians have been traditionally associated with a polygynous mating system, with genetic studies revealing multiple paternity of clutches in several species. The endemic Cuban crocodile, *Crocodylus rhombifer*, is currently listed as Critically Endangered, and is one of the least understood crocodylian species in terms of its mating behavior. Here, we tested a hypothesis of multiple paternity in the Cuban crocodile by collecting genotypic data at nine microsatellite loci for 102 hatchlings from five nests sampled at the Zapata Swamp captive breeding facility and analyzing them in relation to data previously collected for 137 putative parents. All five nests showed evidence of multiple paternity based on the numbers of alleles per locus, with sibship analyses reconstructing all nests as having four to six full-sib family groups. Accordingly, mean pairwise relatedness values per nest ranged from 0.21 to 0.39, largely intermediate between theoretical expected values for half-siblings (0.25) and full-siblings (0.50). It is not possible to differentiate whether the multiple paternity of a nest was due to multiple matings during the same breeding season, or a result of sperm storage. Our results reveal that the *C. rhombifer* mating system is likely best characterized as promiscuous and suggest that the standard practice of enforcing a 1:2 sex ratio at the captive breeding facility should be altered in order to better maintain a demographically and genetically healthy ex situ population.

Keywords: conservation genetics, mating system, polygamy, polygyny, promiscuity.

Introduction

A thorough understanding of factors that can influence the persistence of populations, including reproductive biology and mating system, is important for setting conservation priorities and implementing effective management plans (Dudash and Murren, 2008). The mating system of a species encompasses the behavioral strategies used to obtain mates, the number of mates sought, the presence and characteristics of pair bonding, and the patterns of parental care provided by each sex (Emlen and Oring, 1977). Mating systems in animals can generally be classified into three general types including: 1) monogamy; 2) polygamy including polygyny (male mates with >1 female) and polyandry (female mates with >1 male); and 3) promiscuity where both males and females mate with multiple individuals (Emlen and Oring, 1977; Nunney, 1993). The species of the order Crocodylia have been traditionally associated with a polygynous mating system, with dominant males establishing breeding territories, while excluding other males and mating with multiple females (Grigg and Kirshner, 2015). Yet, genetic studies have revealed multiple paternity of clutches of eggs in several species across four of the nine genera within Crocodylia, including *Alligator* (Davis et al., 2001; Lance et al., 2009; Hu and Wu, 2010), *Caiman* (Amavet et al., 2008, 2012;

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Oliveira et al., 2014), and *Melanosuchus* (Muniz et al., 2011). The remaining genus, *Crocodylus*, contains 12 of the 24 extant crocodylian species, but the mating system of only four (*C. acutus*, Budd et al., 2015; *C. porosus*, Lewis et al., 2013; *C. moreletii*, McVay et al., 2008; and *C. intermedius*, Rossi Lafferriere et al., 2016) has been evaluated using molecular markers.

The endemic Cuban crocodile, *Crocodylus rhombifer*, is currently listed as Critically Endangered (IUCN 2015), and is one of the least understood crocodylians in terms of its reproductive biology (Ramos Targarona, 2013). To study any aspect of the mating system of *C. rhombifer* in the wild presents serious challenges. The range of the species is limited to a small area in Zapata Swamp (core zone of 300 km^2) that is extremely challenging to access, and population sizes are severely reduced due to illegal hunting and habitat degradation (Milian-Garcia et al., 2011, 2015). Consequently, direct observations of *C. rhombifer* reproductive strategy in the wild are scarce. In over 37 years of monitoring the wild population in Zapata Swamp, only 8 nests with eggs have been observed (Ramos Targarona, 2013). Moreover, the wild *C. rhombifer* population is impacted by hybridization with the widespread American crocodile (*Crocodylus acutus*, Milian-Garcia et al., 2015). It is difficult to differentiate hybrids from non-hybrids in the wild based solely on morphological characters, further complicating the study of reproductive behavior based on simple observation.

