POPULATION GENETIC STRUCTURE AND REPRODUCTIVE ECOLOGY OF *CROCODYLUS* ACROSS LOCAL AND REGIONAL SCALES

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ABSTRACT

Population Genetic Structure and Reproductive Ecology of *Crocodylus* across Local and Regional Scales

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New world crocodilians constitute a monophyletic group comprising four species: *Crocodylus rhombifer* (Cuban crocodile), *Crocodylus acutus* (American crocodile), *Crocodylus intermedius* (Orinoco crocodile), and *Crocodylus moreletii* (Morelet's crocodile). All of these are in the IUCN Red list of Threatened Species and exhibit geographic distributions covering small to widespread ranges across the Americas and insular Caribbean. With the overarching goal of generating relevant information for the conservation of endangered new world crocodilians, this dissertation integrates genetic and ecological information to provide a context spanning a scale from the species level to specific populations, to analyses of mating systems and breeding strategies in *Crocodylus*. In addition, my research applies tools of ecological inference to model the influence of environmental factors and natural habitat disturbances in the reproductive success of *Crocodylus* using a long-term dataset. This work uses *C. intermedius* and *C. acutus* as model species to explore four focal questions organized in distinct chapters related to the biology and ecology of crocodilians.

In Chapter I, I compare previously reported reproductive traits among *C. acutus* populations across its geographic range. This comparative analysis reveals a high degree of variability in reproductive traits across *C. acutus* range and provides potential adaptive explanations for the patterns observed. *Crocodylus acutus* appears to be one of the most adaptable of crocodilians in terms of

nesting requirements, total nests per breeding season, nest mode (hole vs. mound), timing of egg-laying, female minimum reproductive size, clutch size, female nest defence behaviour, and female parental care. Besides regional comparisons, this chapter focuses on the largest nesting population of *C. acutus* located in southeastern Cuba, where the species still occurs at its natural population numbers.

In Chapter II, I use molecular tools to elucidate the mating system of the Orinoco crocodile in a reintroduced population in the Llanos of Venezuela. Analyzing 17 polymorphic microsatellite loci from 20 clutches I found multiple paternity in *C. intermedius*, with half of the clutches fathered by two or three males. Sixteen mothers and 14 fathers were inferred by reconstruction of multilocus parental genotypes. Results showed skewed paternal contributions to multiple-sired clutches in four of the clutches (40%), leading to an overall unequal contribution of offspring among fathers with six of the 14 inferred males fathering 90% of the total offspring, and three of those six males fathering more than 70% of the total offspring. Results of this chapter provide the first evidence of multiple paternity occurring in the Orinoco crocodile and confirm the success of reintroduction efforts of this critically endangered species in Venezuela.

In Chapter III, I apply generalized linear mixed models to infer the effect of tropical cyclones and environmental variability on the nesting success of *C. acutus* in the largest nesting population of the species in southeastern Cuba for a period of 21 years. Results of this chapter report the highest-density nesting for the species documented to date, and one of the highest densities of nesting in relation to other crocodilian species, with an average of 164 nests per year and a density of 17 nests per hectare. Two of the five analyzed nesting sites had consistently higher nests and higher nesting success for the whole 21-year period. Much of the temporal variation in nesting success could be explained by the occurrence of tropical cyclones. I found that occurrence of tropical cyclones within a nesting season negatively affected nesting success, whereas the occurrence of tropical cyclones one or two years before the nesting season positively affected nesting success. Additionally, results of this chapter suggest that higher ambient temperature negatively affected nesting success. Higher-intensity tropical cyclones are expected to strike the coasts of Cuba due to climate change, potentially devastating *C. acutus* nests if they occur during the nesting season. As the recruitment of *C. acutus* populations in Cuba heavily rely on nesting success, we propose incorporating information on crocodilian's nesting success and density, as well as the impact of tropical cyclones on the latter, as key components of coastal resilience when designing plans for coastal adaptation in the context of climate change.

In the last chapter, I employed data on mitochondrial DNA (mtDNA) control region and 12 nuclear polymorphic microsatellite loci to assess the degree of population structure of *C. acutus* between and among localities in South America, North America, Central America and the Greater Antilles. All analyses for both mtDNA and nuclear markers show evidence of strong population genetic structure in the American crocodile, with unique populations in each of the sampling localities. My research results reinforce previous findings showing the greatest degree of genetic differentiation between the continental *C. acutus* and the Greater Antillean *C. acutus.* Three new haplotypes unique to Venezuela were reported. These were considerably less distant from Central and North American haplotypes than Greater Antillean haplotypes. Overall evidence of this chapter suggests that Cuban and Jamaican *C. acutus* share a mtDNA haplotype but currently represent at least two different genetic populations when using nuclear, faster evolving, microsatellite markers. Findings of this chapter offer the first evidence of genetic differentiation among the populations of Greater Antillean *C. acutus,* the first ever reported haplotypes for the species in Venezuela, and provide important information for the regional planning and in-situ conservation of the species.

In conclusion, research findings of my dissertation are the product of combining ecological data collected in the field, genetic data generated in the lab, and the use of a suite of classic and inference-based methodological approaches to gain a better understanding of the behavior and evolution of crocodilians. The dissertation presents the first genetic research on *C. intermedius,* shows the importance of coastal mangrove conservation for the persistence of *C. acutus* in Cuba, and depicts phylogeographic linkages among distinct *C. acutus* populations across the Americas and Greater Antilles. The outcomes of this research provide science-based information to influence decision-making processes for the conservation of threatened crocodilians and their habitats across the study areas.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

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Dedication

To my mother, Amelia Sara Lafferriere, and my grandmother, Amelia Angelica Martegani

INTRODUCTION

Integration of Ecological and Evolutionary Approaches for the Conservation of Endangered Crocodilians

Technological advances in the last few decades have made genetic data collection more accessible (e.g., extraction of DNA from different tissues, gathering of DNA from museum specimens, fossil DNA extraction, etc.) and have significantly reduced the cost of obtaining high quality data by using molecular tools [1]. The generation of large amounts of information through molecular techniques (e.g., next generation sequencing) in a very cost-effective way has allowed leveraging the advances of molecular biology and genomics to address questions of the genetic structure and evolutionary history of natural and wild populations [2]. As a consequence, researchers are able to address and integrate important ecological and evolutionary questions on a scale and precision that was unrealistic only a decade ago [2].

In combination with great technological advances, evolutionary approaches have increasingly been applied to the conservation of species and ecosystems [1,3,4]. In a time of accelerated environmental change though pollution, habitat disturbance, climate change and community alterations by invasive species [5,6], linking spatial data on phenotypes, genomes and environmental variables in a phylogenetic context allows researchers to address conservation by tackling important issues such as quantifying taxonomic diversity, defining demographic units for conservation, and inferring species vulnerability to climate change [1]. In addition, the integration of ecological and molecular information can contribute to answering a suite of questions germane to the conservation of individual species. Thus, central research questions focus on defining the number of demographically distinct conservation management units [7], elucidating the patterns of genetic diversity among geographically distinct populations [8], investigating the characteristics and variations of mating systems and breeding strategies [9], and studying the interactions between species [10].

An understanding of the mating strategies that individuals deploy may have a significant impact on the conservation of a species, as these mating strategies affect the long-term maintenance of genetic diversity within and among populations. For instance, multiple paternity (i.e., the existence of more than one father siring one nest) has been shown theoretically to increase effective population size [11], thus potentially increasing the overall genetic diversity of a population. This mating strategy has been reported in different vertebrate groups: fish [12], birds [13], mammals [14,15], amphibians [16], turtles [17], lizards [18], snakes [19], and crocodiles [20-22].

Another important challenge in species conservation is the identification of unique populations that need to be considered as independent management units. These populations are usually defined using information from many sources including: habitat types within the range of the species, local conservation status, threats, enforcement and legislation [23,24], and the patterns of genetic variation found within and among subpopulations. However, even though genetic differentiation has been used widely as a tool for delineating local management units, and work has been pursued on some species of *Crocodylus* [7]*,* there is still a need to incorporate information on intraspecific genetic variation into conservation planning for many species of crocodilians.

Investigating geographic patterns of genetic variation between and among populations across a species' distribution also can be used to help us better understand the ecology and contemporary evolution of the species. Genetic analyses allow us to estimate historical and contemporary levels of gene flow, test models of genetic differentiation, and reconstruct plausible biogeographic scenarios to explain current species distributions [25,26]. For instance, 'isolation by distance' (IBD) models of genetic differentiation [27,28] predict that genetic distances between populations are positively correlated with geographic distances separating them.

Present-day crocodilians (crocodiles, alligators, caimans, and gharials) come from an ancient and diverse lineage, once comprising over 125 genera [29,30]. As top predators and keystone species, crocodilians play an important role in structuring of communities and ecosystems in which they live [31,32], controlling both the flux of energy and the spatial configuration of their landscape, and thus significantly affecting a suite of other species [33-35]. Crocodilians are a much threated taxa: the IUCN Red List of Threatened Species [36] states that of the 23 species of extant crocodilians, six are currently listed as critically endangered, one as endangered, three as vulnerable, twelve as least risk and one as data deficient. In order to assist the conservation of threatened crocodile species, there is a need to better understand the evolutionary relationships within and among species, their reproductive ecology, and their interactions with other species. In addition, information on how these factors interact with, and are affected by, environmental variation makes crocodiles a suitable model for integrating ecological and evolutionary questions in a conservation context.

New world crocodilians include four species: *Crocodylus rhombifer* (Cuban crocodile), *Crocodylus acutus* (American crocodile), *Crocodylus intermedius* (Orinoco crocodile), and *Crocodylus moreletii* (Morelet's crocodile). Phylogenetic studies [37], in combination with known fossil records of *Crocodylus* [38,39], support the hypothesis that all new world crocodilians belong to a monophyletic group. This group is paraphyletic to, and shares a recent common ancestor with eastern populations of *Crocodylus niloticus.* In this context, new world crocodilians appear to have diversified from a founding population or populations of eastern *C. niloticus* that negotiated a trans-Atlantic crossing to the New World during the Pliocene [37]. In addition to the relatively recent diversification of new world crocodilians [37], studies have reported present and historical interspecific hybridization between *C. acutus* and *C. rhombifer* in Cuba [40,41], and between *C. acutus* and *C. moreletii* in the Yucatan Peninsula [42].

Of all new world crocodilians, the American crocodile is the most widely distributed, inhabiting coastal swamps, estuarine rivers, lakes and reservoirs of the Neotropical Region of the Americas and the Insular Caribbean [34]. The geographic range of *C. acutus* extends from Mexico to Peru on the Pacific coast, and from the southern tip of Florida to Venezuela on the Atlantic coast, including most of the continental and insular Caribbean region [34]. The species is categorized globally as “vulnerable” by the IUCN [43] and listed in CITES Appendix I which prohibits commercial trade [44]. The species has a split listing in CITES, with the Cuban population being transferred to Appendix II in 2007 [45]. Although *C. acutus* inhabits mainly brackish water coastal habitats such as the saltwater sections of rivers, coastal lagoons, and mangrove swamps [45], the species has been found across a gradient of freshwater to saltwater and from coastal areas to inland lakes. For instance, there is a significant population in Lago Enriquillo, a landlocked hypersaline lake situated 40 meters below sea level in arid southwestern Dominican Republic [46-48]. The range of *C. acutus* overlaps with three other new world crocodilian species: *C. rhombifer* in the Zapata Swamp, Cuba [40], *C. moreletii* in the Yucatan Peninsula, Mexico [49], Guatemala, and Belize [50] , and *C. intermedius* in Venezuela [51].

The American crocodile exhibits high levels of variation in its reproductive behavior [34]. It appears to be one of the most adaptable of crocodilians in its nesting requirements, nest mode, timing of egg-laying, female minimum reproductive size, female nest defence behavior, and female parental care [34,35,46,52-54]. For instance, although primarily a hole nesting species, *C. acutus* builds mound nests made of sand or gravel in southern Florida [52,55], coastal Belize [53] and Mexico [56,57], probably as an adaptive response to nesting in low-lying areas where the probability of nest flooding is high [34]. As another example, members of *Crocodylus acutus* have been observed to lay eggs in isolated nests [55] or colonially, with up to 90 females laying eggs in a single site [58,59]

In contrast to the widespread and locally abundant American crocodile, the Orinoco crocodile has been reduced to only a few wild populations left in Venezuela and Colombia [60]. One of the most threatened crocodile species in the world, the Orinoco crocodile [61,62] once inhabited large areas of the Llanos of Venezuela and Colombia within the Orinoco basin [63,64]. Extensive hunting until the 1960s and persistent collection of eggs for local consumption decimated its populations in the wild [65,66]. Since the 1990s, efforts have been made to re-establish *C. intermedius* in Venezuela. Through a reintroduction program more than 2000 crocodiles sourced from four captive breeding centers in the country were released at the El Frío Biological Station, one of the few remaining localities within the species range [67]. Until today the Orinoco crocodile remains one of the least studied species within new world crocodilians, and genetic research on this species has been lacking.

Integrating genetic and ecological information for the conservation of endangered crocodilians necessitates a combination of approaches and tools to answer important questions in an evolutionary context. Hence, the key focus of this dissertation is to provide a genetic context spanning a scale from the species level to specific populations, to analyses of mating systems and breeding strategies in *Crocodylus*. The understanding of these evolutionary and ecological interactions, although greatly assisted by traditional ecological methods, can only be elucidated by the use of genetic markers. In addition, my research seeks to apply tools of ecological inference to model the influence of environmental factors and natural habitat disturbances in the reproductive success of *Crocodylus* using a long-term dataset. The results from combined genetic and ecological analyses covering different species, geographies, and time frames can then be interpreted to further our knowledge about crocodilian's natural history, and used to aid conservation and management programs at local and regional scales.

References

1. Losos JB, Arnold SJ, Bejerano G, Brodie III E, Hibbett D, Hoekstra HE, et al. (2013) Evolutionary Biology for the 21st Century. PLoS biology 11: e1001466.

2. Ekblom R, Galindo J (2010) Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity 107: 1-15.

3. Bull J, Wichman H (2001) Applied evolution. Annual Review of Ecology and systematics: 183-217.

4. Thomassen HA, Fuller T, Buermann W, Mila B, Kieswetter CM, Jarrín‐V P, et al. (2011) Mapping evolutionary process: a multi‐taxa approach to conservation prioritization. Evolutionary Applications 4: 397-413.

5. Myers N, Knoll AH (2001) The biotic crisis and the future of evolution. Proceedings of the National Academy of Sciences 98: 5389-5392.

6. Shoo LP, Hoffmann AA, Garnett S, Pressey RL, Williams YM, Taylor M, et al. (2013) Making decisions to conserve species under climate change. Climatic Change: 1-8.

7. Hekkala ER, Amato G, DeSalle R, Blum MJ (2010) Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (*Crocodylus niloticus*) populations. Conservation Genetics 11: 1435-1443.

8. Dever JA, Strauss RE, Rainwater TR, McMurry S, Densmore LD (2002) Genetic diversity, population subdivision, and gene flow in Morelet's

crocodile (Crocodylus moreletti) from Belize, Central America. Copeia: 1078-1091.

9. Griffith SC (2010) The role of multiple mating and extra-pair paternity in creating and reinforcing boundaries between species in birds. Emu 110: 1-9.
10. Rodriguez D, Forstner MRJ, Moler PE, Wasilewski JA, Cherkiss MS, Densmore L (2011) Effect of human-mediated migration and hybridization on the recovery of the American crocodile in Florida (USA). Conservation Genetics 12: 449-459.
11. Sugg DW, Chesser RK (1994) Effective population sizes with multiple paternity. Genetics 137: 1147-1155.
12. Weir LK, Breau C, Hutchings JA, Cunjak RA (2010) Multiple paternity and variance in male fertilization success within Atlantic salmon Salmo salar redds in a naturally spawning population. Journal of Fish Biology 77: 479-493.
13. Dyrcz A, Wink M, Backhaus A, Zdunek W, Leisler B, Schulze-Hagen K (2002) Correlates of multiple paternity in the Aquatic Warbler (Acrocephalus paludicola). Journal Fur Ornithologie 143: 430-439.
14. Borkowska A, Borowski Z, Krysiuk K (2009) Multiple paternity in free-living root voles (Microtus oeconomus). Behavioural Processes 82: 211-213.
15. Gryczynska-Siemiatkowska A, Gortat T, Kozakiewicz A, Rutkowski R, Pomorski J, Kozakiewicz M (2008) Multiple paternity in a wild population

of the yellow-necked mouse Apodemus flavicollis. Acta Theriologica 53: 251-258.

16. Myers E, Zamudio K (2004) Multiple paternity in an aggregate breeding amphibian: the effect of reproductive skew on estimates of male reproductive success. Molecular Ecology 13: 1951-1963.
17. Joseph J, Shaw P (2011) Multiple paternity in egg clutches of hawksbill turtles (Eretmochelys imbricata). Conservation Genetics 12: 601-605.
18. Laloi D, Richard M, Lecomte J, Massot M, Clobert J (2004) Multiple paternity in clutches of common lizard Lacerta vivipara: data from microsatellite markers. Molecular Ecology 13: 719-723.
19. Meister B, Ursenbacher S, Baur B (2012) Frequency of multiple paternity in the grass snake (Natrix natrix). Amphibia-Reptilia 33: 308-312.
20. Davis LM, Glenn TC, Elsey RM, Dessauer HC, Sawyer RH (2001) Multiple paternity and mating patterns in the American alligator, Alligator mississippiensis. Molecular Ecology 10: 1011-1024.
21. Muniz FL, Silveira RD, Campos Z, Magnusson WE, Hrbek T, Farias IP (2011) Multiple paternity in the Black Caiman (Melanosuchus niger) population in the Anavilhanas National Park, Brazilian Amazonia. Amphibia-Reptilia 32: 428-434.
22. McVay JD, Rodriguez D, Rainwater TR, Dever JA, Platt SG, McMurry ST, et al. (2008) Evidence of multiple paternity in Morelet's Crocodile (Crocodylus moreletii) in Belize, CA, inferred from microsatellite markers.

Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 309: 643-648.

23. Sanderson EW, Redford KH, Chetkiewicz CLB, Medellin RA, Rabinowitz AR, Robinson JG, et al. (2002) Planning to save a species: the jaguar as a model. Conservation Biology 16: 58-72.
24. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006) Regional habitat conservation priorities for the American crocodile. Biological Conservation 128: 25-36.
25. Bossart J, Pashley Prowell D (1998) Genetic estimates of population structure and gene flow: limitations, lessons and new directions. Trends in Ecology & Evolution 13: 202-206.
26. Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, et al. (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics: 489-522.
27. Wright S (1943) Isolation by distance. Genetics 28: 114.
28. Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics 145: 1219-1228.
29. Brochu CA (2000) Phylogenetic relationships and divergence timing of Crocodylus based on morphology and the fossil record. Journal Information 2000.
30. Brochu CA (2003) Phylogenetic approaches toward crocodylian history. Annual Review of Earth and Planetary Sciences 31: 357-397.

31. Kohn AJ (1989) Natural history and the necessity of the organism. American Zoologist 29: 1095-1103.

32. Ross CA, Magnusson WE (1989) Living crocodilians.

33. Mazzotti FJ, Brandt LA, Moler P, Cherkiss MS (2007) American Crocodile (*Crocodylus acutus*) in Florida: Recommendations for endangered species recovery and ecosystem restoration. Journal of Herpetology 41: 122-132.

34. Thorbjarnarson J (1989) Ecology of the American Crocodile, Crocodylus acutus. In Crocodiles, Their Ecology, Management and Conservation A Spetial Publication of the Crocodile Specialist Group IUCN Publications NS Gland, Switzerland: 228-258.

35. Alonso Tabet M (2009) Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante & Universidad de Pinar del Rio Hermanos Saiz Montes de Oca.

36. IUCN (2013) IUCN Red List of Threatened Species. Version 2012.2. .

37. Meredith RW, Hekkala ER, Amato G, Gatesy J (2011) A phylogenetic hypothesis for *Crocodylus* (Crocodylia) based on mitochondrial DNA: evidence for a trans-Atlantic voyage from Africa to the New World. Molecular Phylogenetics and Evolution 60: 183-191.

38. Brochu CA, Njau J, Blumenschine RJ, Densmore LD (2010) A new horned crocodile from the Plio-Pleistocene hominid sites at Olduvai Gorge, Tanzania. PLoS One 5: e9333.

39. Brochu CA (2009) Phylogenetic relationships and divergence timing of *Crocodylus* based on morphology and the fossil record.

40. Milian-Garcia Y, Venegas-Anaya M, Frias-Soler R, Crawford AJ, Ramos-Targarona R, Rodriguez-Soberon R, et al. (2011) Evolutionary History of Cuban Crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* Inferred From Multilocus Markers. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 315A: 358-375.

41. Milián-García Y, Ramos-Targarona R, Pérez-Fleitas E, Sosa-Rodríguez G, Guerra-Manchena L, Alonso-Tabet M, et al. (2014) Genetic evidence of hybridization between the critically endangered Cuban crocodile and the American crocodile: implications for population history and in situ/ex situ conservation. Heredity.

42. Cedeño-Vazquez JR, Rodriguez D, Calme S, Ross JP, Densmore LD, Thorbjarnarson JB (2008) Hybridization Between *Crocodylus acutus* and *Crocodylus moreletii* in the Yucatan Peninsula: I. Evidence From Mitochondrial DNA and Morphology. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 309A: 661-673.

43. IUCN (2010) IUCN Red List of Threatened Species. Crocodylus acutus. Crocodile Specialist Group.

44. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006) Regional habitat conservation priorities for the American crocodile. Biological Conservation 128: 25-36.

45. Thorbjarnarson J (2010) American Crocodile *Crocodylus acutus*. Crocodiles Status Survey and Conservation Action Plan. Third Edition ed. Darwin: S.C. Manolis and C. Stevenson. Crocodile Specialist Group. pp. Pp. 46-53.

46. Thorbjarnarson J (1988) The status and ecology of the American crocodile in Haiti. Bulletin of the Florida State Museum of Biological Sciences 33: 1-86.

47. Schubert A, Santana G (1996) Conservation of the American crocodile (*Crocodylus acutus*) in the Dominican Republic. Contributions to Herpetology 12: 425-433.

48. Schubert A (2012) Lagos Enriquillo y Azuei : donde la naturaleza siempre tiene una sorpresa; Espanola EA, editor: LAP LAMBERT Academic Publishing GmbH & Co. KG.

49. Machkour-M'Rabet S, Henaut Y, Charruau P, Gevrey M, Winterton P, Legal L (2009) Between introgression events and fragmentation, islands are the last refuge for the American crocodile in Caribbean Mexico. Marine Biology 156: 1321-1333.

50. Platt SG, Thorbjarnarson JB (2000) Status and conservation of the American crocodile, Crocodylus acutus, in Belize. Biological Conservation 96: 13-20.

51. Thorbjarnarson JB, Hernandez G (1993) Reproductive ecology of the Orinoco crocodile (Crocodylus intermedius) in Venezuela 2. Reproductive and social behavior. Journal of Herpetology 27: 371-379.

52. Mazzotti FJ (1989) Factors affecting the nesting success of the American crocodile, Crocodylus acutus, in Florida Bay. Bulletin of Marine Science 44: 220-228.

53. Platt SG, Thorbjarnarson JB (2000) Nesting ecology of the American crocodile in the coastal zone of Belize. Copeia: 869-873.

54. Alonso Tabet M, Rodriguez Soberón R, Thorbjarnarson J, Ramos Targrarona R (2006) American crocodile (*Crocodylus acutus*) egg losses related to nesting females behaviour, at the Delta del Cauto Wildlife Refuge, Cuba.

55. Ogden JC (1978) Status and Nesting of the American Crocodile, Crocodylus acutus, (Reptilia Crocodilidae) in Florida. . Jour Herpet 12: 183-196.

56. Alvarez del Toro M (1974) Los crocodylia de Mexico. Mexico D.F.

57. Medem F (1981) Los Crocodylia de Colombia In: Colombia UNd, editor. Los Crocodylia de Sur America. Bogota, Colombia. pp. 354

58. Alonso-Tabet M (2009) Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante and Universidad de Pinar del Rio Hermanos Saiz Montes de Oca.