An ex situ population of *C. rhombifer* is housed at the Zapata Swamp captive breeding facility, but certain aspects of reproductive strategy continue to be difficult to elucidate. For example, this species copulates in the water, making the verification of successful mating attempts by direct observation difficult (fig. 1; online supplementary video 1). Harems are formed each reproductive season, but it is uncertain how many females are effectively mated with the dominant male, or how many times the male mates with each female (Ramos Targarona, 2013). There is also the possibility of "sneaker" males that may surreptitiously mate inside the harem of a dominant male. Additionally, some females may move

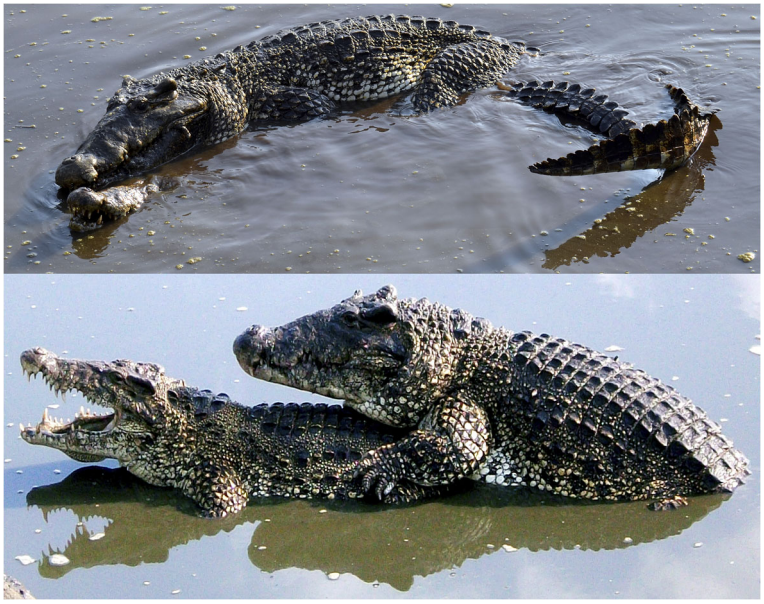

Figure 1. A pair of Cuban crocodiles mating within the breeding pen (C22) at the Zapata Swamp captive breeding facility. Images by YMG. This figure is published in colour in the online version.

among harems within a breeding season (Ramos Targarona, 2013).

The conservation of *Crocodylus* species that inhabit Cuba is limited by this lack of knowledge of their basic biology and reproductive behavior (Ramos Targarona, 2013; Alonso-Tabet et al., 2014). In order to begin to fill this knowledge gap, we tested the hypothesis of multiple paternity in the Cuban crocodile using genotypic data collected for hatchlings and putative parents sampled from multiple nests in the Zapata Swamp ex situ population. We further discuss the importance of understanding the *C. rhombifer* mating system for informing in situ and ex situ conservation strategies.

Materials and methods

Sampling

This study took place at the Zapata Swamp captive breeding facility in Cuba (N 22°22.100′ W 81°09.965′; fig. 2, table 1). This facility is run by the National Enterprise for the Protection of Flora and Fauna in Cuba, the agency in charge of approving all projects involving wildlife within the country. The primary reproductive pen, C22, constitutes a semi-closure of irregular shape (0.6 hectares) containing marshy areas with free movement of water. Areas in which the animals are kept are subject to natural cyclical variations of drought and flood, similar to that taking place in the wild. The C22 breeding pen was founded in 1980 with 100 females and 50 males from a selection of the first generation offspring produced by the founders at the captive breeding facility. The area of the pen that is not flooded is composed of peat mixed with river sand and covered with vegetation to allow nesting, equivalent to that which occurs in the wild. This breeding pen also includes different trees to provide areas of shade and sunlight, enabling the animals to alternate spaces for optimal thermoregulation (Ramos Targarona, 2013). Males and females remain in this area for exhibition and reproduction purposes, with a male-female ratio of 1:2 and maintaining a total adult population size of 150 (Ramos Targarona, 2013).