59. Rodriguez Soberón R, Alonso Tabet M, Álvarez VB, Verdade L, Larriera A (2002) Nidificación del cocodrilo Americano (Crocodylus acutus Cuvier) en el Refugio de Fauna Monte Cabaniguan, Cuba. Conservao e Manejo de Jacarés e Crocodilos da América Latina 2: 135-156.

60. Espinosa-Blanco AS, Seijas AE, Hernández O (2013) Egg collectIon as a conservation tool of Orinoco crocodille (*Crocodylus intermedius*) in the Cojedes river system, Venezuela. Interciencia 38: 358-363.
61. Antelo R, Ayarzagüena J, Castroviejo J (2010) Reproductive ecology of Orinoco crocodiles (*Crocodylus intermedius*) in a newly established population at El Frío Biological Station, Venezuela. The Herpetological Journal 20: 51-58.
62. Barros T, Jiménez-Oraá M, Heredia HJ, Seijas AE (2010) Artificial incubation of wild-collected eggs of American and Orinoco crocodiles (*Crocodylus acutus* and *C. intermedius*), Guárico and Zulia, Venezuela. Conservation Evidence 7: 111-115.
63. Gumilla J (1745) El Orinoco Ilustrado y Defendido, Historia Natural, Civil, y Geographica de este Gran Rio: Manuel Fernandez, editor.
64. Thorbjarnarson JB (1992) Recent investigations of the status and distribution of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. Biological Conservation 62: 179-188.
65. Casal AC, Fornelino MM, Restrepo MFG, Torres MAC, Velasco FG (2013) Uso histórico y actual del caimán llanero (*Crocodylus intermedius*) en la Orinoquia (Colombia-Venezuela). Biota Colombiana 14: 65-82.
66. Medem F (1981) Los Crocodylia de Sur América, Vol. I. Los Crocodylia de Colombia Editorial Carrera 7a Ltda, Bogotá.

67. Antelo R (2008) Biología del Cocodrilo o Caimán del Orinoco (*Crocodylus intermedius*) en la Estación Biológica El Frío, Estado Apure (Venezuela): Tesis Doctoral. Universidad Autónoma de Madrid, Spain.

CHAPTER I

INTRASPECIFIC VARIATION IN THE REPRODUCTIVE ECOLOGY OF THE AMERICAN CROCODILE (*CROCODYLUS ACUTUS*) ACROSS LOCAL AND REGIONAL SCALES

Introduction

Animal mating systems, reproductive strategies and reproductive behavior can vary across space and time, differing among subpopulations within species across local and regional scales [1-6]. Differences encountered across geographically distinct populations have included several aspects of vertebrate's reproductive life histories, such as varying levels of multiple paternity [7], extra pair paternity [8], social monogamy [9], nesting success, female nesting behavior [10-13], and parental care [14].

Environmental and demographic factors such as density of breeding females [15], sex ratios [16], temperature [17], and edaphic features of nesting areas [11, 18] have proven to influence the reproductive ecology of vertebrate taxa. Among abiotic environmental features, temperature has been one of the most common factors directly or indirectly affecting the breeding dynamics of vertebrate species [19, 20] For instance, temperature has been correlated with increasing levels of promiscuity in fish [6] and lizards [17, 21], rates of sperm production in crocodiles [22], and rates of extra pair paternity in birds [23]. As major global environmental changes have been projected for the next half century [24], including average global temperature increases of 2-5°C [25, 26], sea level increases of 80 cm or more [27, 28], changes in global precipitation, and the geographic range, frequency, timing, and intensity of hurricanes [24, 29], a comprehensive understanding of species' reproductive ecology will be crucial for their management and conservation.

Ectothermic taxa (cold-blooded animals) appear to be especially vulnerable to environmental change, as critical ontogenetic, reproductive and life history traits are tightly linked to environmental factors (e.g. environmental sex determination, beginning and duration of breeding season, rate of sperm and egg production, embryo development, etc.) [21, 30-33]. In oviparous vertebrates the environment where the nest is placed impacts embryonic development and survival. And therefore nest-site selection can be an important component of fitness [34]. Nest failure rates for turtles [35] and crocodiles [10, 36] are often as high as 90% and 40% respectively, as many nests succumb to flooding, desiccation and predation. Even for successful nests, edaphic characteristics of the nest and incubation temperature can affect hatchling phenotype, including variations in size, shape, color, sex, locomotor performance, metabolism, growth rates and survival [37-41].

In crocodilians, temperature is highly linked to key ontological traits. Duration of the breeding season, sex ratios at birth, and time of egg and embryo development are at some degree influenced by temperature [42]. In the American alligator (*Alligator mississippiensis*) temperature influences both the initiation and speed of spermatogenesis [22]. For the American crocodile (*Crocodylus acutus*) in Florida Bay, the incubation period is bracketed by the low temperatures of winter and high temperatures of late summer [13]. The precision of this timing allows avoiding the dry season, when desiccation represents a threat for egg development [13]. In the coastal wetlands of southeastern Cuba, nesting areas differing in height, soil substrate, and protection against wave action exhibit different levels of nesting success in *C. acutus* [43]. Sheltered nesting areas with coarse, calcareous sand have more nests and higher hatching rates than those with fine marly sand, with a high degree of compactness and water retention [43].

The American crocodile is primarily a coastal crocodilian widely distributed along the coastal swamps, estuarine rivers, lakes and reservoirs of the Neotropical Region of the Americas [44] (Figure 1). *Crocodylus acutus* plays an important ecologic role as top predator and “landscape engineer” of wetland ecosystems, controlling both the flux of energy and the spatial configuration of the landscape, and thus significantly impacting a suite of other species within it [36, 45, 46]. It extends from Mexico to Peru in the Pacific, and from the southern tip of Florida to Venezuela in the Atlantic, including most of the continental and insular Caribbean [46]. *Crocodylus acutus* overlaps its geographic range with three other new world crocodilian species: the Cuban crocodile (*Crocodylus rhombifer*) in Zapata, Cuba [47], the Morelet’s crocodile (*Crocodylus moreletii*) in the Yucatan Peninsula, Mexico [48] and Belize [49], and the Orinoco crocodile *(Crocodylus intermedius)* in Venezuela [50]; hybridizing in the wild with *C. moreletii* [51, 52] and *C. rhombifer* [47, 53].

Crocodylus acutus is categorized globally as vulnerable by the IUCN [54] and listed in CITES Appendix I [55], except for the Cuban population transferred to Appendix II in 2007 [56]. Although *C. acutus* inhabits mainly brackish water coastal habitats such as the saltwater sections of rivers, coastal lagoons, and mangrove swamps [56], it also occurs in freshwater areas located well inland. Including a significant population from Lago Enriquillo, a landlocked hypersaline lake situated 40 meters below sea level in the arid southwestern Dominican Republic [57-59].

Crocodylus acutus can only mate in water [60], and typically exhibits an annual reproductive season, with females laying eggs annually or bi-annually [61]. Courtship also takes place in water, and is usually initiated by the female, which typically perform a stereotyped sequence of behaviours encompassing snout-lifting, swimming in slow circles around the male, or placing her head on the snout or back of the male [62]. Males, whose displays include a series of one to three headslap, frequently respond to this activity by emitting a very low frequency sound, or sub-audible vibration (SAV) [62]. The SAV is given from a typical "head-emergent tail-arched" posture, and causes the water on the males back to agitate upwards in what has been termed a "water dance" in alligators [63]. Following the SAV are a further series of behaviours that include snout lifting and rubbing, bubbling, and temporary submergences. Copulation is done in shallow water and usually lasts several minutes [62, 64].

Indirect evidence of a polygynous mating system was found in Etang Saumatre, Haiti, [57] studies of captive American crocodiles in Florida [62, 64], and observations made on wild crocodiles in Mexico [65] and the Dominican Republic [58]. According to these observations, dominant males establish breeding territories, exclude other adult males, and mate with multiple females [36, 46]. Male territorial defence is composed of a series of stereotyped postures, frequently followed by actual or mock fighting. Territorial intruders are often approached in a head-emergent, tail-arched posture prior to fighting [64]. Combat frequently includes lunges and chases. Following an aggressive encounter, dominant males commonly assume a raised "inflated posture"[62, 64].

Thorbjarnarson [57, 66] located twenty-seven adult crocodiles in four distinct groups adjacent to the major nesting beaches one month prior to the peak oviposition period in Etang Saumatre, Haiti. The groups were well defined during the day, with a mean intergroup distance of 4.7 kilometres, but at night would break up as individuals dispersed along the shoreline [57, 66]. These groups were inferred to be courtship assemblages, containing one large male, and several smaller adults (presumably females but possibly including subdominant males) [57]. Similar observations done by Alonso Tabet [36] in the Wildlife Refuge Monte Cabaniguán, Cuba, indicate the formation of harems during the months of December and January. Males intercept females in quiet waters of the interior lagoons and reservoirs of the coastal wetland, where food is abundant [36]. Alonso Tabet [36] reported a total of 25 males during the breeding season, 17 of which monopolize at least one female. The number of females found per male varied from one to four, and resulted positively correlated with male size. In spite of observations in the wild [36, 57, 62, 64], genetic analyses of parentage showed multiple paternity in the American alligator [67], Morelet's crocodile [68], broad-snouted caiman (*Caiman latirostris*) [69], and black caiman (*Melanosuchus niger*) [70].

Crocodylus acutus primarily nests in holes, depending on factors supposedly related to nest site conditions; it might also make mound nests [71-73]. For instance, the use of soil or mangrove peat "mound" nests have been documented in Florida [13, 72, 73], as well as reports of *C. acutus* making small mound nests of scraped up vegetative litter. Females build mound nests by digging a shallow hole into which the eggs are laid, afterwards covering it with leaf litter, grass or dead branches, forming a definite mound [65, 74]. Campbell [75] describes that mound nests are found most frequently in species that inhabit low-lying areas, swamps and marshes, whereas hole nests are more common in species where elevated sandy areas or high up river banks are available for nesting.

Across *C. acutus* geographic range, demographic and environmental factors vary considerably [55, 58, 76-78]. Population size estimates range from less than 140 individuals in Portete Bay, Colombia [79], to thousands of individuals in the coastal wetlands of Delta del Cauto, Cuba. Relative abundances range from 7.96 to 16.32 crocodiles per kilometer, and maximum abundances can be as high as 34 crocodiles per kilometer [80]. *Crocodylus acutus* is found in the hypersaline environment (70 parts per million) Lago Enriquillo located in the Dominican Republic 40 meters below sea level [58, 59], as well as offshore cays and coral atolls [46]. These variations can occur in both local and regional scales.

Local comparisons

With an extension of 626 Km², the Wildlife Refuge Monte Cabaniguán (WRMC) encompasses the most important coastal wetland ecosystems of Eastern Cuba [10] (Figure 2). It is located along the littoral zone of the Gulf of Guacanayabo, provinces Las Tunas and Granma, in the lower part of river basins that join to form a continuous and complex delta system of estuaries, lakes and mangrove swamps [80]. This area sustains the largest population of the American crocodile in its entire distribution [78, 81]. More than 150 crocodile nests hatch each year in nesting areas within this wildlife refuge [36].

The formation of harems in the WRMC occurs during the months of December and January. Males defend territories in quiet waters of the interior lagoons and reservoirs of the coastal wetland, which are frequently transit by females [36]. Approximately 25 males establish territories during the breeding season, 68% of which monopolize at least one female. The number of females found per male varies from one to four, being three the most frequent number of females per male [36]. Alonso Tabet [36] reported a strong positive correlation between the size of males and the number of females per harem, with males exceeding total lengths of 290 cm monopolizing the highest number of females [36].These observations suggest the occurrence of males hierarchies, and are in agreement with studies of captive crocodiles in Florida Bay [62, 64], and observations made on wild crocodiles in Mexico [65] , Dominican Republic [58], and Haiti [57, 66].

Crocodylus acutus nesting in this wetland is concentrated on a few beaches scattered along the gulf shoreline, and in a two-hectares patch of raised land within the mangrove swamp, situated 2 kilometers inland [36, 81]. Five of these nesting areas are permanent, used every year for nesting [36]. Given the low availability of nesting areas and high density of crocodiles, nesting is performed in a gregarious manner and at high densities [10]. Additionally, in the WRMC, elevation, levels of exposure to the action of tides, edaphic and other environmental features vary among nesting sites [11].

Crocodylus acutus nesting season at the WRMC takes place from February through July, and includes (a) nest preparation and oviposition (last week of February through March), (b) incubation (February through May), (c) hatching (May through July), and (d) post-hatching [36]. The timing of hatching coincides with the initiation of the rainy season, following the typical pattern of hole-nesting crocodiles which normally nest during periods of falling water levels (e.g. the dry season), with hatching taking place towards the beginning of the rainy season [46].

Nest preparation is usually done in several attempts, where females first dig shallow holes, or “test holes”, at a depth of 5 - 15 centimeters below the surface [36]. Females oviposit in cavities dug in soil along elevated portions of the sandy shoreline beaches and in patches of raised land within the mangrove swamp. Females have reported to be exclusively hole nesters in this locality, as well as the whole Cuban archipelago [80]. Nesting sites differ in height, soil texture and protection against wave action, as well as in disturbance regimes of flooding, high waves and tides [43]. Two of the five nesting sites, Jobabito and Salina (Figure 2), are located in elevated, well-drained nesting substrate consisting mostly coarse, calcareous sand. These sites remain protected from the effects of wind and wave action, and typically exhibit high number of nests and hatching rates. Three other nesting sites, La Jijira, Soloburén and Ojo de Agua (Figure 2), are located in areas of lower elevations, and present obscure, fine marly sand, with a high degree of compactness and water retention. La Jijira and Ojo de Agua constitute sites of low nest productivity, with many nests succumbing to flooding [43].

Besides local environmental differences among nesting sites, nesting female's behavior has proven to affect nest productivity at the WRMC [12]. Alonso Tabet [36, 82] reports an annual average of 31.1 % of the nests and 26.1% of the total egg production being lost between 1993 and 2001 as a result of nesting female's behavior, with some years presenting losses exceeding 50% of the total egg production. These "negative" female behaviors include (a) excavation of prior nests by females, while laying their eggs, or while assisting their hatchlings in nearby nests, (b) laying on the surface of the nesting beach instead of in excavated nests (discarded nests), and (c) females non returning to the nesting beaches to assist hatchlings going out from the nests [12, 36]. Behaviors (b) and (c) are thought to be consequence of high density nesting conditions resulting from a large population having to nest on a limited area of suitable beaches. These results are supported by previous observations made by Varona [83] , which indicate that in Cuba nesting females are territorial, and up to five to six females may compete for one nest site. In other parts of its range, however, such as Florida Bay [71, 84], the Dominican Republic [58] and Haiti [57, 66], females *C. acutus* are apparently less territorial around the nest site and may nest colonially or in small groups.

Incubation time ranges from 75 to 95 days at the WRMC, and nest visits by females are most frequent during the first weeks of the incubation period, after which visits drastically drop until hatching emergence [36]. Female aggression has been reported, with females actively protecting nests and excluding other crocodiles from the nest locale [36]. Although this behavior is individually variable, it has been found in *C. acutus* populations in Mexico [65] and Panama [85]. In turns, in other areas such as Florida Bay [73] and Haiti [57, 66] females provide little or no nest protection. There is no evidence that males play any role in nest defense in the wild.

An average number of 188 nests are laid every year at the WRMC, with values of nesting and hatching success of 64% and 66% respectively [36]. Studies have shown that six major factors adversely affect nesting success and hatching success of crocodilians [13, 57, 66, 73, 86, 87]. These include infertility, predation, temperature extremes, moisture conditions, erosion of nest sites, and human disturbance to nest sites or breeding animals. Differently from other localities such as Florida Bay, where raccoon (*Procyon lotor*) predation is among the main causes of nesting failure [73], at the WRMC is rare [80]. Rodriguez

Soberón [80] reported three nest predation events for the entire period of 1993 to 2003, two of the nests being attacked by ants and one by a turkey Vulture (*Cathartes aura aura*). On the other hand, Rodriguez Soberón [80] found fertility values of approximately 90%, similar to those of Florida Bay [88]. Additionally, due to the high governmental protection and limited access to this wildlife refuge, human disturbance to nest sites or breeding animals is almost inexistent. Main factors affecting nesting success are related to moisture conditions, as many nests succumb to flooding, and “negative” nesting female’s behavior [12, 36]. The latter primarily consisting in excavation of prior nests, discarded nests, and nest desertion [12]. Other factors affecting nesting and hatching success in this locality are given by drastic transformations of the geomorphology of nesting areas caused by severe weather events such as tropical storms and hurricanes [80].

Based on 636 observations of reproductive females upon hatchling emergence, Alonso Tabet [89] concluded that post-hatching care is frequent at the WRMC. When post-hatchling attendance occurs, reproductive females exhibit different behaviors, with the most frequent being: (a) females leaving neonates for two or three days in lagoons or salt marshes adjacent to nesting areas, and then transporting them to estuaries and interior lagoons within the mangrove, (b) females accompanying their offspring through the marshes, upstream, immediately after hatching, and (c) females hiding neonates in red mangrove roots along the estuary shores [36]. High levels of maternal care of recently hatched *C. acutus*, in combination with high density nesting conditions, limited area of suitable nesting sites, elevated rates of egg losses and high hatching success are features that characterize the reproductive ecology of the species at the WRMC [36, 80].

Regional comparisons

Table 1 summarizes *C. acutus* reproductive ecology traits across populations within its geographic range. These are among the best-studied populations where data are available, and include Florida Bay (United States), the Wildlife Refuge Monte Cabaniguán (Cuba), Etang Saumatre (Haiti), Lago Enriquillo (Dominican Republic), Turneffe Atoll (Belize), and Banco Chinchorro Atoll (Mexico).

The timing of nesting of hole-nesting crocodilian species generally follows a typical pattern with nesting occurring during the dry season, and hatching taking place near the beginning of the rainy season [46]. Oviposition during the dry season reduces the probability of nest flooding, and results in neonates emerging during a time of increasing habitat and food availability at the onset of the rainy season [90]. Besides higher resource abundance, rainfall events help rehydrate hatchlings after emergence [91], as neonates cannot maintain body mass under saline conditions [92]. *Crocodylus acutus* nesting season tends to follow this temporal pattern (Table 1) [13, 89, 93], although there is a considerable amount of variation in the timing of egg-laying in relation to rainfall and water level schedules, with nests in some regions hatching prior to the beginning of the rainy season [85, 94], and in others during the early to mid-rainy season [36, 65].

In southern Florida the nesting season takes place from March through August, with egg-laying occurring in April-May somehow delayed by low winter and spring temperatures [13]. Neonates hatch in July-August, at the beginning of the rainy season [13, 73, 88]. Interestingly, captive crocodiles in southern Florida that originated from Jamaica follow the typical Florida nesting schedule with egg-laying taking place in April-May [62], suggesting a degree of plasticity of the timing of nesting with respect to environmental factors. Differently, the islands of Cuba and Hispaniola share a Caribbean climatic pattern, with *C. acutus* laying eggs primarily from February to March and hatching occurring in June-July, during the first half of the rainy season [43, 58, 59, 78]. The notable exception to this is in Etang Saumatre, Haiti, where nests are constructed in late January and early February, and hatch in late April-early May at the very beginning of the rainy season [57, 66].

In Yucatan, Mexico, the Turneffe Atoll, Belize, and along the Caribbean coast south to Costa Rica, *C. acutus* lay eggs from March to May, and eggs hatch during the first half of the rainy season during June-August [65, 95, 96]. In other localities such as Panama, the Pacific coast of Costa Rica and Colombia, crocodiles tend to hatch somewhat earlier than the typical nesting pattern, prior to the initiation of the rain, even though the rainfall patterns are similar [74, 85, 94, 97, 98].

Female minimum reproductive size varies considerably throughout the range of the species (Table 1). In Mexico, Alvarez del Toro [65] reported never finding a reproductive female less than 2.8 meters of total length (TL). Similarly, in Cuba, the reported minimum reproductive size of females is 2.7-3.0 meters TL [83]. South Florida follows a similar trend, with estimations of female minimum reproductive size ranging from 2.25 meters TL [71] to 2.95 meters TL [73]. However, in other areas females make nest at much smaller sizes. For instance, in Etang Saumatre, Haiti, females begin nesting at around 2.2-2.3 meters TL, never exceeding total lengths of 2.5 meters [57, 66]. A similar smaller minimum reproductive size in females was noted for nearby Lago Enriquillo in the Dominican Republic [58, 66]. The extensive variation reported for this trait across *C. acutus* geographic range suggests that minimal size for reproduction should be considered as a population parameter, which could reflect environmental or genetic differences in growth rates, and/or age at sexual maturity of females.

Although *C. acutus* is considered primarily a hole-nesting species and hole nests are found in all nesting localities, regional variation in nesting mode occurs throughout the species range (Table 1). While some localities exhibit an exclusive hole-nesting mode (WRMC, Etang Saumatre, and Lago Enriquillo), others present both hole and mound nests (Florida Bay, Turneffe Atoll, and Banco Chinchorro Atoll). In fact, *C. acutus* appears to be one of the most adaptable of crocodilians in terms of nesting requirements and has been known to nest in a variety of situations [46]. For instance, female *C. acutus* in south Florida use soil or mangrove peat to build mound nests [75, 99, 100]. Ogden [99] reported that 13 of 14 primary nest sites investigated had significant elevations (mounds) above the surrounding terrain. The elevations ranged from 9 to 65 centimetres (mean= 31 centimetres) and were apparently not correlated with location or soil type. Longest diameters of nest mounds ranged from 1 to 4.6 meters (mean= 2.4 meters, N=14). Additionally, eight of the seventeen nests studied by Mazzotti [100] in Florida Bay were classified as "mound" nests. During the course of this study subterranean flooding was found to be a major source of egg mortality. Under these circumstances mounding may be an adaptive feature reducing the probability of nest flooding [100].

The ecological significance of hole versus mound nesting was discussed by Campbell [75], who refuted the phylogenetic importance attributed to nesting mode described by Schmidt (1924) and Greer (1970), in which mound-building habits are indicative of phylogenetic relationship in crocodilians. Campbell (1972) noted that mound nests are found most frequently in species that inhabit low-lying areas, swamps and marshes, and speculate that individual differences in nest morphometrics within a population may reflect the past nesting experience of specific females. However, of even greater important in defining nesting mode is the timing of nesting in relation to rainfall or water level variation [46]. True mound nesters (e.g. the Alligatoridae) typically nest in the rainy season, frequently during peak water levels. Differently, hole nesting crocodiles normally nest during periods of falling water levels (e.g. the dry season), with hatching taking place towards the beginning of the rainy season [61]. However, with a hole nesting species such as *C. acutus*, the tendency to "mound" nests may be an adaptive response to nesting in low-lying areas where the probability of nest flooding is high

Colonial nesting (more than one nest per nesting site) is found across *C. acutus* geographic range (Table 1). Ogden [99] reports the occurrence of nesting sites with up to two nests in Florida Bay. Similarly, five out of twelve nesting areas surveyed in coastal Belize showed evidence of colonial nesting, with nests at each area ranging from one to 10 [95]. However, most localities were colonial nesting is found presents low densities of nests per site. Colonial nesting in the sense of [90], where 20-30 nests could be found within a single nesting site, occurs primarily in the WRMC, Cuba [36, 43], and at a lesser extent in Lago Enriquillo, Dominican Republic [58]. Colonial nesting at the WRMC results from a combination of a large *C. acutus* population having to nest on a limited area of suitable beaches. In this locality, it is possible to observe up to 92 nests in a single nesting site, reaching nesting densities as high as 115 nests per hectare [43]. Besides high density of breeding females, Lang [64]hypothesized that the occurrence of colonial nesting may be influenced by the presence of territorial males near nesting grounds, as well as the quality and quantity of available nesting habitat.