Hatchlings ($n = 102$) were sampled from five nests located in the primary breeding pen (C22) during eclosion time in August 2010. All eggs were removed from each nest (online supplementary fig. S1) and transported to the incubation room available on-site at the facility. Each clutch was incubated separately in boxes made up of foam containing the same substrate available where the nests were built. This incubation strategy ensured no mixing of clutches prior to sampling. Samples consisted of a caudal scale from each hatchling's tail, which is also used as a unique identifier to monitor the nests. All sampling was conducted either directly by or under the supervision of the head veterinarian of the facility (GSR). Each sample was preserved in 95% ethanol until DNA extraction. All hatchlings were classified as *C. rhombifer* based on external morphological characters and type of nest (mound) constructed by the fe-

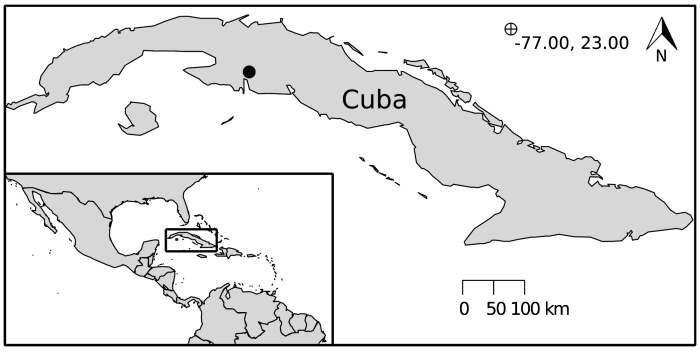

Figure 2. Map of Cuba with inset of Central America; the circle indicates the location of the Zapata Swamp captive breeding facility.

Table 1. Sample size (N = number of hatchlings), mean Queller and Goodnight relatedness (R_{QG}) and associated variance in parentheses, mean number of alleles (N_a), mean observed (H_o) and expected heterozygosity (H_e) for each nest from the ex situ population of *Crocodylus rhombifer* in Zapata Swamp.

	Nest 6	Nest 9	Nest 14	Nest 33	Nest 58
N	19	31	18	23	11
R_{QG}	0.21 (0.08)	0.36 (0.07)	0.39 (0.07)	0.33 (0.07)	0.31 (0.06)
Mean N_a	3.33	2.11	2.89	2.78	2.89
Mean H_o/H_e	0.61/0.47	0.41/0.39	0.55/0.43	0.53/0.44	0.56/0.47

male (fig. S1). One hundred and thirty seven *C. rhombifer* adults were previously sampled at the Zapata Swamp captive breeding facility between 2007-2012 and genotyped at nine microsatellite loci (Dryad Digital Repository: DOI:10.5061/dryad.r86n4) as part of a larger study to investigate hybridization and introgression between *C. rhombifer* and *C. acutus* (Milian-Garcia et al., 2015). A subset of these individuals ($n = 89$) was sampled in the reproductive pen (C22) where the focal nests in this study were laid and constitute the majority of potential parents. All of these individuals have been previously confirmed as *C. rhombifer* based on genetic data (Milian-Garcia et al., 2015). These individuals were sampled prior to the breeding season in order to minimize stress to the animals and for safety reasons, as adult *C. rhombifer* are especially aggressive during this period. For these same reasons, females observed guarding each nest (fig. S1) were identified from a distance outside the pen using the physical mark on their tail (online supplementary fig. S2).