Average number of nests per year ranged from 10.5 in Banco Chinchorro Atoll, Mexico [65, 96] to 188 in the WRMC, Cuba [36, 80] (Table 1). Lago Enriquillo and south Florida show intermediate values, with 36 and 22.5 average number of nests per year respectively [13, 58]. The number of nests laid per year is often used as a proxy for estimating the total population size [73, 80]. As the *C.*

acutus population at the WRMC presents numbers of nests per year exceeding all other populations by one order of magnitude, it is considered the largest and healthiest population across the species entire geographic range [36, 80].

Average clutch size is highest in south Florida (38.0) [13, 99], followed by Cuba (25.4) [89], Belize (22.5) [49], Haiti (22.5) [57], the Dominican Republic (22.3) [58, 59], and Mexico (16.2) [103, 104] (Table 1). Rodriguez Soberón [80] hypothesized that higher clutch sizes of *C. acutus* in Florida may constitute an adaptive strategy of continental populations subjected to more intense pressures of adverse environmental and ecological factors such as extreme temperatures and greater diversity of predators. As opposed to insular populations of *C. acutus* in Cuba and the Dominican Republic, exhibiting lower values of clutch sizes [80]. Additionally, Platt and Thorbjarnarson [49] found that mean clutch size was significantly less in Belize and Haiti than in Florida. Mean egg mass among localities ranges from 80 grams in Mexico [65] to 97 grams in Haiti [57], with Belize (85.6 g) [49] and the Dominican Republic (83.6 g) [58] having similar values to those found in Mexico (80.0 g) [65]; and Cuba (93.9 g) [80] having similar values to those found in Haiti [57]. According to Thorbjarnarson [105], the relationship of clutch size, egg mass, and clutch mass with female body size is highly variable among crocodilians, but these parameters tend to increase with increasing female body size. This trend is observed for *C. acutus* in Belize and the Dominican Republic, which present low values of clutch size, egg mass, and clutch mass and smaller females have been reported [95].

Nesting success is highest among *C. acutus* in Belize (80.0%), followed by Mexico (73.2%), Florida Bay (65-74%), Cuba (64%), and the Dominican Republic (52%) (Table 1). Conversely, hatching success result highest at the Dominican Republic (87%) followed by Cuba (70%), and Florida Bay (48-58%). While percentages of nonviable eggs seem to be similar across nesting localities, causes of nesting failure differ among them (Table 1). In Florida Bay, egg losses due to predation by raccoons and other animals constitute one of the main factors of nesting failure [13, 73]. Differently, egg losses due to negative female nesting behavior (e.g. excavation of prior nests, discarded nests, and nest desertion) in the WRMC, Cuba, can be as high as 50% of the total egg production [12, 36]. Embryonic mortality resulting from flooding and desiccation, and infertility are also common causes of nesting failure across *C. acutus* geographic range [12, 13, 65]. However, Platt and Thorbjarnarson [49], reported negligible losses due to predation and flooding in nesting areas of coastal Belize.

Nest defence behaviour by female *C. acutus* is highly variable [44]. In some localities such as Cuba [106] and Mexico [65] females actively defend nests, whereas in most areas little or no nest protection is provided [13, 57, 66, 95] (Table 1). Similarly, the degree of maternal care for recently hatched *C. acutus* appears to vary considerably throughout the species range. The results of most studies suggest that the formation of distinct pods of young and maternal care of neonates is minimal in this species [13, 66, 95]. However, under certain conditions the formation of pods may be influenced by the habitat into which the neonates are born. In areas exposed to wave action or in hypersaline water the dispersal of hatchlings is almost immediate and there is virtually no group cohesion among the hatchlings. Nevertheless, in some areas such as the WRMC, unusually high levels of maternal care have been reported, with females frequently assisting hatching emergence and transporting neonates to estuaries and interior lagoons within the mangrove, or into red mangrove roots along the estuary shores for protection [36].

Summary

Animal reproductive strategies and behavior show an exceptional degree of variability across space and time, differing among subpopulations within species across local and regional scales [1-6]. This variability is usually related to environmental and demographic factors, as well as traits of the species themselves [15-17]. And it is even more evident in ectothermic taxa (cold-blooded animals) such as crocodilians, where critical ontogenic, reproductive and life history traits are tightly linked to environmental factors (e.g. temperature sex determination, beginning and duration of breeding season, rate of sperm and egg production, embryo development, etc.) [46, 90, 91, 107].

Of all new world crocodilians, the American crocodile exhibits the highest levels of variation in terms of its reproductive ecology [46]. In fact, *C. acutus* appears to be one of the most adaptable of crocodilians in terms of nesting requirements, nest mode, timing of egg-laying, female minimum reproductive size, female nest defence behaviour, and female parental care [12, 13, 36, 46, 57, 95]. For instance, although primarily a hole nesting species, *C. acutus* is able to build mound nests in south Florida [13, 73], coastal Belize [95] and Mexico [65, 74] probably as an adaptive response to nesting in low-lying areas where the probability of nest flooding is high [46]. *Crocodylus acutus* nesting can be done solitary [73] or colonially, with up to 90 females laying eggs in a single site [36, 108]

Reproductive traits vary across local and regional scales for *C. acutus* [109]. For instance, values of nesting and hatching success, as well maternal care of neonates varies across nearby nesting beaches in southeastern Cuba. Average number of nests per year ranges from 10.5 in Banco Chinchorro Atoll [96], Mexico, to 188 in the WRMC, Cuba. Whereas average clutch size is highest in south Florida, followed by Cuba, Belize, Haiti, the Dominican Republic, and Mexico. With higher clutch sizes of *C. acutus* in Florida being attributed to a potential adaptive strategy of continental populations subjected to more intense pressures of adverse environmental and ecological factors such as extreme temperatures and greater diversity of predators, as opposed to insular populations of *C. acutus* in Cuba and the Dominican Republic exhibiting lower values of clutch sizes [80].

Among populations of *C. acutus*, the local population at the WRMC, Cuba, is the largest and healthiest population across the species entire geographic range [36, 80]. High levels of maternal care of recently hatched *C. acutus*, in combination with high density nesting conditions, limited area of suitable nesting sites, elevated rates of egg losses and high hatching success are unique features of this species at this locality [36, 80]. The latter, along with a high population density, undisturbed performance of biologic cycles and minimum anthropogenic disturbance, make this place particularly important for comparative research, management and conservation of *C. acutus* across its geographic range [80].

Major global environmental changes have been projected for the next half century [24], including average global temperature increases of 2-5°C [25, 26], sea level increases of 80 cm or more [27, 28], changes in global precipitation, and the geographic range, frequency, timing, and intensity of hurricanes [24, 29]. In this context, a comprehensive understanding of *C. acutus* reproductive ecology is crucial for their management and conservation. Such information is not only important for better understanding the species reproductive ecology and their adaptability to changing environmental conditions, but also represents an essential element in planning effective conservation strategies for endangered crocodilians [50]. Long-term health of crocodile populations depend on continued nesting success [13, 46]. Finally, as *C. acutus* plays an important ecologic role as top predator and “landscape engineer” of wetland ecosystems [36, 45, 46], maintaining healthy populations of this species will help preserve biodiversity and key ecological processes in these highly valuable ecosystems of the world [110].

References

1. Neff BD, Pitcher TE, Ramnarine IW (2008).Inter-population variation in multiple paternity and reproductive skew in the guppy. Molecular Ecology; 17(12): 2975-84.

2. Hill CE, Gjerdrum C, Elphick CS (2010).Extreme levels of multiple mating characterize the maing system of the saltmarch sparrow (Ammodramus caudatus). Auk; 127(2): 300-7.

3. Ficetola GF, Padoa-Schioppa E, Wang J, Garner TWJ (2010).Polygyny, census and effective population size in the threatened frog, Rana latastei. Animal Conservation; 13: 82-9.

4. Schrader M, Travis J, Fuller RC (2011).Do density-driven mating system differences explain reproductive incompatibilities between populations of a placental fish? Molecular Ecology; 20(19): 4140-51.

5. Mobley KB, Jones AG (2007).Geographical variation in the mating system of the dusky pipefish (Syngnathus floridae). Molecular Ecology; 16(12): 2596-606.

6. Mobley KB, Jones AG (2009).Environmental, demographic, and genetic mating system variation among five geographically distinct dusky pipefish (Syngnathus floridae) populations. Molecular Ecology; 18(7): 1476-90.

7. Yue GH, Li JL, Wang CM, Xia JH, Wang GL, Feng JB (2010).High prevalence of multiple paternity in the invasive crayfish species, *Procambarus clarkii*. International Journal of Biological Sciences; 6(1): 107-15.

8. Olsen BJ, Greenberg R, Fleischer RC, Walters JR (2008).Extrapair paternity in the swamp sparrow, Melospiza georgiana: male access or female preference? Behavioral Ecology and Sociobiology; 63(2): 285-94.

9. McEachern MB, McElreath RL, Van Vuren DH, Eadie JM (2009).Another genetically promiscuous 'polygynous' mammal: mating system variation in Neotoma fuscipes. Anim Behav; 77(2): 449-55.

10. Alonso Tabet M, Rodriguez Soberón R (1998).Observations on nesting behavior of *Crocodylus acutus*. Crocodile Specialist Group Newsletter 17(1): 11-3.

11. Alonso Tabet M, Rodríguez Soberón R, Berovides Alvarez V, Hernández Fuentes CE, editors. Influencia de la geomorfología del hábitat sobre la nidificación de *Crocodylus acutus* en el Refugio de Fauna Monte Cabaniguán, Cuba. Varadero, Cuba2000.

12. Alonso Tabet M, Rodriguez Soberón R, Thorbjarnarson J, Ramos Targrarona R (2006).American crocodile (*Crocodylus acutus*) egg losses related to nesting females behaviour, at the Delta del Cauto Wildlife Refuge, Cuba.

13. Mazzotti FJ (1989).Factors affecting the nesting success of the American crocodile, Crocodylus acutus, in Florida Bay. Bull Mar Sci; 44(1): 220-8.

14. Charruau P, Henaut Y (2012).Nest attendance and hatchling care in wild American crocodiles (Crocodylus acutus) in Quintana Roo, Mexico. Anim Biol; 62(1): 29-51.

15. Stewart SLM, Westneat DF, Ritchison G (2010).Extra-pair paternity in eastern bluebirds: effects of manipulated density and natural patterns of breeding synchrony. Behavioral Ecology and Sociobiology; 64(3): 463-73.

16. Hulin V, Guillon JM (2007).Female philopatry in a heterogeneous environment: ordinary conditions leading to extraordinary ESS sex ratios. Bmc Evolutionary Biology; 7.

17. Olsson M, Wapstra E, Schwartz T, Madsen T, Ujvari B, Uller T (2011).In hot pursuit: fluctuating mating systems and sexual selecion in sand lizards. Evolution; 65(2): 574-83.

18. Soto KH, Trites AW (2011).South American sea lions in Peru have a lek-like mating system. Marine Mammal Science; 27(2): 306-33.

19. Baroiller JF, D'Cotta H, Bezault E, Wessels S, Hoerstgen-Schwark G (2009).Tilapia sex determination: Where temperature and genetics meet. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology; 153(1): 30-8.

20. Lane JE, Forrest MNK, Willis CKR (2011).Anthropogenic influences on natural animal mating systems. Animal Behaviour; 81(5): 909-17.

21. Olsson M, Schwartz T, Wapstra E, Uller T, Ujvari B, Madsen T, et al. (2011).Climate change, multiple paternity and offspring survival in lizards. Evolution; 65(11): 3323-6.

22. Gribbins KM, Elsey RM, Gist DH (2006).Cytological evaluation of the germ cell development strategy within the testis of the American alligator, Alligator mississippiensis. Acta Zool; 87(1): 59-69.

23. Bouwman KM, Komdeur J (2006).Weather conditions affect levels of extra-pair paternity in the reed bunting Emberiza schoeniclus. J Avian Biol; 37(3): 238-44.

24. Michener WK, Blood ER, Bildstein KL, Brinson MM, Gardner LR (1997).Climate Change, Hurricanes and Tropical Storms, and Rising Sea Level in Coastal Wetlands. Ecological Applications; 7(3): 770-801.

25. Mearns LO (1984).Extreme High-Temperature Events: Changes in their probabilities with Changes in Mean Temperature. Journal of climate and applied meteorology; 23(12): 1601-13.

26. Schneider B (2012).Climate model simulation visualization from a visual studies perspective. Wiley interdisciplinary reviews Climate change; 3(2): 185-93.

27. Jevrejeva S (2012).Sea level projections to AD2500 with a new generation of climate change scenarios. Global and planetary change; 80-81: 14-20.

28. Doney SC, Ruckelshaus M, Emmett Duffy J, Barry JP, Chan F, English CA, et al. (2012).Climate Change Impacts on Marine Ecosystems. Annual Review of Marine Science; 4(1): 11-37.

29. Knutson TR (2010).Tropical cyclones and climate change. Nature geoscience; 3(3): 157-63.

30. Janowitz SA, Fischer K (2011).Opposing effects of heat stress on male versus female reproductive success in Bicyclus anynana butterflies. Journal of Thermal Biology; 36(5): 283-7.

31. Sunday JM, Bates AE, Dulvy NK (2011).Global analysis of thermal tolerance and latitude in ectotherms. Proc R Soc B-Biol Sci; 278(1713): 1823-30.

32. Wang T (2011).Gas exchange in frogs and turtles: how ectothermic vertebrates contributed to solving the controversy of pulmonary oxygen secretion. Acta Physiologica; 202(3): 593-600.

33. Weber SB, Blount JD, Godley BJ, Witt MJ, Broderick AC (2011).Rate of egg maturation in marine turtles exhibits universal temperature dependence. Journal of Animal Ecology; 80(5): 1034-41.

34. Freedberg S, Ewert MA, Ridenhour BJ, Neiman M, Nelson CE (2005).Nesting fidelity and molecular evidence for natal homing in the freshwater turtle, Graptemys kohnii. Proceedings of the Royal Society B-Biological Sciences; 272(1570): 1345-50.

35. Wilbur HM, Morin PJ. Life history evolution in turtles Huey GRB, editor. New York: Liss Inc; 1988

36. Alonso Tabet M. Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante & Universidad de Pinar del Rio Hermanos Saiz Montes de Oca; 2009.

37. Nelson NJ, Thompson MB, Pledger S, Keall SN, Daugherty CH (2004).Egg mass determines hatchling size, and incubation temperature influences post-hatching growth, of tuatara Sphenodon punctatus. Journal of Zoology; 263: 77-87.

38. Gutzke WHN, Crews D (1988).Embrionic temperature determines adult sexuality in a reptile. Nature; 332(6167): 832-4.

39. Gutzke WHN, Packard GC (1987).nfluence of the hydric and thermal environments on eggs and hatchlings of bull snakes (Pitouphis melanoleucus). Physiological Zoology; 60(1): 9-17.

40. Gutzke WHN, Packard GC, Packard MJ, Boardman TJ (1987).Influence of hydric and thermal environments on eggs and hatchlings of painted turtles (Chrysemys picta). Herpetologica; 43(4): 393-404.

41. Spotila JR, Zimmerman LC, Binckley CA, Grumbles JS, Rostal DC, List A, Jr., et al. (1994).Effects of incubation conditions on sex determination, hatching success, and growth of hatchling desert tortoises, Gopherus agassizii. Herpetological Monographs; 8: 103-16.

42. Vitt LJ, Caldwell JP. Herpetology. Press/Elsevier A, editor. Boston2009.

43. Rodriguez Soberón R, Alonso Tabet M, Álvarez VB, Verdade L, Larriera A (2002).Nidificación del cocodrilo Americano (Crocodylus acutus Cuvier) en el Refugio de Fauna Monte Cabaniguan, Cuba. Conservao e Manejo de Jacarés e Crocodilos da América Latina; 2: 135-56.

44. Thorbjarnarson J (1989).Ecology of the American Crocodile, *Crocodylus acutus*. In Crocodiles, their Ecology, Management and Conservation IUCN Publications New Series Gland, Switzerland: 228-58.

45. Mazzotti FJ, Brandt LA, Moler P, Cherkiss MS (2007).American Crocodile (*Crocodylus acutus*) in Florida: Recommendations for endangered species recovery and ecosystem restoration. Journal of Herpetology; 41(1): 122-32.

46. Thorbjarnarson J (1989).Ecology of the American Crocodile, *Crocodylus acutus*. In Crocodiles, Their Ecology, Management and Conservation A Spetial

Publication of the Crocodile Specialist Group IUCN Publications NS Gland, Switzerland: 228-58.

47. Milian-Garcia Y, Venegas-Anaya M, Frias-Soler R, Crawford AJ, Ramos-Targarona R, Rodriguez-Soberon R, et al. (2011).Evolutionary History of Cuban Crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* Inferred From Multilocus Markers. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology; 315A(6): 358-75.

48. Machkour-M'Rabet S, Henaut Y, Charruau P, Gevrey M, Winterton P, Legal L (2009).Between introgression events and fragmentation, islands are the last refuge for the American crocodile in Caribbean Mexico. Marine Biology; 156(6): 1321-33.

49. Platt SG, Thorbjarnarson JB, Price A (2000).Nesting ecology of the American crocodile in the coastal zone of Belize. Copeia; 2000(3): 869-73.

50. Thorbjarnarson JB, Hernandez G (1993).Reproductive ecology of the Orinoco crocodile (Crocodylus intermedius) in Venezuela 2. Reproductive and social behavior. Journal of Herpetology; 27(4): 371-9.

51. Cedeño-Vazquez JR, Rodriguez D, Calme S, Ross JP, Densmore LD, Thorbjarnarson JB (2008).Hybridization Between *Crocodylus acutus* and *Crocodylus moreletii* in the Yucatan Peninsula: I. Evidence From Mitochondrial DNA and Morphology. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology; 309A(10): 661-73.

52. Rodriguez D, Cedeno-Vazquez JR, Forstner MRJ, Densmore LD (2008).Hybridization Between *Crocodylus acutus* and *Crocodylus moreletii* in the

Yucatan Peninsula: II. Evidence From Microsatellites. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology; 309A(10): 674-86.

53. Weaver JP, Rodriguez D, Venegas-Anaya M, Cedeno-Vazquez JR, Forstner MRJ, Densmore LD (2008).Genetic characterization of captive Cuban crocodiles (*Crocodylus rhombifer*) and evidence of hybridization with the American crocodile (*Crocodylus acutus*). Journal of Experimental Zoology Part a-Ecological Genetics and Physiology; 309A(10): 649-60.

54. IUCN (2010).IUCN Red List of Threatened Species. Crocodylus acutus. Crocodile Specialist Group.

55. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006).Regional habitat conservation priorities for the American crocodile. Biological Conservation; 128(1): 25-36.

56. Thorbjarnarson J. American Crocodile *Crocodylus acutus*. Crocodiles Status Survey and Conservation Action Plan. Third Edition ed. Darwin: S.C. Manolis and C. Stevenson. Crocodile Specialist Group; 2010. p. Pp. 46-53.

57. Thorbjarnarson J (1988).The status and ecology of the American crocodile in Haiti. Bulletin of the Florida State Museum of Biological Sciences; 33(1): 1-86.

58. Schubert A, Santana G (1996).Conservation of the American crocodile (*Crocodylus acutus*) in the Dominican Republic. Contributions to Herpetology; 12: 425-33.

59. Schubert A. Lagos Enriquillo y Azuei : donde la naturaleza siempre tiene una sorpresa. Espanola EA, editor: LAP LAMBERT Academic Publishing GmbH & Co. KG; 2012.

60. Martin S (2008).Global diversity of crocodiles (Crocodilia, Reptilia) in freshwater. Hydrobiologia; 595: 587-91.

61. Thorbjarnarson JB (1996).Reproductive characteristics of the order Crocodylia. Herpetologica; 52(1): 8-24.

62. Garrick LD, Lang JW (1977).Social Signals and Behaviors of Adult Alligators and Crocodiles. Integrative and Comparative Biology; 17(1): 225-39.

63. Vliet KA (1989).Social displays of the American alligator (*Alligator mississippiensis*). American Zoologist; 29(3): 1019.

64. Lang JW (1976).American crocodile courtship. American Zoologist; 16(2): 197-.

65. Alvarez del Toro M. Los crocodylia de Mexico. Mexico D.F.: 1974.

66. Thorbjarnarson J. Status and Ecology of the American Crocodile in Haiti. Gainesville: University of Florida; 1984.

67. Davis LM, Glenn TC, Elsey RM, Dessauer HC, Sawyer RH (2001).Multiple paternity and mating patterns in the American alligator, Alligator mississippiensis. Molecular Ecology; 10(4): 1011-24.

68. McVay JD, Rodriguez D, Rainwater TR, Dever JA, Platt SG, McMurry ST, et al. (2008).Evidence of Multiple Paternity in Morelet's Crocodile (*Crocodylus moreletii*) in Belize, CA, Inferred From Microsatellite Markers. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology; 309A(10): 643-8.

69. Amavet P, Rosso E, Markariani R, Ignacio Pina C (2008).Microsatellite DNA Markers Applied to Detection of Multiple Paternity in *Caiman latirostris* in

Santa Fe, Argentina. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology; 309A(10): 637-42.

70. Muniz FL, Da Silveira R, Campos Z, Magnusson WE, Hrbek T, Farias IP (2011).Multiple paternity in the Black Caiman (*Melanosuchus niger*) population in the Anavilhanas National Park, Brazilian Amazonia. Amphibia-Reptilia; 32(3): 428-34.

71. Mazzotti FJ. The ecology of Crocodylus acutus in Florida: Pennsylvania State Univ. ; 1983.

72. Campbell HW (1972).Ecological or phylogenetic interpretations of crocodilian nesting habitats. Nature; 238(5364): 404-&.

73. Ogden JC (1978).Status and Nesting of the American Crocodile, Crocodylus acutus, (Reptilia Crocodilidae) in Florida. . Jour Herpet 12(2): 183-96.

74. Medem F. Los Crocodylia de Colombia In: Colombia UNd, editor. Los Crocodylia de Sur America. I. Bogota, Colombia1981. p. 354

75. Campbell HW (1972).Ecological or phylogenetic interpretations of crocodilian nesting habits.

76. Cherkiss MS, Romanach SS, Mazzotti FJ (2011).The American Crocodile in Biscayne Bay, Florida. Estuaries and Coasts; 34(3): 529-35.

77. Hernandez-Hurtado H, Romero-Villaruel JD, Hernandez-Hurtado PS (2011).Population ecology of Crocodylus acutus in the estuarine systems of San Bias, Nayarit, Mexico. Revista Mexicana De Biodiversidad; 82(3): 887-95.

78. Varona L (1987).The Status of *Crocodylus acutus* in Cuba Caribbean Journal of Sciences 23(2): 256-9.

79. Espinosa MI, Bertin A, Gomez J, Mejia F, Guerra M, Baez L, et al. (2012).A three-year mark-recapture study in a remnant population of Crocodylus acutus Cuvier in Portete Bay (Guajira, Colombia). Gayana; 76(1): 52-8.

80. Rodriguez-Soberon R. Situacion, estrategia de conservacion y uso sostenible de *Crocodylus acutus* en Cuba. Republica de Cuba. Republica de Cuba: Universidad de Alicante/Universidad de Pinar del Rio "Hermanos Saiz Montes de Oca" 2009.

81. Rodríguez Soberon R, editor Situación actual de Crocodylus acutus en Cuba. Proceedings of the 15th Working Meeting of the Crocodile Specialist Group, IUCN; 2000; The World Conservation Union, Gland, Switzerland and Cambridge, UK: xvii + 541 p.

82. Alonso-Tabet M, Soberón R, Berovides V, Hernández C, editors. Influence of changes in morphology of nesting habitat on reproduction of *Crocodylus acutus* in the Monte Cabaniguan Wildlife Refuge, Cuba. Crocodiles: Proceedings of the 15th Working Meeting of the Crocodile Specialist Group, IUCNThe World Conservation Union, Gland, Switzerland and Cambridge UK[Links]; 2000.

83. Varona LS (1987).The status of *Crocodylus acutus* in Cuba. Caribbean Journal of Science; 23(2): 256-9.

84. Kushlan JA, Mazzotti FJ (1989).Historical and present distribution of the American crocodilein Florida. Journal of Herpetology; 23(1): 1-7.

85. Dugan BA, Rand AS, Burghardt GM, Bock BC (1981).Interactions between nesting crocodiles and iguanas. Journal of Herpetology: 409-14.

86. Deitz DC, Hines TC (1980).Alligator nesting in north-central Florida. Copeia: 249-58.

87. Jennings ML, Percival H, Abercrombie C, editors. Habitat variables affecting nesting success of the American alligator in Florida. Proceedings of the Annual Conference of Southeast Association Fish and Wildlife Agencies; 1987.

88. Kushlan JA, Mazzotti FJ (1989).Population biology of the American crocodile. Journal of Herpetology; 23(1): 7-21.

89. Alonso-Tabet M. Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante and Universidad de Pinar del Rio Hermanos Saiz Montes de Oca; 2009.

90. Cott H (1961).Scientific results of an inquiry into the ecology and economic status of the Nile crocodile (Crocodilus niloticus) in Uganda and Northern Rhodesia. The Transactions of the Zoological Society of London; 29(4): 211-356.

91. Mazzotti FJ, Bohnsack B, McMahon MP, Wilcox JR (1986).Field and laboratory observations on the effects of high temperature and salinity on hatchling *Crocodylus acutus*. Herpetologica: 191-6.

92. Dunson WA (1982).Salinity relations of crocodiles in Florida Bay. Copeia: 374-85.

93. Thorbjarnarson JB (1988).The status and ecology of the American crocodile in Haiti. Bulletin of the Florida State Museum Biological Sciences (USA).

94. Breder CM (1946).Amphibians and reptiles of the Rio Chucunaque drainage, Darien, Panama, with notes on their life histories and habits. Bulletin of the AMNH; v. 86, article 8.

95. Platt SG, Thorbjarnarson JB (2000).Nesting ecology of the American crocodile in the coastal zone of Belize. Copeia; (3): 869-73.

96. Charruau P, Thorbjarnarson JB, Henaut Y (2010).Tropical cyclones and reproductive ecology of Crocodylus acutus Cuvier, 1807 (Reptilia: Crocodilia: Crocodylidae) on a Caribbean atoll in Mexico. Journal of Natural History; 44(11-12): 741-61.

97. Espinal M, Escobedo-Galvan AH (2011).Population status of the American crocodile (Crocodylus acutus) in El Cajon Reservoir, Honduras Southwestern Naturalist; 56(2): 212-5.

98. Rodda GH (1984).Movements of Juvenile American crocodiles in Gatun Lake, Panama. Herpetologica; 40(4): 444-51.

99. Ogden JC (1978).Status and nesting biology of the American crocodile, *Crocodylus acutus*,(Reptilia, Crocodilidae) in Florida. Journal of Herpetology: 183-96.

100. Mazzotti FJ. The ecology of *Crocodylus acutus* in Florida: Pennsylvania State University.; 1983.

101. Mazzotti F, Kushlan J, Dunbar-Cooper A (1988).Desiccation and cryptic nest flooding as probable causes of egg mortality in the American Crocodile, *Crocodylus acutus*. Everglades National Park, Florida Florida Scientist; 51: 65-72.

102. Crespo R, editor American crocodile (*Crocodylus acutus*) in Florida: the recovery of a threatened species and
its role as indicator for the restoration of the Everglades wetlands of southern Florida. IX International Wetland Symposium
November 12-15, 2013, ; 2013; Ciénaga de Zapata, Cuba.

103. Charruau P (2012).Microclimate of American crocodile nests in Banco Chinchorro biosphere reserve, Mexico: Effect on incubation length, embryos survival and hatchlings sex. Journal of Thermal Biology; 37(1): 6-14.

104. Charruau P, Hénaut Y (2012).Nest attendance and hatchling care in wild American crocodiles (*Crocodylus acutus*) in Quintana Roo, Mexico. Animal Biology; 62(1): 29-51.

105. Thorbjarnarson JB (1996).Reproductive characteristics of the order Crocodylia. Herpetologica: 8-24.

106. Varona L. The status of Crocodylus acutus and Crocodylus rhombifer in Cuba [Unpubl. Ms.]1980.

107. Lang JW, Andrews HV (2005).Temperature‐dependent sex determination in crocodilians. Journal of Experimental Zoology; 270(1): 28-44.

108. Soberón RR, Tabet MA, Alvarez VB, Verdade L, Larriera A (2002).Nidificación del cocodrilo americano (Crocodylus acutus Cuvier) en el refugio de fauna “Monte Cabaniguan”, Cuba. Conservaçaoe Manejo de Jacarés e Crocodilos da América Latina; 2: 135-56.

109. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006).Regional habitat conservation priorities for the American crocodile. Biological Conservation; 128(1): 25-36.

110. Fisher B, Bradbury RB, Andrews JE, Ausden M, Bentham-Green S, White SM, et al. (2011).Impacts of species-led conservation on ecosystem services of wetlands: understanding co-benefits and tradeoffs. Biodivers Conserv; 20(11): 2461-81.

Table 1. Reproductive ecology traits of *Crocodylus acutus* across the regional scale, including populations of Florida Bay (United States), the Wildlife Refuge Monte Cabaniguán (Cuba), Etang Saumatre (Haiti), Lago Enriquillo (Dominican Republic), Turneffe Atoll (Belize), and Banco Atoll (Mexico).

	Nesting season	**Nest mode**	**Colonial / solitary nesting**	**Avg. nests per year**	**Female min. TL (m)**	**Avg. clutch size**	**Avg. egg mass (gr)**	**Avg. clutch mass (kg)**	**Nesting success (%)**	**Hatching success**	**Pb. of hatching**	**Non-viable eggs (%)**	**Main causes of nesting failure**	**Parental care**	**Ref.**
Florida Bay and upper Keys, United States	March trough August	Hole and mound nests	Solitary, colonial in low densities	20	2.95	45.2 ± 17.2 N=20	N/A	N/A	65 N=140	48 N=333	0.312	12.5	Predation, embryonic mortality	N/A	[62]
Florida Bay (Everglades National Park), United States	March trough August	Hole and mound nests	Solitary, colonial in low densities	23	2.25	38 ± 9.4 N = 46	N/A	N/A	74 N=104	58 N=271	0.43	10	Predation, flooding, desiccation, and infertility	No evidence of maternal care of neonates	[32, 64]
Florida Bay (Everglades National Park), United States	March trough August	Hole and mound nests	Solitary, colonial in low densities	100-120	2.25	30	N/A	N/A	N/A	90	50-60	N/A	Flooding, some predation	No evidence of maternal care of neonates	[65]
Wildlife Refuge Monte Cabaniguán Cuba	February trough July	Hole nests	Colonial at high densities	188	2.7-3.0	25.4 ± 1.6 N=410	93.9 ± 2.9 Range : 87.5-97 N=10	2.7 ± 0.1 Range: 2.5-3 N=10	64 N: 1210	70 N=10390	0.45	10.3	Dropped nests and nest destruction by other females, flooding and desiccation	Females transport neonates to shallow, brackish interior lagoons after hatching	[4, 39]
Etang Saumatre, Haiti	Late January trough May	Hole nests	Mostly solitary	17	2.2-2.3	22.5 ± 2.7 N= 14	97.0 ± 8.1 Range : 80-116 N=68	2.18	N/A	N/A	N/A	9.9	No predation observed	Females transport neonates to water after hatching	[15, 24]

Table 1 (cont.)

	Nesting season	**Nest mode**	**Colonial / solitary nesting**	**Avg. nests per year**	**Female min. TL (m)**	**Avg. clutch size**	**Avg. egg mass (gr)**	**Avg. clutch mass (kg)**	**Nesting success (%)**	**Hatching success**	**Pb. of hatching**	**Non-viable eggs (%)**	**Main causes of nesting failure**	**Parental care**	**Ref.**
Lago Enriquillo, Dominican Republic	February trough June	Hole nests	Colonial in high densities	36	2.2-2.3	22.3 ± 9.5 Range: 9-35 N=8	83.6 ± 6.5 Range: 78.3-88.2 N=178	N/A	52 N=8	87 N=178	N/A	13	Flooding, infertility	N/A	[16, 17]
Turneffe Atoll, Belize	March trough July	Hole and mound nests	Solitary, colonial in low densities	up to 15	1.8-2	22.3 ± 6.0 Range: 12-32 N=14	85.6 ± 9.7 Range: 61.5-111 N=280	1.8 ± 0.8 Range: 0.9-3.1 N= 13	80 N: 10	N/A	N/A	8.7	Negligible losses due to nest predation and flooding	Minimal maternal care of neonates	[24]
Atoll, Mexico	March trough August	Hole and mound nests	Mostly solitary	10.5	2.8	16.2 ± 4.6 Range: 9-27 N=30	80.0 ± 7.2 Range: 70-102 N=322	1.4 ± 0.4 Range: 0.9–2.3 N=11	73.2 N=4	N/A	N/A	13.2	Flooding	Some evidence of female nest defense behavior, and minimal maternal care of neonates	

Nesting success: percentage of nests producing at least one hatchling; hatching success: percentage of eggs in successful nests producing hatchlings; probability of hatching: product of nesting success times hatching success, also defined as the proportion of all eggs laid that hatch

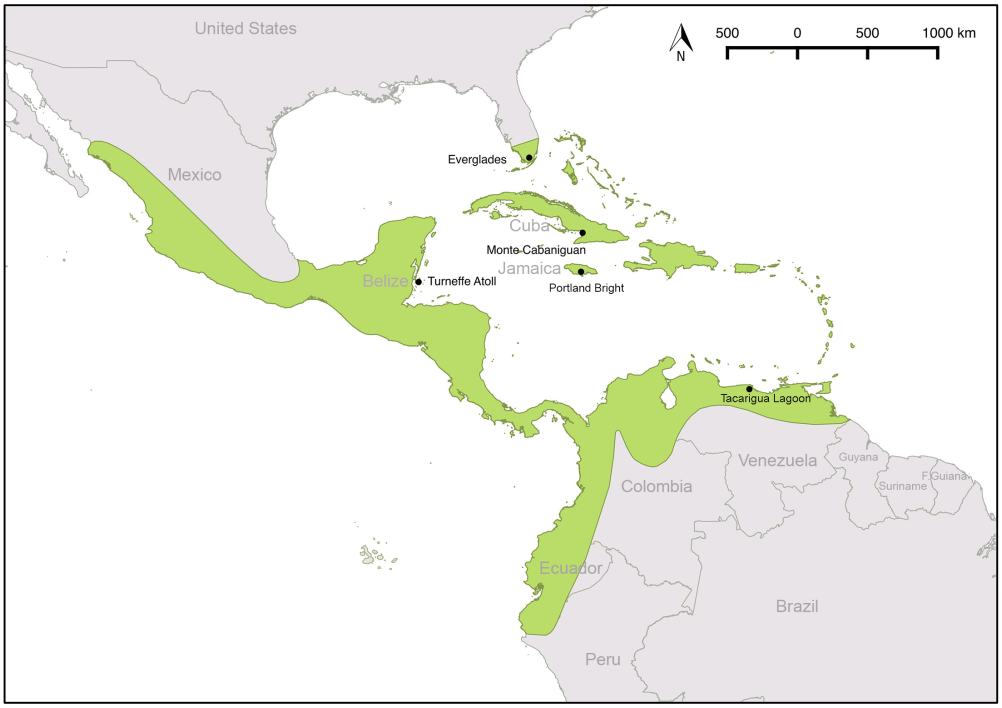

Figure 1. Geographic range of the American crocodile (*Crocodylus acutus*)

(green layer)

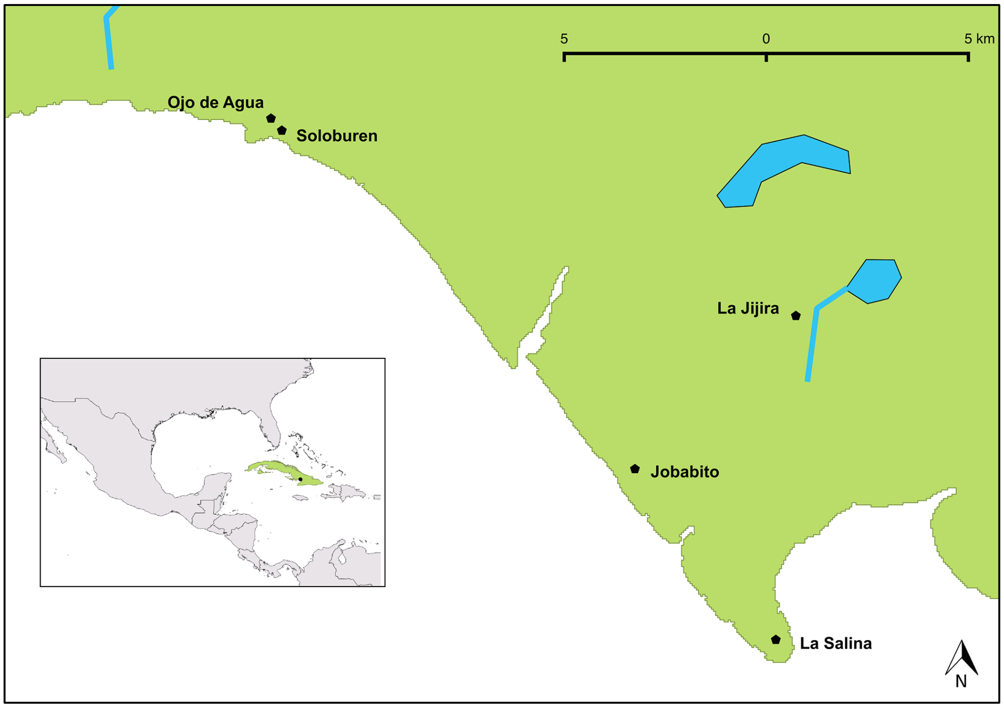

Figure 2. *Crocodylus acutus* permanent nesting areas in the Wildlife Refuge

Monte Cabaniguán-Ojo de Agua, Cuba

CHAPTER II

MULTIPLE PATERNITY IN A REINTRODUCED POPULATION OF THE ORINOCO CROCODILE (*CROCODYLUS INTERMEDIUS*) AT THE EL FRÍO BIOLOGICAL STATION, VENEZUELA

Abstract

The success of a reintroduction program is determined by the ability of individuals to reproduce and thrive. Hence, an understanding of the mating system and breeding strategies of reintroduced species can be critical to the success, evaluation and effective management of reintroduction programs. As one of the most threatened crocodile species in the world, the Orinoco crocodile (*Crocodylus intermedius*) has been reduced to only a few wild populations in the Llanos of Venezuela and Colombia. One of these populations was founded by reintroduction at Caño Macanillal and La Ramera lagoon within the El Frío Biological Station, Venezuela. Twenty egg clutches of *C. intermedius* were collected at the El Frío Biological Station for incubation in the lab and release of juveniles after one year. Analyzing 17 polymorphic microsatellite loci from 335 hatchlings we found multiple paternity in *C. intermedius*, with half of the 20 clutches fathered by two or three males. Sixteen mothers and 14 fathers were inferred by reconstruction of multilocus parental genotypes. Our findings showed skewed paternal contributions to multiple-sired clutches in four of the clutches (40%), leading to an overall unequal contribution of offspring among fathers with six of the 14 inferred males fathering 90% of the total offspring, and three of those six males fathering more than 70% of the total offspring. Our results provide the first evidence of multiple paternity occurring in the Orinoco crocodile and confirm the success of reintroduction efforts of this critically endangered species in the El Frío Biological Station, Venezuela.

Introduction

Successful reproduction is critical to the recovery of endangered species. Many factors may limit reproduction, but in a small, newly established population knowledge of a species' mating system can be of particular importance to understanding the effective population size and the factors that drive reproductive success more generally [1]. Mating systems and their potential influence in the effective population size are also of critical importance for the design of *ex situ* conservation strategies for the recovery of endangered species [2]. Reintroduction of captive populations has proven challenging with approximately one-third of reintroductions failing [3,4] due to one or a combination of factors including poor habitat quality [5], altered behaviors (e.g. nest site selection, courtship rituals), and depleted genetic diversity [6].

For many species it is difficult, if not impossible, to regularly observe matings. Over the last thirty years, genetic tools have provided an increasingly accurate, and subtle, means to assess mating systems and to examine the result of differential mating success among both males and females [7]. Genetic tools have revealed that, throughout the animal kingdom, females of many species pursue a variety of alternative reproductive strategies exercising a more active role in mate choice [8,9], often mating with more than one male [10,11]. The result of this discovery of widespread polyandry is a better understanding of the frequency of extra pair paternity [12], female cryptic choice [13], and multiple paternity of a clutch or litter of offspring [11].

Multiple paternity of a single clutch, whereby offspring in that clutch are fathered by different males, can be the result of multiple mating within a single breeding season [13] or fertilizations from sperm stored from matings in previous seasons [14]. The evolution of female multiple mating (polyandry), and multiple paternity, can be driven by direct and indirect benefits. Direct benefits include provisioning of resources to the mother and paternal care of offspring [15,16]. Indirect benefits that may accrue include: improved quality of mate resulting in transfer of “good genes” or offspring of higher fitness [17,18], maximization of genetic diversity among the offspring resulting in bet-hedging against variation in the environment in successive generations [19,20], and improved genetic compatibility between mating pairs [21,22]. Mate encounter rates within a reproductive season also have been shown to influence both the occurrence and frequency of multiple paternity [23]. By increasing the number of males that contribute offspring to successive generations, and by changing the frequency of such contributions multiple paternity has also been shown, both theoretically [24] and empirically [25], to increase the effective population size [24], potentially increasing the overall genetic diversity of a population [26].

As a group, crocodilians were long believed to be polygynous and monandrous, with dominant males establishing breeding territories, excluding other adult males, and mating with multiple females [27-29]. This belief was primarily based on observations of crocodilians in captivity, supported with a few, mostly anecdotal, accounts of wild animals [30]. Multi-locus, highly variable, codominant markers such as microsatellites are a powerful genetic tool for parentage analysis [31]. They can be used for reconstruction of full-sibling and half-sibling families and inference of parental genotypes using maximum likelihood frameworks [32]. The use of molecular markers has shown multiple paternity to be an ubiquitous phenomenon [33-39].

Using these methods and data, evidence of multiple paternity has been observed in many crocodilian species, including members of families Crocodylidae [34-37] and Alligatoridae [38,39]. The frequency of multiple-sired nests ranges from 30% to 90% [34-37]. Across studies, multiply sired clutches were fathered by two to four males [34-37]. Paternal contributions to multiply sired clutches were found to be skewed in the American alligator (*Alligator mississippiensis*) [38] and spectacled caiman (*Caiman crocodilus*) [37], with some males significantly contributing more than others to the total reproductive output. Overall, across all species of Crocodylia, these studies reveal high variability both in the prevalence of multiple paternity, measured as the percentage of multiple-sired clutches, and in the relative paternal contribution of males. This variability suggests that the incidence of multiple paternity is likely influenced by a variety of environmental factors as well as species behavior and life history traits.

Captive breeding and reintroduction has been used worldwide as a tool to recover critically endangered species of crocodilians including the Chinese alligator (*Alligator sinensis*) [40], the Cuban crocodile (*Crocodylus rhombifer*) [41], the Philippine crocodile (*Crocodylus mindorensis)* [42], the Siamese crocodile (*Crocodylus siamensis*) [43,44], the gharial (*Gavialis gangeticus*) [45], and the Orinoco crocodile (*C. intermedius*) [46]. With the exception of *A. sinensis* [40], information about mating systems and occurrence of multiple paternity based on genetic data is lacking. Genetic investigations of reproductive success could improve our understanding of crocodilian reproductive ecology and greatly aid *ex-situ* and *in-situ* conservation strategies, in particular the demographic and population structure of crocodilian reintroduction efforts.

One of the most threatened crocodile species in the world, the Orinoco crocodile [46,47] once inhabited large areas of the Llanos of Venezuela and Colombia within the Orinoco basin [48,49]. Extensive hunting until the 1960s and persistent collection of eggs for local consumption decimated its populations [50,51], with only few wild populations remaining in both countries [52]. In Venezuela, these include principally natural populations at Capanaparo, Cojedes and Manapire river systems [52], and a reintroduced population at Caño Macanillal and La Ramera lagoon within the El Frío Biological Station (EFBS) (Fig 1), and the adjacent Caño Guaritico Wildlife Refuge [53]. The original population at EFBS was extirpated during the 20thcentury. From 1990-2006 efforts were made to re-establish this population by the reintroduction of more than 2000 crocodiles raised in four captive breeding centers in Venezuela [53].

Post reintroduction, several management interventions were made to improve the survival of the *C. intermedius* at EFBS: river sand was supplemented along the shores of rivers and lagoons to facilitate the excavation of nests by females, eggs were collected and artificially incubated to prevent losses from nest predation, with the primary nest predator being the Tegu lizard (*Tupinambis teguixin*), juveniles were retained in captivity in a "head-start" program and released as one year-old juveniles to avoid the highest mortality rates which occur during the first year of life [46,53,54].

The occurrence of male hierarchies in Orinoco crocodiles during the breeding season was observed in previous studies [55,56]. However, both the extent to which males are able to monopolize breeding, and the occurrence of multiple mating, were unknown. Here, we use 17 polymorphic microsatellite loci to determine the occurrence of multiple paternity in 20 clutches of *C. intermedius* collected in EFBS, Venezuela. Specifically, we investigated the incidence of multiple paternity over three breeding seasons, used a maximum likelihood approach for sibship and parental inference, and assessed the degree of reproductive skew among inferred fathers.

Materials and Methods

Sampling

Twenty clutches of *C. intermedius* were collected during three consecutive years (n=1 in 2004; n=7 in 2005; and n=12 in 2006) along artificial nesting sites placed in the wetland shorelines of Caño Macanillal (7° 50' 11.45"N, 68° 55'

43.33"W) and La Ramera lagoon (7° 50' 5.65"N, 68° 55' 38.14"W) in the EFBS, Venezuela (Fig 1). Clutches were transported to the EFBS and incubated in artificially constructed nests as part of a captive breeding and reintroduction program for this species. Upon hatching, skin tissue samples were taken from all hatched crocodiles (n=335). Samples were removed from the dorsal section of the base of the tail and stored at 8 °C in 95 % ethanol, following sampling protocols described in [36].

The Oficina Nacional de Diversidad Biológica, Ministerio del Ambiente y de los Recursos Naturales de Venezuela, granted tissue sample collection permits (Permit number 5-0358) and endorsed all other research activities of this project. CITES export permit number 1395/VE9120190 allowed *C. intermedius* skin tissue samples to be transported to Uppsala University, Sweden, and Museo Nacional de Ciencias Naturales (CSIC) in Madrid, Spain, to conduct genetic laboratory work.

Genotyping

Genomic DNA was extracted from preserved scales using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following manufacturer protocols. A panel of 17 microsatellite markers previously developed for *Crocodylus*, including C391, Cj16, Cj18, Cj101, Cj122, Cj127, CUJ131, Cu5123 [57], Cj109 [58], CpDi13, CpP302, CpP305, CpP314, CpP801, CpP1409, CpP1610, CpP3216 [59], was used to genotype all samples. Microsatellites were amplified in 4 multiplex PCRs (Mix1: CpP302, CpP305, CpP314, CpP1409, CpP3216, CpP1610; Mix2: C391, Cj16, Cj122, CUJ-131; Mix3: Cj18, Cj109, Cu5-123; Mix4: Cj101, Cj127, Cp801, CpDi13) using the Qiagen Multiplex PCR kit (Qiagen, Valencia, CA) for 30 cycles and two different annealing temperatures (57 °C for Mix1, Mix2 and Mix4, and 60 ºC for Mix3). Reactions were prepared in a final volume of 12.5 µL including: 6.25 µL of Qiagen 2X PCR Master Mix, 2.5 µL of 10X primer mix (final concentration: 2 µM each, except for Cj16, Cj127 and CpP801, which were used at 4 µM), 2 µL DNA (ca. 10 ng/µL) and 1.75 µL of RNase-free H_2O. Fluorescently labeled PCR products were electrophoresed on an ABI 3730 XL DNA analyzer with GS500 (-250) ROX size standard (Applied Biosystems, Foster City, CA) and allele sizes were determined using GeneMapper 3.7 software (Applied Biosystems Foster City, CA). Genetic laboratory work was conducted at Uppsala University, Sweden, and Museo Nacional de Ciencias Naturales, Spain.