DNA extraction and genotyping

DNA was isolated from the 95% ethanol-preserved tissue using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) following manufacturer's protocols. All sampled hatchlings were genotyped at the same nine microsatellite loci as in (Milian-Garcia et al., 2015): Cj16, Cj18, Cj20, Cj35, Cj109, Cj119, Cj127, Cj131 and CU5-123 (FitzSimmons et al., 2000; Dever and Densmore, 2001). Forward primers were 5′-tailed with an M13 sequence [5′-TCCCAGTCACGA-CGT -3′] to facilitate automated genotyping. Specifically, the M13-tailed forward primer was used in combination with an M13 primer of the same sequence 5′-labeled with one of four fluorescent dyes (6-FAM, VIC, NED, PET) to incorporate the fluorescent label into the resulting PCR amplicon (Schuelke, 2000). All PCRs were performed on an ABI Veriti thermal cycler in 12.5 μl reactions containing: ∼20-50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.08 μM of the M13-tailed forward primer, 0.8 μM of each of the reverse primer and the M13 fluorescent dye-labeled primer, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems). Reaction conditions for all primers were as follows: 95°C (2 min), 8 cycles of 95°C (30 s), 59°C (30 s), 72°C (45 s), followed by 32 cycles of 95°C (30 s), 51°C (30 s), 72°C (45 s) and a final extension of 72°C (10 min). Fragments were separated on an ABI 3130XL Genetic Analyzer and scored using GENEMAPPER 4.0 (Applied Biosystems).

Data analysis

An earlier study involving putatively unrelated *C. rhombifer* adults sampled at the Zapata Swamp captive breeding facility and in the wild detected no evidence of null/false alleles at the sampled microsatellite loci (Milian-Garcia et al., 2015). Furthermore, linkage disequilibrium at one locus pair (Cj127/Cj131) and deviation from Hardy-Weinberg equilibrium at a single locus (Cj119) was detected in the Zapata Swamp captive population, but these patterns were not consistent across sites. As in Milian-Garcia et al. (2015), all downstream analyses in the current study were based on genotypic data at all nine microsatellite loci.

Observed (H_o) and expected heterozygosity (H_e), and allelic richness were calculated at each locus for each nest using GENALEX 6.5 (Peakall and Smouse, 2006, 2012). Exclusion probabilities for each locus were calculated assuming none, one or two parents known (PE0, PE1, and PE2 respectively) using the software COANCESTRY (Wang, 2011).

The hypothesis of multiple paternity of each nest was evaluated using four independent approaches. First, the number of alleles per locus in a nest was calculated to determine if it exceeded the maximum number of expected alleles under the hypothesis of single paternity. If all of the individuals in a nest were full siblings, the maximum number of alleles per locus would be four assuming both parents are heterozygous for different alleles. This expectation would result in all individuals in a nest also being heterozygous, so by evaluating patterns of homozygosity in hatchlings, a different maximum number of expected alleles less than four can be determined.

Second, we calculated average pairwise relatedness within and among nests. As the statistical properties of various relatedness estimators are known to differ in accordance with dataset-specific characteristics (Blouin, 2003), we initially evaluated the performance of eight pairwise relatedness coefficients using irelr (Goncalves da Silva and Russello, 2011). The highest ranked relatedness estimator was the symmetric version of Queller and Goodnight (Queller and Goodnight, 1989) as identified based on a composite score that incorporates estimates of bias, variance, skewness, and kurtosis. We then evaluated our power to distinguish full-siblings (FS) from half-siblings (HS) using the Queller and Goodnight (1989) symmetric estimator and the allele frequency distribution calculated using all sampled *C. rhombifer* adults following Blouin et al. (1996). Specifically, we simulated 1000 dyads each of full-siblings and half-siblings, and estimated the probability that a dyad of one type of relationship (e.g. FS) would be misclassified as the other type of relationship (e.g. HS and vice versa) by using the midpoint between the means of the two distributions as implemented in irelr (Goncalves da Silva and Russello, 2011). Observed mean relatedness and associated variances were subsequently calculated for all hatchlings within and among nests using the symmetric Queller and Goodnight (1989) estimator as implemented in COANCESTRY (Wang, 2011).

As a third line of evidence, full-sibling groups within each nest were reconstructed using the maximum likelihood approach implemented in KINGROUP (Konovalov et al., 2004). The analysis was run constraining the individuals in a nest to be at least maternal siblings. The latter assumption was warranted due to the sampling design associated with nest selection and strict incubation of all eggs from the same nest separately.

Lastly, full- and half-sibling relationships were reconstructed in COLONY (Jones and Wang, 2010; Wang, 2013) using the genotyped captive adults ($n = 137$) as potential parents. The allele frequency distribution from the adult

samples was used as the known distribution. The probability of having sampled the true mother was set to 0.65 since 65 of the ~100 females in the reproductive pen were genotyped, and the true father to 0.5, since 24 of the ~50 males were genotyped. Adults sampled in other reproductive pens ($n = 48$) were still included as potential parents since it is possible that they were moved during or just previous to the study period. Both sexes were assumed to be polygamous, and runs were completed using the full-likelihood model and the medium run length option. COLONY analyses were run three times to evaluate the robustness of the inferred relationships.

Results

All nine microsatellite loci were polymorphic in the hatchling crocodiles (table 2). The overall exclusion probabilities were high, particularly when one or both parents were known (⩾0.97, table 3). Between one and six alleles per locus were found in each nest, with observed and expected heterozygosity ranging from 0 to 1 and 0 to 0.71, respectively (table 2). Three loci (Cj109, Cj119 and Cj131) showed more than four alleles in a nest, the maximum number of expected alleles assuming that both parents are heterozygous for different alleles. When taking into account patterns of homozygosity in hatchlings, discrepancies between the maximum expected and observed number of alleles per locus were found at one or more loci in all five nests, and in all but one locus overall (Cj35, table 3).

The average relatedness of all individuals within nests ranged from 0.21 (Nest 6) to 0.39 (Nest 14) (table 1), while average among nest

Table 2. The per locus number of alleles (N_a), observed (H_o) and expected heterozygosity (H_e) for each nest and for all sampled adults from the ex situ population of *Crocodylus rhombifer* in Zapata Swamp. Loci with number of alleles greater than four are highlighted in grey.

Locus	Nest 6		Nest 9		Nest 14		Nest 33		Nest 58		Adults	
	N_a	H_o/H_e	N_a	H_o/H_e	N_a	H_o/H_e	N_a	H_o/H_e	N_a	H_o/H_e	N_a	H_o/H_e
Cj16	4	0.68/0.62	2	0.65/0.44	4	1.00/0.65	3	0.57/0.42	3	0.55/0.42	9	0.68/0.72
Cj18	4	1.00/0.65	1	0.00/0.00	2	0.39/0.49	3	0.61/0.44	4	0.55/0.55	7	0.69/0.66
Cj20	3	0.84/0.61	2	0.55/0.50	3	0.61/0.44	3	0.57/0.50	2	1.00/0.50	7	0.67/0.66
Cj35	2	0.05/0.05	1	0.00/0.00	1	0.00/0.00	1	0.00/0.00	2	0.36/0.30	2	0.07/0.10
Cj109	1	0.00/0.00	2	0.57/0.41	5	0.50/0.63	2	0.91/0.50	3	0.64/0.46	4	0.48/0.48
Cj119	6	0.89/0.71	3	0.48/0.59	3	0.33/0.32	4	0.57/0.67	3	0.09/0.37	12	0.51/0.76
Cj127	3	0.79/0.51	3	0.45/0.51	2	0.17/0.15	2	0.50/0.49	3	0.64/0.48	7	0.48/0.55
Cj131	5	0.79/0.65	3	0.74/0.64	3	0.94/0.56	3	0.52/0.49	3	0.73/0.65	10	0.68/0.75
CU5-123	2	0.42/0.43	2	0.29/0.45	3	1.00/0.62	4	0.52/0.41	3	0.45/0.48	10	0.66/0.70

Table 3. Expected number of alleles per locus per nest based on homozygous genotypes found in the offspring compared to the observed number of alleles per locus per nest. Exclusion probabilities considering none, one or two parents known (PE0, PE1 and PE2 respectively) are also presented. Discrepancies between expected and observed number of alleles are indicated in grey.