The number of alleles, observed and expected heterozygosities, polymorphic information content, exclusion probabilities, allele frequencies, and tests for presence of null alleles were calculated in CERVUS 3.0.7 [60] for a sub-sample of one randomly selected individual per nest (n=20). CERVUS 3.0.7 [60] uses genetic data from codominant markers to estimate allele frequencies and calculate various summary statistics and exclusion probabilities for each locus, assuming autosomal markers, linkage equilibrium between genetic markers, and diploid species. Conformity to Hardy-Weinberg (HW) expectations for each locus and genotypic linkage disequilibrium (LD) between pairs of loci were tested in GENEPOP 4.3 [61]. Significance levels (p=0.05) for departure from HW and LD were corrected for multiple comparisons with a sequential Bonferroni correction [62].

Contamination and primer-site mutations may result in the incorrect assignment of microsatellite genotypes (genotyping errors), potentially biasing the results of parentage analysis [63]. Additionally, DNA degradation, low DNA concentration, and primer-site mutations can potentially cause non-amplified alleles (null alleles), which have proven to introduce errors in parentage analysis leading to high frequencies of erroneous parentage exclusions [64]. Potential genotyping inconsistencies including the presence of null alleles, large allele dropout, scoring of stutter peaks and typographic errors, were assessed in Micro-checker [65] and specific allele dropout rates were estimated in Microdrop 1.10 [66].

Paternity analysis

Sibship and parental inference analyses were conducted using a maximum likelihood approach implemented in COLONY 2.0 [32]. COLONY assigns individuals into full-sib and half-sib families using multi-locus genetic marker data with or without the imputing of parental information [32], and allowing for the incorporation of various types of genotyping errors [67]. By clustering offspring into full- and half-sib families COLONY is able to infer parental genotypes, when unknown, and calculate paternity assignment likelihoods, assuming Mendelian segregation and no maximum limit on the numbers of contributing parents. Ten replicate runs of “long” length and “high” likelihood precision were carried out in COLONY using the same dataset assuming an error rate of 0.04 for allelic dropout and 0.05 for genotyping error (based on the highest error rate per locus obtained in Microdrop 1.10 [68] (Appendix 1) and suggested genotyping error rates by [67]). Polygamy was assumed for males and females and a full likelihood method was implemented. Offspring genotypes and known maternal sibship for each clutch were inputted into COLONY.

We used GERUD 2.0 [69] as alternative analysis package for the detection of multiple paternity. GERUD uses an exhaustive algorithm to determine the minimum number of fathers that can explain a full or half-sib progeny array when one or neither parent is known [69]. Because GERUD can only incorporate up to 10 loci, we used the 10 loci with highest exclusion probabilities (Table 1).

Probabilities of detecting multiple paternity were estimated using the PrDM software [70] (http://publish.uwo.ca/~bneff/software.html) for scenarios of equal (50:50) or skewed (90:10) paternal contribution by two males, averaged across 10 replicate runs, and using an average of 17 hatchlings per clutch sampled in this study . The degree of reproductive skew (B) was assessed for clutches exhibiting multiple paternity by calculating the binomial skew index B [71-73] in SKEW CALCULATOR 2003 (https://www.eeb.ucla.edu/Faculty/Nonacs/PI.html). Significant levels of B were calculated by simulation with 100,000 permutations. Significant positive values of B indicate a skewed paternal contribution to a given clutch, significant negative values of B indicate an excessively equal paternal contribution, and non-significant values do not show differences from a random paternal contribution [71]. A Pearson's product moment correlation test between number of alleles per locus and exclusion probabilities, clutch size and number of fathers, and clutch size and probability of detecting multiple paternity (PrDM) was conducted using the package stats in R version 3.1.2 [74].

Results

Characterization of microsatellite markers

We identified a total of 90 alleles averaging 5.3 alleles per locus among a subsample of 20 Orinoco crocodile individuals, taking a random individual from each clutch. Observed and expected heterozygosities ranged from 0.143 and 0.135 for CpP1610, respectively, to 0.821 and 0.862 for CpP302, exhibiting overall values of 0.524 and 0.544, respectively. Our analysis did not detect significant ($p<0.05$) deviations from Hardy-Weinberg expectations and linkage equilibrium at any locus (Appendix 1), or evidence of null alleles.

The discriminating power of the 17 markers combined was high (Table 1), with a probability of excluding a potential parent when neither parent is known (PE) of 0.976 and probabilities of detecting multiple paternity (PrDM) given 17 offspring sampled per clutch and two fathers contributing 50:50 and 90:10 of 0.999 and 0.876, respectively. However, exclusion probabilities (PE) of individual markers were mostly low, ranging from 0.012 to 0.559, and were highly correlated with the number of alleles per locus ($r=0.831$).

On a clutch-by-clutch basis, probabilities of detecting multiple paternity

(PrDM) remained relatively high (p>0.830) for all clutch sizes under the equal paternal contribution scenario, and ranged from 0.587 to 0.852 under a skewed paternal contribution (90:10) (Table 2).

Paternity analysis

We obtained complete multilocus genotypes for all of the 17 loci and for more than 95% of the total individuals (335) sampled within a total of 20 clutches. Genotype data can be accessed at the Dryad Digital Repository under the DOI: 10.5061/dryad.n2114. Multiple paternity and parental reconstruction results were consistent over 10 COLONY replicate runs, assuming error rates of 0.04 for allelic dropout and 0.05 for genotyping error, and using different random seed numbers for each run. Our analyses revealed evidence of multiple paternity, with 10 (50%) out of 20 clutches fathered by two or three males (Table 2). We found multiple paternity in two of the three breeding seasons analyzed (2005 and 2006), with four out of seven clutches (57%) in 2005, and six out of 12 clutches (50%) in 2006 being fathered by two or three males. The only nest from 2004 was single-sired. GERUD and COLONY inferred multiple fathers for the same 10 clutches. The number of fathers remained the same using both programs for all but one clutch (C6), for which GERUD determined a minimum of three fathers whereas COLONY determined a minimum of two fathers. Due to the different number of markers used (a maximum of 10 loci for GERUD and all 17 for COLONY), the estimate offered by COLONY is considered more reliable.

For 90% of clutches, configurations of full-sib families inferred by COLONY exhibited high probabilities of both including all full-sib individuals in a given paternal family (Prob Inc.), and excluding all non full-sibs from each paternal family (Prob Exc.). Clutches 5 and 20 showed the lowest probabilities for full-sib paternal family configurations. Potential causes of these low values may be associated with mislabeling of hatchlings or mutations at one or several of the microsatellite loci that might have generated mismatches among sib hatchling genotypes.

Of the 10 multiple-sired clutches, four (40%) were significantly skewed from equal paternal contributions (Table 2). Four additional clutches (C6, C7, C14, C20) failed to show significant skew between fathers, which may be explained by small clutch size (n<10) hindering the degree of reproductive skew. We did not find significant correlations between clutch size and number of fathers (t=-1.639, df=21, p=0.116), and clutch size and probability of multiple paternity (PrDM) (t=-1.328, df=21, p=0.199).

COLONY reconstructed 16 maternal and 14 paternal multilocus genotypes (Appendix 2). Four of the 16 inferred mothers laid eggs on consecutive nesting seasons (M1, M5, M6, and M7), with two of these (M1 and M6) returning to the same geo-referenced nesting site (data not shown). Full siblings were observed in different nests when females mated with the same male in successive years. Some of these females laying eggs over consecutive seasons mated exclusively with the same male (M5), but the majority mated with more than one male in either one (M1, M7) or two (M6) consecutive breeding seasons.

Shared paternity across clutches was inferred by COLONY, with highly skewed contributions among identified males (Fig 2). Six of the 14 inferred males

(F2, F4, F7, F9, F12 and F13) fathered 90% of the total offspring, and three of those six males (F2, F4 and F7) fathered more than 70% of the total offspring.

Discussion

Our results report for the first time a polygamous mating system for *C. intermedius*, with females mating with more than one male in 10 out of 20 clutches studied. *Crocodylus intermedius* at the EFBS exhibited both single-fathered and multiple-fathered clutches, indicating that females can employ different reproductive strategies. Results of this study accord with other studies of multiple paternity in crocodilians including 30% multiple-sired clutches in *A. sinensis* [39], 32% in *A. mississippiensis* [38], 50% in *Crocodylus moreletii* [33], 50% in *Caiman latirostris* [34], 69% in *Crocodylus porosus* [36], 90% in *Melanosuchus niger* [35], and 95% in *C. crocodilus* [37]. In these studies, two to four males fathered multiply sired clutches.

Observed differences in the incidence of multiple paternity within and among crocodilian species could be influenced by one or a combination of factors, including: density of breeding individuals, mate encounter rates, sex ratio of sexually mature individuals, differences in mating behavior and reproductive strategies (e.g. ability of males to monopolize matings, male territory defense during the breeding season, male harassment, female choice, timing of copulation), and habitat type and spatial configuration of the environment (e.g. mangrove swamps, internal rivers and lagoons, small streams, ponds). Relatively small sample sizes, and a dearth of comparative data, make it difficult to identify which of these factors come in to play between and among these studies.

Multiple paternity in *C. intermedius* could have resulted from multiple mating within a single breeding season and/or fertilizations from sperm stored in females' reproductive tract within one or more breeding seasons. Sperm storage has proven an effective strategy to ensure fertilization and lengthen the breeding season in taxa where rates of encounter between the sexes are low, multiple clutches are laid within a single reproductive season, and/or where high asynchrony in gonadal cycles between males and females occurs [38,75,76]. Although sperm storage has not been reported in *C. intermedius*, sperm was found in the oviducts of female *A. mississippiensis* [77] and possibly occurred in a captive female of *Paleosuchus palpebrosus* which laid fertile eggs a long time after being separated from a male [78]. The absence of sperm from the oviduct of female American alligators during non-reproductive periods, however, suggested that alligator sperm may be retained within a reproductive season but is unlikely to be retained from one year to the next [77]. Female crocodilians generally congregate in breeding areas with groups of males during the reproductive season [79-81], lay one clutch per reproductive season on an annual or bi-annual basis [30], and some species showed a degree of synchrony in gonadal cycles between males and females (*A. mississippiensis* [82] and *C. niloticus* [83]). Given low population numbers, female sperm storage in *C. intermedius* (if it does occur) would most likely be associated with low frequency of mate encounter and low mate availability. Female cryptic choice (female selection for a particular male's sperm [84]) and the assurance of paternal diversity within clutches [85] could also potentially result in sperm storage in this species.

Our results, and observations from previous studies documenting male hierarchies in Orinoco crocodiles during the breeding season [55,56], suggest unequal distribution of matings. In this study shared paternity across clutches revealed highly skewed contributions among identified males. Similarly, paternal contributions of multiply sired clutches were skewed in *A. mississippiensis* [86] and *C. crocodilus* [37]. Skewed paternal contributions to clutches may arise by one or a combination of different mechanisms, such as: female preference for a “dominant” male, higher success for copulations occurring closest to female ovulation, sperm storage and female cryptic choice, and competition among male’s sperm in female’s reproductive tract (sperm competition [87]).

Four of the 16 identified mothers returned to lay eggs in two consecutive years, with two of them laying eggs in the same nesting site on the second year. In this recently reintroduced population, because of a lack of appropriate habitat, nesting sites have been artificially supplemented by providing river sand along the shores of rivers and lagoons to facilitate the excavation of nests by females. Despite the artificial construction of nest sites, two females showed site fidelity, a phenomenon not uncommon to crocodilians [44]. Of the four returning females, two exhibited a switch from a single-sired clutch in one breeding season to a multiple-sired clutch in the subsequent breeding season, whereas the other two maintained either single-sired or multiple-sired clutches in both breeding seasons (Table 2). Hence, in this small sample, mating strategy, nesting site, and site fidelity, do not appear correlated, although further research is required to parse out the reasons for site fidelity.

A recent study of *C. acutus* [88] reporting nests with full siblings in successive mating seasons suggests the occurrence of mate fidelity in this species. In this study, we found that all four females that laid clutches on two consecutive seasons mated with at least one of the same males. Of these, only one mated exclusively with a single male, whereas the other three had multiple mates in either one or two breeding seasons. These findings could be a consequence of female choice and/or male dominance within this population.

Inference of parental genotypes from offspring multilocus marker data and derived interpretations needs to be carefully assessed. Maximum likelihood approaches for sibship and parental inference implemented by COLONY 2.0 [32] have been shown to overestimate parent numbers in both empirical [89] and simulated marker datasets [90] with low to intermediate levels of polymorphism. Nonetheless, parentage studies and family reconstructions of simulated datasets identified number and diversity of loci as main factors determining accuracy of results, with COLONY outperforming most available methods of parentage analysis [91]. In this study, the resolving power of the combined marker set was high. In addition, convergence of multiple runs in COLONY and high values of full-sib family exclusion and inclusion probabilities for the majority of the clutches suggested that our marker set could be sufficient for accurate parental reconstruction. The alternative method used to assess multiple paternity (GERUD) accorded with COLONY and confirmed the robustness of our analysis.

Information on the mating system and breeding strategies of reintroduced populations is critical to their success, evaluation and effective management [1]. The occurrence of multiple paternity may accelerate the recovery and resilience of reintroduced populations of *C. intermedius* by increasing genetic diversity among siblings, potentially increasing the effective population size [24], and overall genetic diversity of the population [26]. However, the dominance of certain males could potentially have the opposite effect by reducing the number of contributing males and increasing the variance in their contribution to the gene pool of next generations. In this study, we report heterozygosity values similar to those of other crocodilian populations in the wild [92-94]. The latter suggests that the genetic makeup and/or rapid population growth after individuals were reintroduced may have quickly compensated any potential founder effect, despite biases in the contribution of the different males.

Before the first reintroductions of *C. intermediu*s, the species had been depleted from the El Frío Biological Station and surrounding areas [53] that fall within the historical range of the species [48,49]. After first reintroduction efforts took place in 1990, a combination of management interventions resulted in a successful population recovery with increasing nesting numbers adding up to at least 93 nests between 1996 and 2007 [53]. Nesting at the EFBS lays within nesting numbers previously reported at other sites: in the Capanaparo river system there were 11 nests in 1991 [81], 14 nests in 2001, 20 nests in 2002 [47], and 25 nests in 2013 [95]; during the 2009 nesting season 13 nests were laid in the Cojedes [52] and four nests in the Manapire river systems [47]. In the context of the few remaining natural populations of *C. intermedius* in Venezuela, the EFBS reproductive output makes it a very important population for the recovery of the species.

Successful reproduction is a necessary first step in the recovery of the Orinoco crocodile. However, other conservation actions will be critical to the continued and sustained growth of this and other populations. Conservation actions include mitigating the threat of poaching and habitat protection [50]. Continued involvement and support from the governmental nature conservation authorities is essential for the long-term sustainability of these initiatives. Political changes in Venezuela led to a reduction in conservation and research activities at the El Frío Biological Station in 2009. Since then, the fate of the reintroduced Orinoco crocodile population, one of the last populations of C. *intermedius*, is little known. The limited information published in the popular press in recent years suggests that this population, and the species more generally, continues to be in need of urgent conservation actions [96].

Future research should aim to better elucidate the factors that influence multiple paternity in crocodilian species, looking at environmental (e.g. latitude, temperature, habitat type and configuration), demographic and species-specific traits. Research on specific mechanisms involved in producing multiple-fathered clutches, potential long-term adaptive advantages of multiple paternity for crocodilians, and impacts of multiple paternity on the overall genetic diversity of populations will further our knowledge on crocodilian mating systems and can aid conservation strategies for endangered crocodilian species.

References

1. Schmidt F, Franke F, Shirley M, Vliet K, Villanova V (2015) The importance of genetic research in zoo breeding programmes for threatened species: the African dwarf crocodiles (genus *Osteolaemus*) as a case study. International Zoo Yearbook 49: 125-136.
2. Willoughby JR, Fernandez NB, Lamb MC, Ivy JA, Lacy RC, DeWoody J (2015) The impacts of inbreeding, drift and selection on genetic diversity in captive breeding populations. Molecular Ecology 24: 98-110.
3. Fischer J, Lindenmayer DB (2000) An assessment of the published results of animal relocations. Biological Conservation 96: 1-11.
4. Pérez I, Anadón JD, Díaz M, Nicola GG, Tella JL, Giménez A (2012) What is wrong with current translocations? A review and a decision-making proposal. Frontiers in Ecology and the Environment 10: 494-501.
5. Moorhouse T, Gelling M, Macdonald D (2009) Effects of habitat quality upon reintroduction success in water voles: evidence from a replicated experiment. Biological Conservation 142: 53-60.
6. Jamieson IG, Lacy RC (2012) Managing genetic issues in reintroduction biology. Reintroduction Biology: Integrating Science and Management 12: 441.
7. DeWoody J, Avise J (2001) Genetic perspectives on the natural history of fish mating systems. Journal of Heredity 92: 167-172.
8. Clutton-Brock T, McAuliffe K (2009) Female mate choice in mammals. The Quarterly Review of Biology 84: 3-27.

9. Jennions MD, Petrie M (1997) Variation in mate choice and mating preferences: a review of causes and consequences. Biological Reviews 72: 283-327.

10. Reynolds JD (1996) Animal breeding systems. Trends in Ecology and Evolution 11: 68-72.

11. Taylor ML, Price TA, Wedell N (2014) Polyandry in nature: a global analysis. Trends in Ecology and Evolution 29: 376-383.

12. Adams EM, Jones AG, Arnold SJ (2005) Multiple paternity in a natural population of a salamander with long‐term sperm storage. Molecular Ecology 14: 1803-1810.

13. Canal D, Jovani R, Potti J (2012) Multiple mating opportunities boost protandry in a pied flycatcher population. Behavioral Ecology and Sociobiology 66: 67-76.

14. Brante A, Fernández M, Viard F (2011) Microsatellite evidence for sperm storage and multiple paternity in the marine gastropod *Crepidula coquimbensis*. Journal of Experimental Marine Biology and Ecology 396: 83-88.

15. Richardson DS, Burke T, Komdeur J (2002) Direct benefits and the evolution of female-biased cooperative breeding in Seychelles warblers. Evolution 56: 2313-2321.

16. Moller AP (2000) Male parental care, female reproductive success, and extrapair paternity. Behavioral Ecology 11: 161-168.

17. Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. Biological Reviews 75: 21-64.

18. Yasui Y (1998) The 'genetic benefits' of female multiple mating reconsidered. Trends in Ecology and Evolution 13: 246-250.

19. Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. Conservation Biology 17: 230-237.

20. Bach L, Dahllöf I (2012) Local contamination in relation to population genetic diversity and resilience of an arctic marine amphipod. Aquatic Toxicology 114: 58-66.

21. Petrie M, Doums C, Moller AP (1998) The degree of extra-pair paternity increases with genetic variability. Proceedings of the National Academy of Sciences of the United States of America 95: 9390-9395.

22. Rubenstein DR (2007) Female extrapair mate choice in a cooperative breeder: trading sex for help and increasing offspring heterozygosity. Proceedings of the Royal Society B: Biological Sciences 274: 1895-1903.

23. Boomer JJ, Harcourt RG, Francis MP, Walker TI, Braccini JM, Stow AJ (2013) Frequency of multiple paternity in gummy shark, *Mustelus antarcticus*, and rig, *Mustelus lenticulatus*, and the implications of mate encounter rate, postcopulatory influences, and reproductive mode. Journal of Heredity 104: 371-379.

24. Sugg DW, Chesser RK (1994) Effective population sizes with multiple paternity. Genetics 137: 1147-1155.

25. Martinez J, Moran P, Perez J, De Gaudemar B, Beall E, Garcia‐Vazquez E (2001) Multiple paternity increases effective size of southern Atlantic salmon populations. Molecular Ecology 9: 293-298.

26. Rafajlović M, Eriksson A, Rimark A, Hintz-Saltin S, Charrier G, Panova M, et al. (2013) The effect of multiple paternity on genetic diversity of small populations during and after colonisation. PloS One 8: e75587.

27. Thorbjarnarson J (1989) Ecology of the American Crocodile, *Crocodylus acutus*. In Crocodiles, their Ecology, Management and Conservation IUCN Publications New Series Gland, Switzerland: 228-258.

28. Alonso-Tabet M (2009) Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante and Universidad de Pinar del Rio Hermanos Saiz Montes de Oca.

29. Grigg G, Kirshner D (2015) Biology and Evolution of Crocodylians: CSIRO PUBLISHING.

30. Thorbjarnarson JB (1996) Reproductive characteristics of the order Crocodylia. Herpetologica: 8-24.

31. Jones AG, Ardren WR (2003) Methods of parentage analysis in natural populations. Molecular Ecology 12: 2511-2523.

32. Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. Molecular Ecology Resources 10: 551-555.

33. McVay JD, Rodriguez D, Rainwater TR, Dever JA, Platt SG, McMurry ST, et al. (2008) Evidence of multiple paternity in Morelet's crocodile (*Crocodylus moreletii*) in Belize, CA, inferred from microsatellite markers. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 309A: 643-648.

34. Amavet P, Rosso E, Markariani R, Ignacio Pina C (2008) Microsatellite DNA Markers Applied to Detection of Multiple Paternity in *Caiman latirostris* in Santa Fe, Argentina. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 309A: 637-642.

35. Muniz FL, Da Silveira R, Campos Z, Magnusson WE, Hrbek T, Farias IP (2011) Multiple paternity in the Black Caiman (*Melanosuchus niger*) population in the Anavilhanas National Park, Brazilian Amazonia. Amphibia-Reptilia 32: 428-434.

36. Lewis JL, FitzSimmons NN, Jamerlan ML, Buchan JC, Grigg GC (2013) Mating systems and multiple paternity in the estuarine crocodile (*Crocodylus porosus*). Journal of Herpetology 47: 24-33.

37. Oliveira DP, Marioni B, Farias IP, Hrbek T (2014) Genetic evidence for polygamy as a mating strategy in *Caiman crocodilus*. Journal of Heredity 105: 485-492.

38. Davis LM, Glenn TC, Elsey RM, Dessauer HC, Sawyer RH (2001) Multiple paternity and mating patterns in the American alligator, *Alligator mississippiensis*. Molecular Ecology 10: 1011-1024.

39. Hu Y, Wu X-B (2010) Multiple paternity in Chinese alligator (*Alligator sinensis*) clutches during a reproductive season at Xuanzhou Nature Reserve. Amphibia-Reptilia 31: 419-424.

40. Wang Z, Yao H, Ding Y, Thorbjarnarson J, Wang X (2011) Testing reintroduction as a conservation strategy for the critically endangered Chinese alligator: Movements and home range of released captive individuals. Chinese Science Bulletin 56: 2586-2593.

41. Rodríguez-Soberón R, Ramos R, McMahan W, Ross J (1996) Reintroduction of the Cuban crocodile on the Isle of Pines. Crocodile Specialist Group Newsletter 15: 10-11.

42. Van Weerd M, Guerrero J, Balbas M, Telan S, Ven W, Rodriquez D, et al. (2010) Reintroduction of captive-bred Philippine Crocodiles. Oryx 44: 13.

43. Daltry J, Starr A (2010) Development of a re-introduction and re-enforcement program for Siamese crocodiles in Cambodia. In: Global Re-introduction Perspectives: Additional Case Studies from Around the Globe (ed Soorae P), pp 118-123 Re-introduction Specialist Group and Environment Agency, Abu Dhabi, UAE: 118.