Locus	Maximum number of expected alleles per locus/ observed number of alleles per locus							
	Nest 6	Nest 9	Nest 14	Nest 33	Nest 58	PE0	PE1	PE2
Cj16	3/4	3/2	4/4	3/3	3/3	0.29	0.46	0.64
Cj18	4/4	3/1	2/2	3/3	3/4	0.01	0.23	0.38
Cj20	2/3	2/2	3/3	2/3	4/2	0.21	0.35	0.50
Cj35	3/2	3/1	3/1	3/1	4/2	0.00	0.00	0.00
Cj109	3/1	3/2	1/5	3/2	3/3	0.14	0.23	0.35
Cj119	3/6	2/3	3/3	2/4	2/3	0.24	0.41	0.59
Cj127	3/3	2/3	3/2	2/2	3/3	0.12	0.22	0.33
Cj131	3/5	3/3	3/3	3/3	2/3	0.31	0.49	0.66
CU5-123	2/2	2/3	4/3	3/4	3/3	0.20	0.35	0.51
Overall						0.84	0.97	1.00

Table 4. The best configuration of groups in each nest and number of hatchlings per full-sib group for *C. rhombifer* from the sibship analysis implemented in KINGROUP (Konovalov et al., 2004).

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Nest 6	15	2	1	1		
Nest 9	24	3	2	2		
Nest 14	8	5	2	1	1	1
Nest 33	16	3	2	1	1	
Nest 58	8	1	1	1		

relatedness ranged from a minimum of −0.20 (N6/N33) to a maximum of 0.04 (N6/N58). Based upon the simulated relatedness distributions, we estimated moderate misclassification rates (29.3% FS misclassified as HS; 29.2% HS misclassified as FS), suggesting reasonable power in assigning dyads as either full-siblings or half-siblings.

Multiple runs of KINGROUP (Konovalov et al., 2004) resolved the same best configuration of full-sib families per nest. All of the nests were reconstructed to have between four and six full-sib groups (table 4, supplementary table S1). Four of the nests contained two or more full-sib groups each containing two or more individuals (table 4).

Multiple runs of COLONY resolved the same best configuration of sibships and parentage. Many of the assigned parents were hypothetical individuals despite the fact that ∼59% of putative parents in the reproductive pen where the nests were located were genotyped. Two of the nests were assigned a known female as the mother (online supplementary table S2); in both cases, the assigned female was the one observed guarding the nests. The females observed guarding the other three nests had a 0 probability of maternity assigned to those nests. All of the nests were reconstructed to have more than one father, with two to six males being assigned paternity in a nest (table S2). Five males sired offspring with multiple females (table S2).

Discussion

In the present study, the nine microsatellite loci proved to be useful in testing a hypothesis of multiple paternity. The heterozygosity values (tables 1 and 2) are among the highest reported for any *Crocodylus* species (Milian-Garcia et al., 2011, 2015). Moreover, the exclusion probabilities (table 3) are also among the highest on record reported for crocodylians in the wild or captivity (Lance et al., 2009; Oliveira et al., 2014), affirming the quality of the markers used in this study.

Interpretations of patterns reconstructed from the genotypic data assumed that all individuals sampled from a nest were maternal siblings. This assumption was based on field observations that found no suspicious patterns of oversized nests with substantially more eggs than the average, nests with more than one egg chamber, or more than one female defending the nest during sampling. In addition, no significant variation was observed in the shape among the eggs inside each clutch or different embryonic stages within clutches at the moment of collecting the eggs (EPF, pers. obs.). The latter issues are common when opportunistic females take advantage of a mound nest built by another female to lay their eggs (EPF, pers. obs.). In other words, our sampling design ensured that individuals from a clutch shared just one mother and minimized the probability that multiple females used the same nest. It was, however, curious that the females observed guarding the five nests exhibited allele transmission discrepancies at one or more loci (table S1), results not expected for the genetic mothers of the emerging hatchlings. Tissue sampling for adults occurred before the breeding/nesting season so as not to disturb behavior during this time. Also, adults are particularly aggressive during the breeding/nesting

season, making it unsafe to approach for identifying marks and sampling tissue. Consequently, the tail marks must be observed from a distance outside the pen to identify guarding females, which can be challenging given the marking system employed (fig. S2). Thus, misidentification likely led to the discrepancy between observational and genetic results.

Overall, we detected multiple paternity in all five nests based on independent lines of evidence. The observed numbers of alleles per locus exceeded the number of alleles expected under the hypothesis of single paternity in all sampled nests (table 3). In addition, mean pairwise relatedness values per nest (ranged from 0.21 to 0.39, table 1) were largely intermediate between theoretical expected values for half-siblings (0.25) and full-siblings (0.50). Importantly, we had reasonable power for differentiating full-siblings from lower orders of relatedness based on our genotypic data and the symmetric Queller and Goodnight (Queller and Goodnight, 1989) estimator. In contrast, hatchlings among nests were characterized by exceptionally low relatedness estimates (−0.20 – 0.04). Moreover, four to six full-sib groups were reconstructed in each nest, with high variability in the number of members (ranging from 1-24; table 4). Although the reconstructed patterns for each nest suggested a single, predominant sire with smaller contributions from secondary and/or tertiary sires (table 4), all but one nest had multiple full-sib groups that consisted of 2 or more members. The results of sibship reconstruction yielded by ML-RELATE and COLONY were largely congruent in terms of the number of groups per nest, increasing confidence in an interpretation of multiple paternity (table 4, table S2). Unfortunately, it is not possible to differentiate whether the multiple paternity of a nest is due to multiple matings during the same breeding season, or a result of sperm storage, as has been reported for other crocodylians (Gist et al., 2008). Direct observations have suggested that multiple matings during the same breeding season do occur (YMG, pers. obs.), but the success rate is difficult to assess since *C. rhombifer* mates in the water.

From a conservation perspective, mating strategies need to be taken into account when designing in situ and ex situ management plans. Polygamous mating can play a significant role in limiting inbreeding as well as in maintaining genetic diversity and increasing effective population size (Sugg and Chesser, 1994). This is particularly important in small populations where the probability of mating among relatives is higher. Our results demonstrate, for the first time, multiple paternity in *C. rhombifer*. Moreover, the recovered patterns suggest that the mating system of this species is likely best characterized as promiscuous as has been found in *C. porosus* (Lewis et al., 2013). It is important to note that captive conditions may have potentially altered natural behavior, yet detailed studies in the wild are likely not feasible. Assuming our findings in the ex situ population are reflective of *C. rhombifer* more generally, this new knowledge of mating system can be important if reintroduction ever becomes warranted, especially when considering optimal sex ratios and choosing males of high breeding potential (Rowe and Hutchings, 2003; Sigg et al., 2005). In terms of ex situ management, imposing polygyny by enforcing a 1:2 male to female sex ratio in captive breeding pens is no longer justifiable. Although the optimal sex ratio for Cuban crocodiles remains unknown, the equalization of males and females to the extent possible taking into account behavioral and logistical considerations may best contribute to the maintenance of a demographically and genetically healthy ex situ population. Moreover, our results further punctuate the need for more intensive surveys of the wild population to enhance understanding of basic biology, demography and life history under natural conditions to better inform interactive in situ and ex situ conservation strategies.

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