44. Elsey RM, Trosclair III PL, Glenn TC (2008) Nest-site fidelity in American alligators in a Louisiana coastal marsh. Southeastern Naturalist 7: 737-743.

45. Ballouard JM, Priol P, Oison J, Ciliberti A, Cadi A (2010) Does reintroduction stabilize the population of the critically endangered gharial (*Gavialis

gangeticus, Gavialidae) in Chitwan National Park, Nepal? Aquatic Conservation: Marine and Freshwater Ecosystems 20: 756-761.

46. Antelo R, Ayarzagüena J, Castroviejo J (2010) Reproductive ecology of Orinoco crocodiles (*Crocodylus intermedius*) in a newly established population at El Frío Biological Station, Venezuela. The Herpetological Journal 20: 51-58.
47. Barros T, Jiménez-Oraá M, Heredia HJ, Seijas AE (2010) Artificial incubation of wild-collected eggs of American and Orinoco crocodiles (*Crocodylus acutus* and *C. intermedius*), Guárico and Zulia, Venezuela. Conservation Evidence 7: 111-115.
48. Gumilla J (1745) El Orinoco Ilustrado y Defendido, Historia Natural, Civil, y Geographica de este Gran Rio: Manuel Fernandez, editor.
49. Thorbjarnarson JB (1992) Recent investigations of the status and distribution of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. Biological Conservation 62: 179-188.
50. Casal AC, Fornelino MM, Restrepo MFG, Torres MAC, Velasco FG (2013) Uso histórico y actual del caimán llanero (*Crocodylus intermedius*) en la Orinoquia (Colombia-Venezuela). Biota Colombiana 14: 65-82.
51. Medem F (1981) Los Crocodylia de Sur América, Vol. I. Los Crocodylia de Colombia Editorial Carrera 7a Ltda, Bogotá.
52. Espinosa-Blanco AS, Seijas AE, Hernández O (2013) Egg collectIon as a conservation tool of Orinoco crocodiIle (*Crocodylus intermedius*) in the Cojedes river system, Venezuela. Interciencia 38: 358-363.

53. Antelo R (2008) Biología del Cocodrilo o Caimán del Orinoco (*Crocodylus intermedius*) en la Estación Biológica El Frío, Estado Apure (Venezuela): Tesis Doctoral. Universidad Autónoma de Madrid, Spain.

54. Ramo C (1992) Breeding and rearing of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. Biological Conservation 60: 101-108.

55. Seijas A, Chávez C (2002) Reproductive status and nesting ecology of the Orinoco crocodile (*Crocodylus intermedius*) in the Cojedes River System, Venezuela. Vida Silvestre Neotropical 11: 23-32.

56. Thorbjarnarson JB, Hernández G (1993) Reproductive ecology of the Orinoco Crocodile (*Crocodylus intermedius*) in Venezuela. I. Nesting ecology and egg and clutch relationships. Journal of Herpetology: 363-370.

57. FitzSimmons N, Tanksley S, Forstner M, Louis E, Daglish R, Gratten J, et al. Microsatellite markers for *Crocodylus*: new genetic tools for population genetics, mating system studies and forensics; 2001. Surrey Beatty & Sons. pp. 51-57.

58. Dever JA, Densmore LD (2001) Microsatellites in Morelet's crocodile (*Crocodylus moreletii*) and their utility in addressing crocodilian population genetics questions. Journal of Herpetology 35: 541-544.

59. Miles L, Isberg S, Moran C, Hagen C, Glenn T (2009) 253 Novel polymorphic microsatellites for the saltwater crocodile (*Crocodylus porosus*). Conservation Genetics 10: 963-980.

60. Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16: 1099-1106.

61. Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Molecular Ecology Resources 8: 103-106.

62. Rice WR (1989) Analyzing tables of statistical tests. Evolution: 223-225.

63. Hoffman J, Amos W (2005) Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Molecular Ecology 14: 599-612.

64. Dakin E, Avise J (2004) Microsatellite null alleles in parentage analysis. Heredity 93: 504-509.

65. Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535-538.

66. Wang C, Schroeder KB, Rosenberg NA (2012) A maximum-likelihood method to correct for allelic dropout in microsatellite data with no replicate genotypes. Genetics 192: 651-669.

67. Wang J (2004) Sibship reconstruction from genetic data with typing errors. Genetics 166: 1963-1979.

68. Wang C, Rosenberg NA (2012) MicroDrop: a program for estimating and correcting for allelic dropout in nonreplicated microsatellite genotypes version 1.01.

69. Jones AG (2005) GERUD 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Molecular Ecology Notes 5: 708-711.

70. Neff B, Pitcher T (2002) Assessing the statistical power of genetic analyses to detect multiple mating in fishes. Journal of Fish Biology 61: 739-750.

71. Nonacs P (2000) Measuring and using skew in the study of social behavior and evolution. The American Naturalist 156: 577-589.

72. Nonacs P (2003) Measuring the reliability of skew indices: is there one best index? Animal Behaviour 65: 615-627.

73. Liu J-X, Avise JC (2011) High degree of multiple paternity in the viviparous Shiner Perch, *Cymatogaster aggregata*, a fish with long-term female sperm storage. Marine Biology 158: 893-901.

74. R Development Core Team (2010). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing

75. Pearse D, Avise J (2001) Turtle mating systems: behavior, sperm storage, and genetic paternity. Journal of Heredity 92: 206-211.

76. Uller T, Olsson M (2008) Multiple paternity in reptiles: patterns and processes. Molecular Ecology 17: 2566-2580.

77. Gist DH, Bagwill A, Lance V, Sever DM, Elsey RM (2008) Sperm storage in the oviduct of the American alligator. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 309: 581-587.

78. Davenport M (1995) Evidence of possible sperm storage in the caiman, *Paleosuchus palpebrosus*. Herpetological Review 26: 14-14.

79. Garrick LD, Lang JW (1977) Social signals and behaviors of adult alligators and crocodiles. American Zoologist 17: 225-239.

80. Vliet K (2001) Courtship behaviour of American alligators *Alligator mississippiensis*. Crocodilian Biology and Evolution: 383-408.

81. Thorbjarnarson JB, Hernández G (1993) Reproductive ecology of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. II. Reproductive and social behavior. Journal of Herpetology: 371-379.

82. Lance VA (1989) Reproductive cycle of the American alligator. American Zoologist 29: 999-1018.

83. Kofron CP (1990) The reproductive cycle of the Nile crocodile (*Crocodylus niloticus*). Journal of Zoology 221: 477-488.

84. Birkhead T (2000) Defining and demonstrating postcopulatory female choice–again. Evolution 54: 1057-1060.

85. Calsbeek R, Bonneaud C, Prabhu S, Manoukis N, Smith TB (2007) Multiple paternity and sperm storage lead to increased genetic diversity in *Anolis* lizards. Evolutionary Ecology Research 9: 495.

86. Davis LM (2001) Multiple paternity and mating patterns in the American alligator, *Alligator mississippiensis*. Molecular Ecology 10: 1011-1024.

87. Andersson M, Iwasa Y (1996) Sexual selection. Trends in Ecology & Evolution 11: 53-58.

88. Budd KM, Spotila JR, Mauger LA (2015) Preliminary Mating Analysis of American Crocodiles, *Crocodylus acutus*, in Las Baulas, Santa Rosa, and Palo Verde National Parks, Guanacaste, Costa Rica. South American Journal of Herpetology 10: 4-9.

89. Jones B, Grossman GD, Walsh DC, Porter BA, Avise JC, Fiumera AC (2007) Estimating differential reproductive success from nests of related individuals, with application to a study of the mottled sculpin, *Cottus bairdi*. Genetics 176: 2427-2439.

90. Sefc KM, Koblmüller S (2009) Assessing parent numbers from offspring genotypes: the importance of marker polymorphism. Journal of Heredity 100: 197-205.

91. Harrison HB, Saenz-Agudelo P, Planes S, Jones GP, Berumen ML (2013) Relative accuracy of three common methods of parentage analysis in natural populations. Molecular Ecology 22: 1158-1170.

92. Dever JA, Strauss RE, Rainwater TR, McMurry ST, Densmore III LD, Wood R (2002) Genetic diversity, population subdivision, and gene flow in Morelet's crocodile (*Crocodylus moreletii*) from Belize, Central America. Copeia 2002: 1078-1091.

93. Farias IP, Silveira RD, de Thoisy B, Monjeló LA, Thorbjarnarson J, Hrbek T (2004) Genetic diversity and population structure of Amazonian crocodilians. Animal Conservation 7: 265-272.

94. Milián-García Y, Venegas-Anaya M, Frias-Soler R, Crawford AJ, Ramos-Targarona R, Rodríguez-Soberón R, et al. (2011) Evolutionary history of

Cuban crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* inferred from multilocus markers. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 315: 358-375.

95. Hernández O, Boede E, Amauci J (2014) Evaluation of the Orinoco crocodile nesting (*Crocodylus intermedius*) in Capanaparo River, Santos Luzardo National Park, Apure State, Venezuela Boletín de la Academia de las Ciencias Físicas, Matemáticas y Naturales 2: 53-59.
96. Neuman W, Ramon P (December 26 2013) Venezuela's Fitful Effort to Save a Scaly Predator. The New York Times. pp. A4.

Appendix 1

Additional information on 17 microsatellite loci used in *C. intermedius* paternity analysis

Locus	**Primer sequences (5’-3’)**	**Multiple x Mix**	**Ta (˚C)**	**n**	**HW (p-value)**	**SE**	**Allele dropout rate**
CpP1409	F: GTTTATGCCCTACTGGTTATCTATC	1	57˚C	25	0.44370	0.00000	0.00000
	R: CAGTCGGGCGTCATCAGGGAAGGGGATTTAATAAT						
CpP1610	F: CAGTCGGGCGTCATCATAGAGGGATTTTGACTGT	1	57˚C	25	1.00000	0.00000	0.00004
	R: GTTTGATTATTTTGTCTGGGTTCTT						
CpP302	F: GTTTGGAACCCAAGAACTTACAAC	1	57˚C	25	0.26360	0.00160	0.00000
	R: CAGTCGGGCGTCATCATTGGGTTTAGTCAGCACATA						
CpP305	F: GTTTGTAGCTGGAACCTGATAGTG	1	57˚C	25	0.27330	0.00000	0.00000
	R: CAGTCGGGCGTCATCAGGTTAACACGTGGTAACTACA						
CpP314	F: GTTTGAAATGCCACTAATACACACA	1	57˚C	25	0.11840	0.00000	0.00323
	R: CAGTCGGGCGTCATCACCAATTCTTCAGGTCCTTAT						
CpP3216	F: CAGTCGGGCGTCATCAGATTAATTCATTGGCTCTC	1	57˚C	25	0.71670	0.00000	0.00016
	R: GTTTATGCCTTTGCCTTTAG						
C391	F: ATGAGTCAGGTGGCAGGTTC	2	57˚C	25	0.57390	0.00700	0.00000
	R: CATAAATACACTTTTGAGCAGCAG						
CUJ131	F: GTCCCTTCCAGCCCAAATG	2	57˚C	25	0.70140	0.00000	0.00164
	R: CGTCTGGCCAGAAAACCTGT						
Cj122	F: GTTTCATGCTGACTGTTTCTAATCACC	2	57˚C	25	0.58420	0.00000	0.04368
	R: GGAACTACAATTGGTCAACCTCAC						
Cj16	F: CATGCAGATTGTTATTCCTGATG	2	57˚C	25	0.92570	0.00040	0.00729
	R: TGTCATGGTGTCAATTAAACTC						
Cj109	F: GTATTGTCAACCCCACCGTGTC	3	60˚C	25	0.21000	0.00270	0.00000
	R: GTTTCCCCTCCACAGATTTACTTGC						
Cj18	F: ATCCAAATCCCATGAACCTGAGAG	3	60˚C	25	0.72870	0.00100	0.00000
	R: CCGAGTGCTTACAAGAGGCTGG						
Cu5123	F: GGGAAGATGACTGGAAT	3	60˚C	25	1.00000	0.00000	0.00000
	R: AAGTGATTAACTAAGCGAGAC						
Cj101	F: ACAGGAGGAATGTCGCATAATTG	4	57˚C	25	0.58650	0.00000	0.03461
	R: GTTTATACCGTGCCATCCAAGTTAG						
Cj127	F: CCCATAGTTTCCTGTTACCTG	4	57˚C	25	1.00000	0.00000	0.00000
	R: GTTTCCCTCTCTGACTTCAGTGTTG						
CpDi13	F: GTTTGTGTCAGCCTATACATGTT	4	57˚C	25	1.00000	0.00000	0.00000
	R: CAGTCGGGCGTCATCAGTCTCAGAGTATGCCTAGAA						
CpP801	F: CAGTCGGGCGTCATCATTGGCATTAGATTGGTAGAC	4	57˚C	25	0.07370	0.00060	0.00041
	R: GTTTCTATGCCAAAGCTACAAC						

Ta, annealing temperature; HW, p-value estimates for Hardy-Weinberg equilibrium test; SE, standard error for p-value estimates of Hardy-Weinberg equilibrium test.

Appendix 2

Table 1. Parental genotypes reconstructed for 20 *C. intermedius* clutches across 17 polymorphic microsatellite loci.

Father (F) or Mother ID (M)	CpP1419	CpP1610	CpP302	CpP305	CpP314	CpP3216	C391	CUJ131	Cj122	Cj16	Cj109	Cj18	Cu5123	Cj101	Cj127	CpDi13	CpP801	p
F1	231/235	278/278	189/200	178/180	238/246	125/125	170/182	202/202	392/405	170/187	403/405	230/230	215/215	378/382	353/353	345/345	172/172	0.917
F2	235/235	278/278	183/189	176/180	246/246	125/125	180/192	208/208	398/402	187/191	386/388	227/230	215/215	387/387	353/353	347/347	168/168	1.000
F3	235/235	278/278	189/202	180/180	242/246	121/125	182/182	208/208	405/405	170/187	388/398	227/232	211/215	382/387	353/353	345/347	168/172	0.630
F4	231/235	278/278	185/202	180/180	246/246	121/125	170/182	202/208	392/405	160/187	386/388	227/230	215/215	382/382	353/353	345/345	168/172	0.703
F5	235/235	278/278	189/189	180/180	246/246	121/125	182/182	208/208	392/392	187/187	388/386	227/227	215/215	382/382	353/353	345/347	164/164	0.926
F6	235/235	278/278	168/202	160/180	242/246	125/125	170/182	208/208	405/405	160/187	388/390	227/227	215/215	382/387	353/353	345/345	168/172	0.632
F7	231/235	278/278	189/200	178/180	238/246	125/125	170/182	202/202	405/405	170/187	403/405	230/230	215/215	372/382	353/353	345/345	172/172	1.000
F8	235/235	278/278	193/202	180/178	238/246	125/125	176/182	208/208	392/405	187/187	386/388	227/227	211/215	382/382	353/353	345/345	168/168	0.950
F9	231/235	278/278	189/200	178/180	238/246	121/125	180/182	202/208	392/405	170/187	403/405	225/230	215/215	382/387	353/353	345/347	168/172	1.000
F10	235/235	278/278	185/202	180/180	238/242	121/125	188/196	202/208	402/402	187/187	386/388	227/227	215/215	387/387	353/353	345/347	164/168	0.782
F11	235/235	278/278	183/189	176/180	246/246	125125	183/189	208/208	398/402	187/191	386/388	227/230	215/215	387/387	353/353	347/347	168/168	0.848
F12	231/235	278/278	202/202	160/178	242/242	121/125	170/184	210/210	392/392	160/187	398/398	227/230	215/215	382/382	353/353	345/345	168/172	0.631
F13	235/235	278/278	185/202	180/180	246/246	121/125	180/182	208/208	392/405	170/187	396/388	227/227	215/215	382/382	353/356	345/347	168/172	0.903
F14	231/235	278/278	202/202	160/180	242/246	125/125	182/182	208/208	402/402	187/187	386/398	227/232	211/215	382/382	353/356	345/347	168/168	0.950

Table 1 (cont.)

M1	235/235	278/278	168/189	160/180	238/246	121/121	180/182	202/208	392/405	160/170	388/388	227/227	211/215	382/387	353/353	345/345	168/172	0.908
M2	231/235	278/278	183/185	178/180	242/246	121/125	170/182	202/208	398/398	187/189	380/396	227/230	200/215	382/382	353/353	345/345	168/176	0.966
M3	231/235	278/278	189/200	178/180	238/246	125/125	180/182	202/208	392/405	170/187	403/405	225/230	215/215	382/387	353/353	345/347	168/172	0.703
M4	235/235	278/278	168/202	180/182	238/246	121/121	182/182	208/208	392/398	160/187	388/388	227/227	215/215	382/387	353/353	345/345	168/172	0.930
M5	231/235	274/278	202/204	178/180	238/246	121/121	180/182	208/208	392/405	170/187	380/388	227/227	215/215	382/387	353/353	345/345	168/172	0.982
M6	235/235	278/278	187/189	180/182	238/246	125/125	166/166	202/208	392/392	170/187	386/386	227/227	215/215	382/387	353/356	345/345	168/172	0.972
M7	231/231	278/278	189/202	178/180	238/250	125/125	166/182	208/208	398/405	160/187	388/388	230/232	211/215	382/387	353/353	345/347	168/172	0.999
M8	235/235	278/278	183/185	180/180	242/246	121/125	166/170	202/208	392/392	160/160	386/388	227/230	215/215	382/382	353/356	345/347	168/172	0.935
M9	231/235	278/278	189/202	160/160	246/246	121/125	170/182	208/210	392/402	187/187	384/388	230/230	211/215	382/382	353/356	345/347	164/172	0.999
M10	235/235	278/278	168/200	180/180	242/242	121/125	166/180	208/208	392/392	170/170	386/388	230/232	215/215	382/382	353/353	347/347	168/172	0.761
M11	231/235	278/278	189/191	160/180	238/246	121/125	176/182	202/202	405/405	160/160	388/407	227/230	211/215	382/382	353/353	345/345	164/172	0.999
M12	235/235	278/278	187/189	180/182	238/246	125/125	166/166	202/208	392/392	170/187	386/386	227/227	211/215	382/387	353/356	345/345	168/172	0.631
M13	231/235	278/278	185/193	160/178	246/246	121/125	176/182	208/208	392/402	187/187	386/405	227/227	215/215	382/382	353/353	345/347	164/176	0.991
M14	235/235	278/278	185/202	180/180	246/246	121/121	166/176	208/208	392/405	160/187	386/386	227/227	215/215	382/387	353/353	347/347	164/168	0.745
M15	235/235	274/278	189/202	180/180	246/246	121/125	182/192	208/210	392/392	187/187	398/405	230/232	215/215	382/387	353/356	347/347	168/176	0.999
M16	235/235	278/278	193/202	180/180	238/246	121/125	170/182	202/208	392/392	187/187	388/405	230/230	211/215	382/382	353/353	345/347	164/176	0.801

p, maximum probability obtained in COLONY 2.0 for parental genotype inference averaged across 17 loci.

Table 1. Characterization of 17 microsatellite loci in *Crocodylus intermedius* at the El Frío Biological Station, Venezuela

Locus	n	Allele size range (bp)	No. Alleles	H_o	H_E	PIC	PE	PrDM
CpP1409	20	231-238	4	0.464	0.371	0.310	0.073	
CpP1610	20	274-278	2	0.143	0.135	0.124	0.012	
CpP302	20	168-204	10	0.821	0.862	0.829	0.559	
CpP305	20	160-182	5	0.536	0.531	0.484	0.109	
CpP314	20	236-250	6	0.500	0.592	0.516	0.184	
CpP3216	20	121-125	2	0.464	0.503	0.372	0.120	
C391	20	164-196	11	0.815	0.807	0.762	0.362	
CUJ131	20	202-210	4	0.536	0.551	0.474	0.154	
Cj122	20	392-406	4	0.667	0.713	0.645	0.250	
Cj16	20	160-194	7	0.679	0.722	0.660	0.264	
Cj109	20	380-408	11	0.750	0.774	0.733	0.317	
Cj18	20	225-235	5	0.571	0.573	0.481	0.167	
Cu5123	20	200-218	4	0.286	0.249	0.215	0.030	
Cj101	20	378-388	4	0.464	0.551	0.442	0.119	
Cj127	20	351-357	3	0.179	0.166	0.149	0.024	
CpDi13	20	342-348	3	0.500	0.499	0.370	0.085	
CpP801	20	164-184	5	0.538	0.651	0.571	0.270	
Overall				0.524	0.544	0.479	0.976	0.999/0.876

H_0: observed heterozygosity; H_E: expected heterozigosity; PIC: polymorphic information content; PE: probability of excluding a potential parent when neither parent is known; PrDM: probability of detecting multiple paternity given 17 progeny sampled per clutch and two fathers contributing 50:50 / 90:10.

Table 2. Paternity analyses for 20 clutches of *Crocodylus intermedius* from the El Frío Biological Station, Venezuela.

				GERUD	COLONY								
Clutch ID	Year	N	PrDM	Number of inferred fathers	Inferred mother ID	Number of inferred fathers	Paternal sibship	Inferred father ID	N full siblings	Prob (Inc.)	Prob (Exc.)	*B* value*	p
C1	2004	3	0.747/0.546	1	M1	1	1A	F1	3	1.000	1.000		
C2	2005	16	0.952/0.788	2	M2	2	2A	F2	15	1.000	1.000	0.3516	0.0005
							2B	F3	1	1.000	1.000		
C3	2005	32	0.925/0.744	1	M3	1	3A	F4	32	1.000	1.000		
C4	2005	19	0.983/0.801	2	M4	2	4A	F5	13	1.000	1.000	0.0416	0.1679
							4B	F1	6	0.986	0.986		
C5	2005	19	0.972/0.776	1	M5	1	5A	F2	19	0.604	0.604		
C6	2005	8	0.944/0.734	3	M1	2	6A	F1	7	1.000	1.000	0.2188	0.0684
							6B	F6	1	1.000	1.000		
C7	2005	8	0.971/0.768	2	M6	2	7A	F2	7	0.999	0.999	0.2188	0.0708
							7B	F3	1	1.000	1.000		
C8	2005	42	0.996/0.852	1	M7	1	8A	F2	42	1.000	1.000		
C9	2006	14	0.972/0.971	3	M8	3	9A	F7	9	0.949	0.904		
							9B	F8	4	0.811	0.797		
							9C	F2	1	1.000	1.000		
C10	2006	24	0.957/0.743	1	M9	1	10A	F2	24	1.000	1.000		
C11	2006	8	0.980/0.798	1	M5	1	11A	F2	8	1.000	1.000		
C12	2006	22	0.931/0.706	2	M10	2	12A	F9	21	1.000	1.000	0.3905	0.0000
							12B	F10	1	1.000	1.000		
C13	2006	23	0.940/0.718	1	M11	1	13A	F7	23	1.000	1.000		
C14	2006	3	0.929/0.746	2	M6	2	14A	F2	2	0.999	0.999	−0.1111	1.0000
							14B	F6	1	1.000	1.000		
C15	2006	13	0.983/0.807	3	M7	3	15A	F2	11	1.000	1.000	0.3432	0.0007
							15B	F3	1	1.000	1.000		
							15C	F11	1	1.000	1.000		
C16	2006	19	0.897/0.653	1	M12	1	16A	F12	19	0.998	0.998		
C17	2006	16	0.954/0.745	1	M13	1	17A	F7	16	1.000	1.000		
C18	2006	23	0.902/0.677	3	M14	3	18A	F13	21	1.000	1.000	0.4751	0.0000
							18B	F7	1	1.000	1.000		
							18C	F14	1	1.000	1.000		
C19	2006	16	0.950/0.738	1	M15	1	19A	F7	16	1.000	1.000		
C20	2006	7	0.830/0.587	2	M16	2	20A	F10	4	0.706	0.706	−0.0612	1.0000
							20B	F11	3	0.549	0.549		

N: number of offspring used in paternity analysis; PrDM: probability of detecting multiple paternity given two fathers contributing 50:50/90:10, averaged over 10 replicate simulations; Prob (Inc.): probability that all individuals of a given full-sib paternal family are full-sibs; Prob (Exc.): probability that no other individuals are full-sibs within this paternal family; B value: binomial skew index; p: significance level of B value.

*Significant values of B are represented in bold.

Figure 1. Location of the El Frío Biological Station (EFBS), and three additional localities where last remaining populations of *Crocodylus intermedius* are found in Venezuela

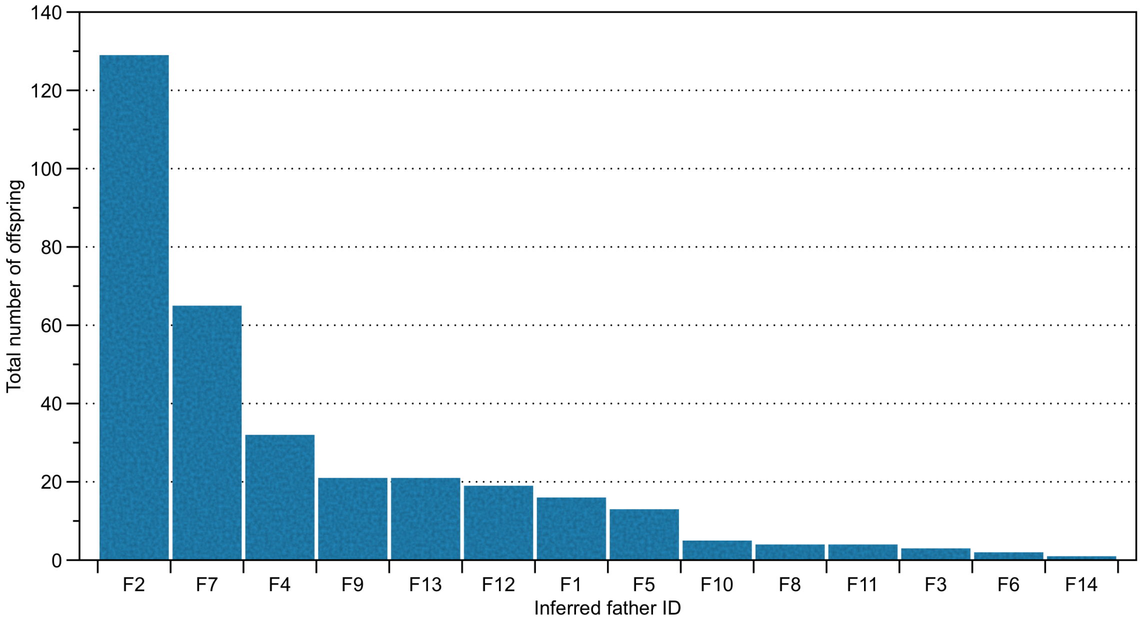

Figure 2. Paternal contributions from 14 fathers inferred by COLONY for 335 hatchlings of *Crocodylus intermedius*.

CHAPTER IIII

EFFECT OF TROPICAL CYCLONES AND ENVIRONMENTAL VARIABILITY ON THE NESTING SUCCESS OF THE AMERICAN CROCODILE (*CROCODYLUS ACUTUS*) IN THE COASTAL WETLANDS OF SOUTHEASTERN CUBA

Abstract

In Cuba, American crocodiles thrive in extensive coastal mangroves that provide suitable habitat for reproduction, growth and survival. The majority of nesting occurs within a single protected area located in the Southeast of the archipelago: the Wildlife Refuge Monte Cabaniguán. Within this cyclone-prone area, crocodiles hatch yearly in five nesting sites located near the Caribbean coast. We used generalized linear mixed models to investigate the effect of tropical cyclones and environmental variability on the nesting success of *C. acutus* for a period of 21 years. Our results revealed significant spatial and temporal variation in nesting success. Overall, we report the highest-density nesting for the species documented to date, and one of the highest densities of nesting in relation to other crocodilian species, with an average density of 17 nests per hectare. Two of the five nesting sites had consistently greater nest densities and higher nesting success for the period analyzed. Much of the temporal variation in nesting success could be explained by the occurrence of tropical cyclones. We found that occurrence of tropical cyclones within a nesting season negatively affected nesting success, whereas the occurrence of tropical cyclones one or two years before the nesting season positively affected nesting success. Additionally, our results suggested that higher ambient temperature negatively affected nesting success. Higher-intensity tropical cyclones are expected to strike the coasts of Cuba due to climate change, potentially devastating *C. acutus* nests if they occur during the nesting season. As the recruitment of *C. acutus* populations in Cuba heavily rely on nesting success, we propose incorporating information on crocodilian’s nesting success and density, as well as the impact of tropical cyclones on the latter, as key components of coastal resilience when designing plans for coastal adaptation in the context of climate change.

Introduction

Vertebrate taxa exhibit a myriad of adaptations to cope with predictable [1] and unpredictable [2] environmental variation. Adaptive responses can be as diverse as adjusting the timing of breeding in accordance to seasonal patterns of precipitation [2], manipulating sex ratios according to environmental temperature [3], or evolving developmental plasticity that allows the production of environmentally induced phenotypes in response to local conditions [4]. Among vertebrates, ectotherms have shown exceptional plasticity for adjusting and responding to changes in the environment [2,5].

In crocodilians, a diversity of critical ontogenic and reproductive traits are tightly linked to environmental factors. These include, but are not limited to, environmental sex determination, sex ratios at birth, beginning and duration of breeding season, rate of sperm and egg production, and embryo development [6-

8]. In particular, nest site selection can be an important component of fitness because the environment in which the nest is placed can affect embryonic development and survival [9]. Nest failure rates for crocodiles can be as high as 40% [10,11], as many nests succumb to flooding, desiccation and predation. Even for successful nests, edaphic characteristics of the nest and its influence on incubation temperature and water and gas exchanges between the egg and its surrounding environment can affect many aspects of hatchling phenotype such as size, shape, color, sex, locomotor performance, metabolism, growth rates and survival [12-14].

In addition to relatively predictable environmental variability, crocodile populations that nest in beaches and coastal dunes, wetlands and rivers are exposed to large-scale natural habitat disturbances caused by tropical cyclones [15,16]. The impact of tropical cyclones on crocodilians vary greatly according to their scale, frequency, intensity, severity and duration [15,17]. On islands in particular, tropical cyclones can have important consequences for nesting success by drastically reducing recruitment through cooling and flooding of clutches within the nest [15,18], and, at a lesser degree, diminishing breeding populations by direct adult mortality [10].

For species that nest in coastal environments such as crocodiles and sea turtles, the degree of overlap between the timing of nesting and tropical cyclone season influences the severity of cyclones on reproductive output [15,17]. Higher overlap translates into lower reproductive output, as storm surges produced by tropical cyclones lower the number of nests that hatch and the number of hatchlings that emerge from the nest [15,17]. For coastal crocodilians, however, the effects of tropical cyclones beyond the nesting season have not yet been clearly established [15]

The recovery of depleted populations after the impact of tropical cyclones depends on levels of recruitment in subsequent years [16] and immigration of new individuals from adjacent unaffected populations in continental environments [15,19]. In contrast, isolated island populations rely almost entirely on recruitment for recovery.

The coastline of the Cuban archipelago is dominated by wetlands [20]. These ecosystems, which comprise 70% of the coastline, are prime habitat for the American crocodile (*Crocodylus acutus*). *Crocodylus acutus* is primarily a coastal crocodilian widely distributed along the coastal swamps, estuarine rivers, and lakes of the Neotropical region of the Americas [21]. In Cuba, the largest numbers of *C. acutus* occur in the Southeastern coasts of the archipelago [22]. The majority of nesting takes place every year, from February through July, within a single protected area: the Wildlife Refuge Monte Cabaniguán (WRMC) [23]. Dense mangroves offer suitable habitats for *C. acutus* to thrive: they provide food such as invertebrates and fish, substrate for the construction of sheltered caves, and sandy areas suitable for nesting [23]. Suitable nesting sites in coastal mangroves of Cuba, however, are scattered and limited [24].

Crocodylus acutus nesting season at the WRMC includes (i) nest preparation and oviposition (last week of February through March), (ii) incubation (February through May), (iii) hatching (May through July), and (iv) post-hatching

[10]. The timing of hatching coincides with the initiation of the rainy season, following the typical pattern of hole nesting crocodiles which normally nest during periods of falling water levels (i.e. the dry season), with hatching taking place towards the beginning of the rainy season [25].

Given the low availability of nesting areas and high density of crocodiles at the WRMC, crocodiles nest in communal sites [11]. Communal nesting at high densities is rare in extant crocodilians [26-28]. In the WRMC aggregations of gravid females in certain nesting sites was attributed to the quality of substrate for nesting rather than the size of the site [29]. Among suitable nesting sites, smaller areas with calcareous gravel soils have approximately five times more nests than larger areas containing clay soils [29].

As a consequence of communal nesting, some nests are destroyed by other females (interference) [30]. This occurs when females make new nests on top on existing ones, or by excavating nearby nests when they retrieve their recently emerged hatchlings [30]. Predation of eggs is extremely rare, and has been observed only once in ten years [11]. Other causes of mortality include flooding, droughts, and the impact of tropical cyclones (tropical storms and hurricanes) [11].

The building of long-term datasets of species nesting information is critical to assess the effects of tropical cyclones in cyclone-prone islands and archipelagos. These datasets are useful tools for assessing the effect of tropical cyclones on reproductive output when they strike, but also years after the extreme-weather event occurred.

In this study, we investigate the effect of tropical cyclones (tropical storms and hurricanes) and environmental variability on the nesting success of *C. acutus* in the wetlands of Southeastern Cuba. We look at the spatial and temporal variation in the nesting success of *C. acutus* in the largest breeding population of the species within Cuba over a period of 21 years. Specifically, we i) compare *C. acutus* total number of nests and nesting success across five permanent nesting sites; ii) assess the overall nesting success over the period 1993-2013; iii) analyze the influence of environmental factors on nesting success; and iv) investigate the short (during the nesting season) and long term (one and two years before the nesting season) effects of tropical cyclones on nesting success.

Materials and Methods

Study site

We conducted research from 1993 to 2013 in the Wildlife Refuge Monte Cabaniguán (WRMC; 20º40’53” N, 77º17’46” W) (Fig 1), in Southeastern Cuba. Located along the littoral zone of the Gulf of Guacanayabo, within the provinces of Las Tunas and Granma, the WRMC comprises a continuous and complex delta system of estuaries, lakes and mangrove swamps [24]. With an extension of 626 Km^2, this area constitutes one of the most important coastal wetland ecosystems of southeastern Cuba and sustains the largest population of *C. acutus* in its entire range of distribution [31,32]. Nesting by *C. acutus* in this wetland is concentrated on a few beaches scattered along the gulf shoreline, and in a patch of raised land within the mangrove swamp, situated 2.5-kilometer inland [10,31] (Fig 1). Five of these nesting areas, covering a combined surface area of 9.4 hectares, are used every year (Table 1).

Data collection

Nesting success

From late February to early August of 1993-2013, we monitored *C. acutus* nesting activity at five nesting sites (Table 1) at the WRMC. For each year and nesting site, we recorded: total number of nests, total number of successful nests (producing at least one hatchling), dates of first and last nests laid, and dates of first and last nest hatched. We calculated the duration of the nesting season as the period between the first nest laid and the last nest hatched, and the nesting success as the proportion of successful nests.

Environmental variables

We obtained environmental data from the Instituto de Meteorología de Cuba and the Anuario Estadístico de Cuba for the period of 1993-2013. These included monthly maximum and minimum temperatures, monthly total rainfall, and date of occurrence , duration, and intensity of tropical storms and hurricanes. All data were available at the provincial (Las Tunas) and municipality (Jobabo) levels. We used the latter in our analyses.

Data analysis

Across the years 1993-2013 we evaluated changes in: the total number of nests; nesting success by nesting site; and nesting success by year using non-parametric Kruskal-Wallis tests and post hoc Dunn tests with the Bonferroni adjustment for pairwise multiple comparisons between nesting sites. In order to assess the potential association between density of breeding females and nesting site area we conducted a Pearson's product-moment correlation test. All tests were implemented in the software R 3.2.2 for Mac [33].

We used generalized linear mixed models (GLMMs)[34] to explore the influence of environmental variables on *C. acutus* reproductive success. GLMM models were implemented in STATA 14 software [35] employing a binomial error structure with a logit link function to model proportion data (nesting success) with non-constant variance, and non-normal error distributions.

Our GLMMs had *C. acutus* nesting success as response variable, and nesting site as random effect. Fixed effect variables included maximum temperature during the nesting season, minimum temperature during the nesting season, total rainfall during the nesting season, distance from coastline ("far from beach"), tropical cyclone occurring during nesting season (t_0), tropical cyclone occurring the year before (t_1), and tropical cyclone occurring two years (t_2) before the nesting season as explanatory variables. The variable "far from beach" was computed as "zero" for nesting sites located less than 25 meters away from the coast, and "one" for nesting sites located more than 25 meters from the coast. We computed occurrence of tropical cyclones as a one if it occurred within (t_0), one year before (t_1), or two years before (t_2) the nesting season.

In order to examine nesting success before and after the occurrence of a tropical cyclone, we used our fitted GLMM to estimate nesting success one and two years before the event to examine the impact of time lags on the behavior of the crocodiles. We evaluated model fit and parsimony using the Akaike information criterion (AIC), calculating delta AIC (ΔAIC) and Akaike weights (ω_i) [36]. The AIC statistic accounts simultaneously for the degrees of freedom used and the goodness of the fit, with more parsimonious models having lower AICs. We selected most informative and parsimonious models. We made all figures using R 3.2.2 for Mac [33] and STATA 14 software [35].

This project was carried out in collaboration with the Wildlife Refuge Monte Cabaniguán, Empresa Nacional para la Proteccion de la Flora y la Fauna (ENPFF), Universidad de la Habana, Wildlife Conservation Society, and American Museum of Natural History. The Centro de Inspección y Control Ambiental of the Ministerio de Ciencia, Tecnología y Medio Ambiente (CITMA) authorized all research activities.

Results

There were significant differences in the total number of nests and nesting success among nesting sites during the period 1993-2013 (Kruskal-Wallis chi-squared = 86.059 and 21.832, df = 4 and 4, and p-value=0.0000 and 0.0002 for total nests and nesting success, respectively). Nesting sites Jobabito and La Salina showed the largest number of nests per year, with median values of 63 and 49, respectively. These sites also had the highest nesting success, reaching median values of 0.63 in and 0.64, respectively (Fig 3). Nesting sites Soloburén, Ojo de Agua and Jijira showed considerable fewer nests per year, with median values of four, eight, and 24 nests, respectively (Fig 2). Nesting success in these three nesting sites was highly variable. Whereas the inland nesting site La Jijira showed the lowest median nesting success (0.04), the most weather-exposed sites of Soloburén and Ojo de Agua exhibited higher values of nesting success (0.25 and 0.56, respectively) (Fig 3). In addition, we found a negative association between density of breeding females and nesting site area (r=-0.786, p=0.000), indicating that smaller areas had higher densities of breeding females.

Pairwise comparisons between *C. acutus* nesting sites (Table 2) revealed significant differences in the total number of nests laid for all but a few site-pairs. These pairs corresponded to sites with similarly high or low values of nesting success, and with similar nesting substrates (pairs of Jobabito-La Salina, Ojo de Agua-La Jijira, and Soloburén-Ojo de Agua). For the nesting success, as expected, sites exhibiting higher nesting success (Jobabito and Salina), showed significant differences with those having lower nesting success (Jijira and Soloburén) (Table 2). Ojo de Agua was the only site that did not exhibit significant differences in nesting success when compared with all other sites.

Overall nesting success across all sites also varied significantly over the period 1993-2013 (Kruskal-Wallis chi-squared = 360.0359, df = 20, p-value = 0.000), exhibiting a bi-modal pattern with two distinctive intervals of high nesting success (1997-2000 and 2008-2011) separated by an interval of low nesting success (2001-2007) (Fig 4). Specifically, years of low nesting success included 1993, 1994, and the period 2001-2008 (Fig 4: unweighted nesting success). Peak years of nesting success included, in descending order, 1997, 1999, 2010, 1998, 2011, and 2008 (Fig 4: unweighted nesting success). Among all nesting sites and for all 21 years of data, Jobabito and La Salina showed the highest peaks of nesting success, as well as numbers of total nests, with 101, 97, and 94 nests laid in Jobabito in 1998, 1995, and 1994 and 94 nests laid in La Salina in 1994. These high nesting values in relatively small areas reveal the highest-density nesting for the species documented to date [21,27,37-40], and one of the highest densities of nesting in relation to other crocodilian species [41].

During 1993-2013, nine tropical cyclones impacted the WRMC: the "Storm of the Century" in 1993, tropical storm Alberto in 1994, hurricane Lily in 2007, tropical storm Barry in 2007, tropical storm Noel in 2007, hurricane Ike in 2008, hurricane Sandy in 2012, and tropical storm Isaac in 2012 (Fig 4). Some of these occurred during June-July, overlapping *C. acutus* nesting season (February to July), and others between August and October, once the nesting season was over. The severity of these tropical cyclones was different for each event, but for most cases we recorded flooding of coastal areas, heavy winds, destruction of coastal mangrove vegetation, movement of sediments due to wave action, and destruction and flooding of coastal nests when cyclones occurred within *C. acutus* nesting season.

The GLMM analyses included nine models, all with medium to low ω_i values (Table 3). The most parsimonious model (lowest AIC value) included the variables tropical cyclones within the nesting season, tropical cyclones one, and two years before the nesting season, and maximum temperature within the nesting season. This model had an intermediate to high Akaike weight (0.59), explaining a relatively large proportion of the variance in the dataset. All other models were outperformed, with AIC values greater than two.

In our model, nesting site (random effect) explained 28.9% of the variation in nesting success. When analyzing the influence from the different variables in this model we found that the occurrence of tropical cyclones within a nesting season negatively affected nesting success, whereas the occurrence of tropical cyclones one or two years before the nesting season positively affected nesting success. Additionally, our results suggested that higher ambient temperature negatively affected nesting success (Table 4). Predicted nesting success before and after the occurrence of a tropical cyclone (Fig 5) by our model confirmed a positive increase in nesting success for the first nesting season after the hurricane, and in a lesser degree, for the second season.

Discussion

In Cuba, American crocodiles thrive in extensive coastal mangroves that provide suitable habitat for reproduction, growth and survival [23]. The majority of nesting occurs within a single protected area located in the Southeast of the archipelago: the Wildlife Refuge Monte Cabaniguán [23]. Within this area, crocodiles hatch yearly in five nesting sites located close or near the Caribbean coast. Our results revealed significant spatial and temporal variation in nesting success. Approximately 75% of the total nesting for the period 1993-2013 took place in two nesting sites that have a relatively small combined area of 2.8 hectares (Jobabito and La Salina). The remaining 25% was distributed among three sites, one inland (Jijira) and two coastal (Soloburén and Ojo de Agua). Overall, we report the highest-density nesting for the species documented to date

[21,27,37-40], and one of the highest densities of nesting in relation to other crocodilian species [41], with an average of 164 nests per year and a density of 17 nests per hectare. Jobabito and La Salina consistently exhibited the largest number of nests per year, and the highest nesting success for all years.

Differences in nesting success among sites could be partially attributed to differences in substrate quality [29]. Most successful sites have soils with high content of calcareous gravel, of larger grain size, which allow more effective water, heat and gas exchanges between the egg and the nest environment [29,42-44]. Less successful sites have soils mainly containing fine sand and clay, less suitable for embryo survival [25]. In addition, Jobabito and La Salina are located in elevated ground, sheltered by surrounding vegetation, non-exposed to wave action [29]. Nest losses among nesting sites of different substrate quality, however, are relatively stable throughout the years [10,11] . Substantial declines in nesting success of up to 50% are principally associated with extreme weather events, particularly the strike of tropical cyclones [24]

For a hole nesting species such as *C. acutus*, suitable substrate for nesting may be a limiting resource [45]. The high population density and limited availability of suitable nesting sites in the WRMC could intensify nest-site selection behavior and competition for sites among reproductive females. We found highest success at greatest densities, and a negative relation between density of breeding females and area of nesting site, which suggests that site selection by females would not only be associated with suitable area but also with other features. High densities would most likely be the result of females selecting the best “quality” site (with higher success). Even if these sites have highest mortality due to overcrowding, where other females laying their own eggs destroy nests, nesting success continues to be higher than other nesting sites with larger areas but poorer substrates.

The timing of nesting of *C. acutus* in Cuba overlaps partially with tropical cyclone season [23]. Tropical cyclones have shown to negatively impact beach-nesting species such as sea turtles [17,46,47] and crocodiles [15,48,49]. Intense storms associated with hurricanes can cause mortality of sea turtle nests by flooding, and later suffocation of hatchlings due to sand being moved towards existing nesting beaches [46]. Hurricanes can also erode coasts provoking significant changes in beach morphology, which can negatively affect nesting success [47]. Hurricane Dean (2007) and tropical storm Arthur (2008) caused high mortality of eggs of *C. acutus* by cooling and flooding clutches in Banco Chinchorro atoll, Mexico [15]. Besides the negative short-term impacts on nesting success of these tropical cyclones, however, they can potentially produce longer-term benefits for coastal crocodilians by creating and maintaining sandy nesting areas, and decreasing salinity in crocodile nursery sites due to their heavy rainfalls [15].

Our 21-year *C. acutus* nesting success dataset at the WRMC allowed us to examine in greater detail the impact of cyclones on nesting success. Nine tropical cyclones occurred in the study area within the period we examined, some occurring during, and others after, *C. acutus* nesting season. Our most parsimonious GLMM model predicted that much of the temporal variation in nesting success over the period 1993-2013 can be explained by the occurrence of tropical cyclones (tropical storms and hurricanes). Nesting site also proved to be an important factor, explaining approximately 30% of the total variance in nesting success. It is not surprising that different nesting sites had different nesting success, as the soil quality varies across sites and seems to be one important factors influencing nesting success [29]

Whereas tropical cyclones occurring within the nesting season negatively affect nesting success, these events positively affect nesting success after one and two years of striking the coasts of the WRMC. The increase of nesting density and nesting success after the occurrence of tropical cyclones may suggest that these weather phenomena indirectly benefit crocodile’s breeding and/or their ability to nest. Tropical cyclones may benefit crocodile nesting in subsequent years by moving sediments across the landscape, which can improve existing nesting sites and/or create new ones [15]. Tropical cyclones could also potentially enhance the productivity of coastal mangroves allowing crocodiles to reproduce more often (e.g annually instead of every two or three years) and/or achieve higher reproductive outcomes.

For all years where hurricanes occurred during the nesting season (1993,1994, and 2007) nesting success in the coastal areas was zero. However, nesting sites (Jobabito and La Salina) sheltered by mangrove vegetation produced surviving hatchlings even in years impacted by these large-scale weather events. We hypothesize that the protection of these sites by dense mangrove vegetation constitutes one of the main factors allowing crocodile hatchling survival. Mangroves offer the first barrier to buffer heavy storms associated with tropical cyclones, offering shelter for nests, juvenile and adult crocodiles.

In our GLMM, high ambient temperatures negatively influenced nesting success. There is extensive research on the effect of incubation temperatures on nesting success and fitness metrics in crocodilians [50-55] and other related-taxa [56,57] which suggests the link between environmental ambient temperature and nesting success has not been clearly established. Many factors can influence how ambient temperatures affect the temperature within a nest; hence ambient temperature may not accurately reflect the temperature within a nest. Substrate structure and porosity can have an influence on temperature directly (differential insulation) and on the influence of water and evaporative cooling. In Cuba, *C. acutus* nesting season takes place during the hottest months of the year. The small, but significant, negative effect of maximum temperature in *C. acutus* nesting success that we observe suggests that peak ambient temperatures may trigger embryo mortality. Previous findings in *Crocodylus porosus* showed that nest temperatures between 33.1°C and 35°C can cause high mortality of clutches, and higher nest temperatures (>35°C) can induce deformities in embryos [50]. We identified maximum temperature as a critical factor to monitor more closely into the future, and to expand monitoring protocols for systematic measurements of nest temperatures in addition to ambient temperatures. Our findings are especially important as potentially increases in temperature as a result of climate change in the following decades could not only affect sex ratios of crocodilians in the wild [58], but also their nesting success.

In our GLMM, total rainfall did not significantly influence nesting success. One of the main causes of clutch mortality in the WRMC is flooding (predation of eggs is practically non-existent), but in this area the initiation of the rainy season overlaps with the end of the nesting season. Hence, the mortality caused by flooding is not driven by the average and predictable rainfall associated with the wet season, but potentially by significant increases in rainfall during the nesting season that is mainly associated with tropical cyclones.

Estimates of sea level rise for Southeastern Cuba derived from climate change projections [59,60] report 15 centimeters increase by 2030, with up to 40% of the coastal areas of WRMC likely to be subject to inundation in the future [61]. These changes in the landscape will likely have detrimental effects on current *C. acutus* nesting sites, especially those located along or close to the coastline. Because Jobabito and Salina are the smallest sites, with the highest density of crocodiles, and the highest nesting success, our findings clearly show that *C. acutus* can thrive even when limited suitable nesting sites are available. Loss of habitat, while potentially significant, need not be catastrophic if management strategies for *C. acutus* in the WRMC incorporate information on nesting success derived from this study. Areas that will not be inundated, but which have the right properties for nesting, should be protected as potential climate refugia for the species. In addition, the construction of artificial nesting sites of calcareous gravel soils in high terrain, sheltered from the action of waves, might be required. Long-term conservation and restoration of coastal mangroves will also be critical to maintain crocodile nesting and will serve as a significant buffer against the broader impact of tropical cyclones.

Predictions indicate that human induced climate change will cause an increase in the intensity of tropical cyclones at the global scale [62-64], with substantial increases in the precipitation rate within 100 kilometers of the cyclone center [62]. The Caribbean region is expected to be amongst the most severely impacted by climate change over the course of this century [65,66]. Even though tropical cyclones can be beneficial for crocodile nesting populations in years after its occurrence; higher-intensity tropical cyclones can be devastating when they strike during the nesting season. In archipelagos and islands, the recruitment of these populations relies entirely on nesting success. As plans for coastal adaptation in the context of climate change are developed in Cuba, we suggest incorporating information on crocodilians and their habitats, as they will be key components of coastal resilience.

References

1. Ylönen H, Ronkainen H (1994) Breeding suppression in the bank vole as antipredatory adaptation in a predictable environment. Evolutionary Ecology 8: 658-666.
2. Shine R, Brown GP (2008) Adapting to the unpredictable: reproductive biology of vertebrates in the Australian wet–dry tropics. Philosophical Transactions of the Royal Society of London B: Biological Sciences 363: 363-373.
3. Bull JJ (2015) Evolution: Reptile sex determination goes wild. Nature 523: 43-44.
4. Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. Molecular Ecology 20: 1347-1363.
5. Seebacher F, White CR, Franklin CE (2015) Physiological plasticity increases resilience of ectothermic animals to climate change. Nature Climate Change 5: 61-66.
6. Woodward DE, Murray JD (1993) On the effect of temperature-dependent sex determinaion on sex-ratio and survirvorship in crocodilians. Proceedings of the Royal Society of London Series B-Biological Sciences 252: 149-155.
7. Lance VA (2003) *Alligator* physiology and life history: the importance of temperature. Experimental Gerontology 38: 801-805.

8. Gribbins KM, Elsey RM, Gist DH (2006) Cytological evaluation of the germ cell development strategy within the testis of the American alligator, *Alligator mississippiensis*. Acta Zoologica 87: 59-69.

9. Somaweera R, Shine R (2012) Nest-site selection by crocodiles at a rocky site in the Australian tropics: Making the best of a bad lot. Austral Ecology.

10. Alonso Tabet M (2009) Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante & Universidad de Pinar del Rio Hermanos Saiz Montes de Oca.

11. Alonso Tabet M, Rodriguez Soberón R (1998) Observations on nesting behavior of *Crocodylus acutus*. Crocodile Specialist Group Newsletter 17: 11-13.

12. Marcó MVP, Piña CI, Simoncini M, Alejandro L (2010) Effects of Incubation and Rearing Temperatures on *Caiman latirostris* Growth. Zoological Studies 49: 367-373.

13. Charruau P (2011) Microclimate of American crocodile nests in Banco Chinchorro biosphere reserve, Mexico: Effect on incubation length, embryos survival and hatchlings sex. Journal of Thermal Biology.

14. López-Luna MA, Hidalgo-Mihart MG, Aguirre-León G, González-Ramón MdC, Rangel-Mendoza JA (2015) Effect of nesting environment on incubation temperature and hatching success of Morelet's crocodile (*Crocodylus moreletii*) in an urban lake of Southeastern Mexico. Journal of thermal biology 49: 66-73.

15. Charruau P, Thorbjarnarson JB, Henaut Y (2010) Tropical cyclones and reproductive ecology of *Crocodylus acutus* Cuvier, 1807 (Reptilia: Crocodilia: Crocodylidae) on a Caribbean atoll in Mexico. Journal of Natural History 44: 741-761.
16. Lande R (1993) Risks of population extinction from demographic and environmental stochasticity and random catastrophes. American Naturalist: 911-927.
17. Pike DA, Stiner JC (2007) Sea turtle species vary in their susceptibility to tropical cyclones. Oecologia 153: 471-478.
18. Milton SL, Leonekabler S, Schulman AA, Lutz PL (1994) Effects of hurricane Andrew on the sea turtle nesting beaches of South Florida. Bulletin of Marine Science 54: 974-981.
19. Michener WK, Blood ER, Bildstein KL, Brinson MM, Gardner LR (1997) Climate change, hurricanes and tropical storms, and rising sea level in coastal wetlands. Ecological Applications 7: 770-801.
20. ONEI (2013) Anuario Estadistico de Cuba. Republica de Cuba: Oficina Nacional de Estadistica e Informacion.
21. Thorbjarnarson J (1989) Ecology of the American Crocodile, *Crocodylus acutus*. In Crocodiles, their Ecology, Management and Conservation IUCN Publications New Series Gland, Switzerland: 228-258.
22. Soberón RR, Tabet MA, Alvarez VB, Verdade L, Larriera A (2002) Nidificación del cocodrilo americano (Crocodylus acutus Cuvier) en el

refugio de fauna “Monte Cabaniguan”, Cuba. Conservaçaoe Manejo de Jacarés e Crocodilos da América Latina 2: 135-156.

23. Alonso-Tabet M (2009) Comportamiento del cocodrilo americano (*Crocodylus acutus*) en el Refugio de Fauna Monte Cabaniguan, Cuba. Republica de Cuba: Universidad de Alicante and Universidad de Pinar del Rio Hermanos Saiz Montes de Oca.
24. Rodriguez-Soberon R (2009) Situacion, estrategia de conservacion y uso sostenible de *Crocodylus acutus* en Cuba. Republica de Cuba. Republica de Cuba: Universidad de Alicante/Universidad de Pinar del Rio "Hermanos Saiz Montes de Oca"
25. Thorbjarnarson J (1989) Ecology of the American Crocodile, *Crocodylus acutus*. In Crocodiles, Their Ecology, Management and Conservation A Spetial Publication of the Crocodile Specialist Group IUCN Publications NS Gland, Switzerland: 228-258.
26. Swanepoel D, Ferguson N, Perrin M (2000) Nesting ecology of Nile crocodiles (*Crocodylus niloticus*) in the Olifants River, Kruger National Park. Koedoe 43: 35-46.
27. Mazzotti FJ (1989) Factors affecting the nesting success of the American crocodile, *Crocodylus acutus*, in Florida Bay. Bulletin of Marine Science 44: 220-228.
28. Thorbjarnarson J (1988) The status and ecology of the American crocodile in Haiti. Bulletin of the Florida State Museum of Biological Sciences 33: 1-86.

29. Alonso-Tabet M, Soberón R, Berovides V, Hernández C. Influence of changes in morphology of nesting habitat on reproduction of *Crocodylus acutus* in the Monte Cabaniguan Wildlife Refuge, Cuba; 2000. pp. 42-58.

30. Alonso Tabet M, Rodriguez Soberón R, Thorbjarnarson J, Ramos Targrarona R (2006) American crocodile (*Crocodylus acutus*) egg losses related to nesting females behaviour, at the Delta del Cauto Wildlife Refuge, Cuba.

31. Rodríguez Soberon R. Situación actual de *Crocodylus acutus* en Cuba; 2000; The World Conservation Union, Gland, Switzerland and Cambridge, UK: xvii + 541 p. pp. Pp.17-32.

32. Varona L (1987) The Status of *Crocodylus acutus* in Cuba Caribbean Journal of Sciences 23: 256-259.

33. Team RC (2015) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

34. Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, et al. (2009) Generalized linear mixed models: a practical guide for ecology and evolution. Trends in ecology & evolution 24: 127-135.

35. StataCorp. (2015) Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.

36. Sakamoto Y, Ishiguro M, Kitagawa G (1986) Akaike information criterion statistics. Dordrecht, The Netherlands: D Reidel.

37. Platt SG, Thorbjarnarson JB, Price A (2000) Nesting ecology of the American crocodile in the coastal zone of Belize. Copeia 2000: 869-873.

38. Mazzotti FJ (1999) The American crocodile in Florida Bay. Estuaries 22: 552-561.

39. Balaguera-Reina SA, Venegas-Anaya M, Sanjur OI, Lessios HA, Densmore III LD (2015) Reproductive Ecology and Hatchling Growth Rates of the American Crocodile (*Crocodylus acutus*) on Coiba Island, Panama. South American Journal of Herpetology 10: 10-22.

40. Charruau P, Hénaut Y (2012) Nest attendance and hatchling care in wild American crocodiles (*Crocodylus acutus*) in Quintana Roo, Mexico. Animal Biology 62: 29-51.

41. Thorbjarnarson JB (1996) Reproductive characteristics of the order Crocodylia. Herpetologica 52: 8-24.

42. Deeming DC, Thompson MB (1991) Gas exchange across reptilian eggshells. Egg incubation: its effects on embryonic development in birds and reptiles Cambridge University Press, Cambridge: 277-284.

43. Lutz PL, Dunbar-Cooper A (1984) The nest environment of the American crocodile (*Crocodylus acutus*). Copeia: 153-161.

44. Ackerman RA, Seagrave RC, Dmi'el R, Ar A (1985) Water and heat exchange between parchment-shelled reptile eggs and their surroundings. Copeia: 703-711.

45. Ogden JC (1978) Status and nesting biology of the American crocodile, *Crocodylus acutus*,(Reptilia, Crocodilidae) in Florida. Journal of Herpetology: 183-196.

46. Milton SL, Leone-Kabler S, Schulman AA, Lutz PL (1994) Effects of Hurricane Andrew on the sea turtle nesting beaches of South Florida. Bulletin of Marine Science 54: 974-981.

47. Long TM, Angelo J, Weishampel JF (2011) LiDAR-derived measures of hurricane-and restoration-generated beach morphodynamics in relation to sea turtle nesting behaviour. International journal of remote sensing 32: 231-241.

48. Lance VA, Elsey RM, Butterstein G, Trosclair PL, Merchant M (2010) The effects of hurricane Rita and subsequent drought on alligators in southwest Louisiana. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 313: 106-113.

49. Elsey RM, Aldrich C (2009) Long-distance displacement of a juvenile alligator by Hurricane Ike. Southeastern Naturalist 8: 746-749.

50. Webb GJ, Cooper-Preston H (1989) Effects of incubation temperature on crocodiles and the evolution of reptilian oviparity. American Zoologist 29: 953-971.

51. Webb GJ, Smith AM. Sex ratio and survivorship in the Australian freshwater crocodile *Crocodylus johnstoni*; 1984. pp. 319-355.

52. Piña CI, Larriera A, Cabrera MR (2003) Effect of incubation temperature on incubation period, sex ratio, hatching success, and survivorship in Caiman latirostris (Crocodylia, Alligatoridae). Journal of herpetology 37: 199-202.

53. Woodward D, Murray J (1993) On the effect of temperature-dependent sex determination on sex ratio and survivorship in crocodilians. Proceedings of the Royal Society of London B: Biological Sciences 252: 149-155.

54. Charruau P (2012) Microclimate of American crocodile nests in Banco Chinchorro biosphere reserve, Mexico: Effect on incubation length, embryos survival and hatchlings sex. Journal of Thermal Biology 37: 6-14.

55. Hutton J (1987) Incubation temperatures, sex ratios and sex determination in a population of Nile crocodiles (*Crocodylus niloticus*). Journal of Zoology 211: 143-155.

56. Spencer R-J (2002) Experimentally testing nest site selection: fitness trade-offs and predation risk in turtles. Ecology 83: 2136-2144.

57. Booth DT (2006) Influence of Incubation Temperature on Hatchling Phenotype in Reptiles*. Physiological and Biochemical Zoology 79: 274-281.

58. Campos Z (1993) Effect of habitat on survival of eggs and sex ratio of hatchlings of *Caiman crocodilus* yacare in the Pantanal, Brazil. Journal of Herpetology: 127-132.

59. Jevrejeva S (2012) Sea level projections to AD2500 with a new generation of climate change scenarios. Global and planetary change 80-81: 14-20.

60. Doney SC, Ruckelshaus M, Emmett Duffy J, Barry JP, Chan F, English CA, et al. (2012) Climate Change Impacts on Marine Ecosystems. Annual Review of Marine Science 4: 11-37.

61. Alvarez AF, Mercadet A (2011) El sector forestal cubano y el cambio climatico; Cuba IdIA-FdMdlAd, editor. Cuba.

62. Knutson TR, McBride JL, Chan J, Emanuel K, Holland G, Landsea C, et al. (2010) Tropical cyclones and climate change. Nature Geoscience 3: 157-163.

63. Pachauri RK, Allen M, Barros V, Broome J, Cramer W, Christ R, et al. (2014) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.

64. Emanuel K (2005) Increasing destructiveness of tropical cyclones over the past 30 years. Nature 436: 686-688.

65. Rhiney K (2015) Geographies of Caribbean Vulnerability in a Changing Climate: Issues and Trends. Geography Compass 9: 97-114.

66. IPCC (2014) Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; Barros VR, C.B. Field, D.J. Dokken, M.D. Mastrandrea, K.J. Mach, T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea, and L.L. White, editor. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press. 688 p.

Table 1. Characterization of nesting sites at the WRMC

Nesting site ID	Area (hectares)	Location	Distance from coastline (m)	Elevation (cm above sea level)	Predominant soil/nesting substrate	Exposure to wave action
La Jijira	2.0	Inland	2500	>45	Fine sand and clay	No
La Salina	1.2	Near the coast	245	>45	Calcareous gravel	Low
Jobabito	1.6	Near the coast	199	>45	Calcareous gravel	Low
Soloburén	2.7	Coastal	23	<40	Fine sand and clay	High
Ojo de Agua	1.9	Coastal	17	<40	Fine sand and clay	High

Table 2. Pairwise comparisons among *C. acutus* nesting sites in the Wildlife Refuge Monte Cabaniguán

Nesting site ID	Jijira	Salina	Jobabito	Soloburén	Ojo de Agua
Jijira	-	-3.168 (**0.008**)	-4.171 (**0.000**)	3.302 (**0.004**)	2.301 (0.107)
Salina	-3.284 (**0.005**)	-	1.003 (1.000)	6.470 (**0.000**)	-5.469 (**0.000**)
Jobabito	-3.795 (**0.001**)	0.512 (1.000)	-	7.473 (**0.000**)	6.472 (**0.000**)
Soloburén	-0.608 (1.000)	2.675 (**0.037**)	3.187 (**0.007**)	-	1.001 (1.000)
Ojo de Agua	-2.329 (0.099)	-0.954 (1.000)	1.466 (0.713)	1.721 (0.426)	-

Table 3. Results from GLMM analysis showing variation in nesting success in C. acutus in the WRMC (n= 3450 observations from 21 years in 5 nesting sites). The table shows variable included in the models, and the influence from each model on nesting success. Model selection is based on ΔAIC. TC: tropical cyclones; ΔAIC = AIC(i) - AIC(min); ω_i = Akaike weights explaining total variance. The most parsimonious model is highlighted in bold

Models	**K**	**AIC**	**ΔAIC**	ω_i
TC (t_0) + TC (t_1)+ TC (t_2) + Tmax	**6**	**4420.71**	**0.00**	**0.59**
TC (t_0) + TC (t_1)+ TC (t_2)	5	4423.05	2.34	0.18
TC (t_0) + TC (t_1)+ TC (t_2) + far from beach	6	4425.02	4.30	0.07
TC (t_0) + TC (t_1)+ TC (t_2) + Tmin	6	4425.03	4.31	0.07
TC (t_0) + TC (t_1)+ TC (t_2) + Tmax + Tmin + rainfall + far_from_beach	9	4425.79	5.07	0.05
*TC (t_0) + TC (t_1)+ TC (t_2) * far from beach*	7	4425.99	5.28	0.04
rainfall	3	4466.18	45.46	~ 0.00
temperature (Tmax + Tmin)	4	4486.29	65.58	~ 0.00
intercept	2	4486.42	65.71	~ 0.00

Table 4. Parameter estimates (β) from the most parsimonious model in Table 3 explaining variation in *C. acutus* nesting success.

Parameter	**Estimate (β)**	**SE**	**z-value**	**Pr(β>|z|**
(Intercept)	1.833	1.111	1.650	0.099
tropical cyclone_t_0	-0.690	0.115	-5.975	< 0.001
tropical cyclone_t_1	0.403	0.085	4.408	< 0.001
tropical cyclone_t_2	0.191	0.088	2.164	0.030
T_{max}	-0.056	0.031	-1.826	0.047

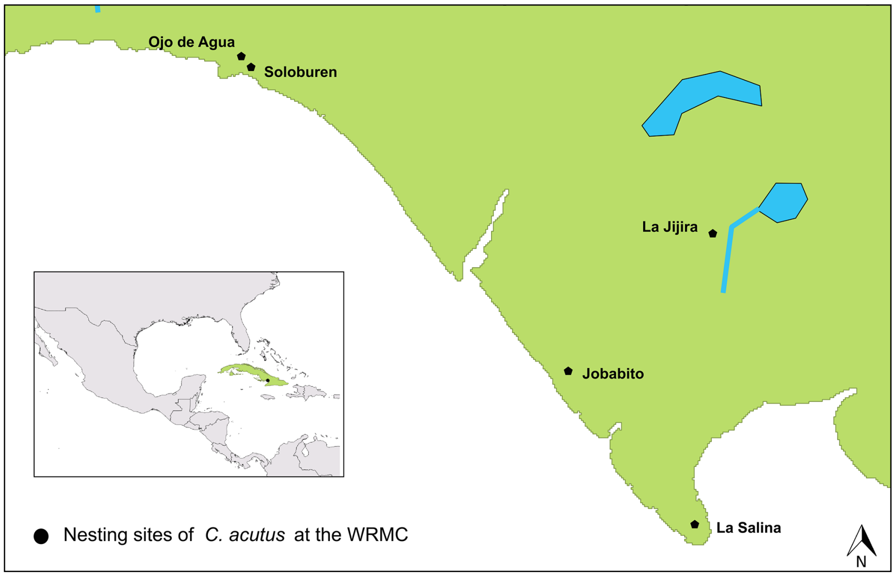

Figure 1. Location of WRMC and five permanent *C. acutus* nesting sites

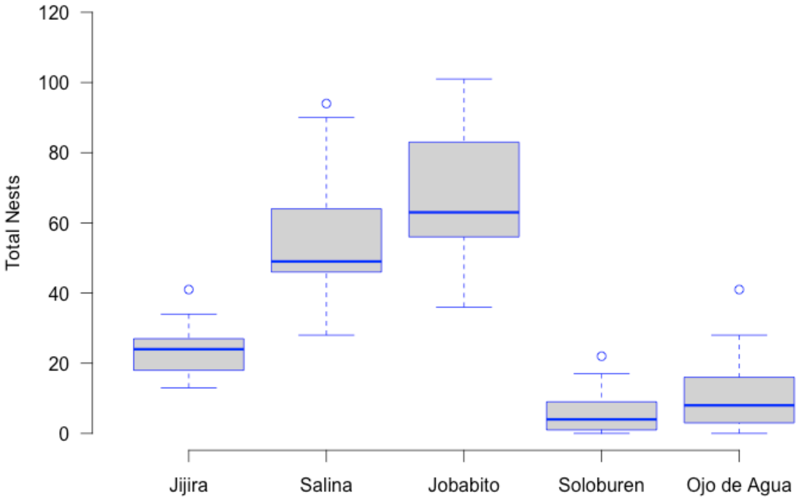

Figure 2. Boxplots depicting the total number of nests across five permanent nesting sites in the Wildlife Refuge Monte Cabaniguán for the period of 1993-2013

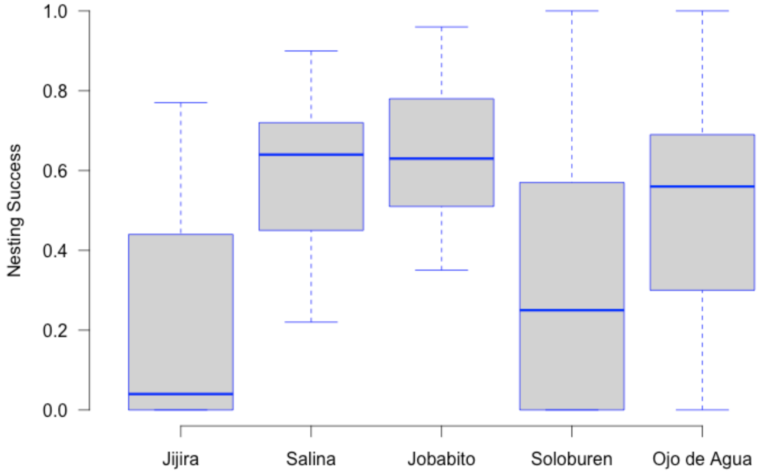

Figure 3. Boxplots depicting *C. acutus* nesting success across five permanent nesting sites in the Wildlife Refuge Monte Cabaniguán for the period of 1993-2013

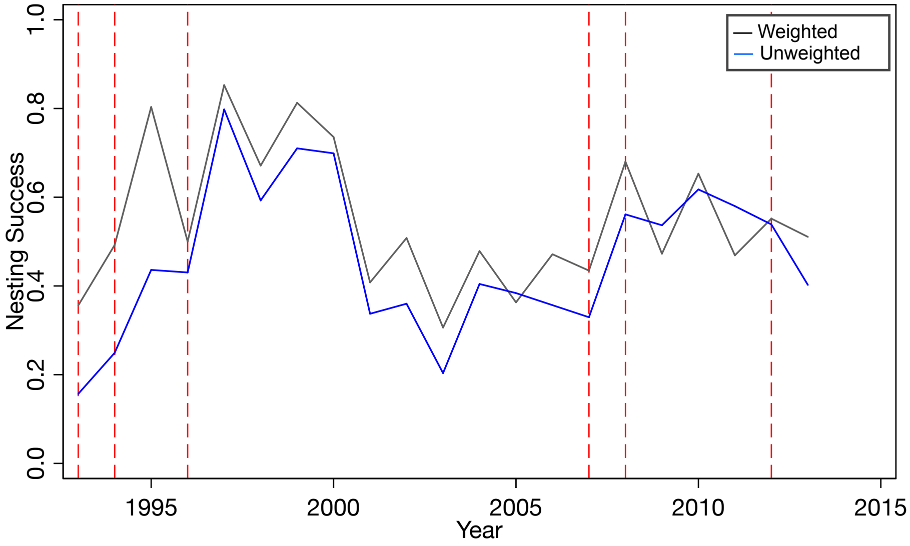

Figure 4. Trend of nesting success by year for the period of 1993-2013 for five permanent nesting sites combined at the Wildlife Refuge Monte Cabaniguán Dashed-red-lines indicate years of occurrence of a hurricane/tropical storm; blue lined indicated unweighted nesting success; and black line indicates weighted nesting success

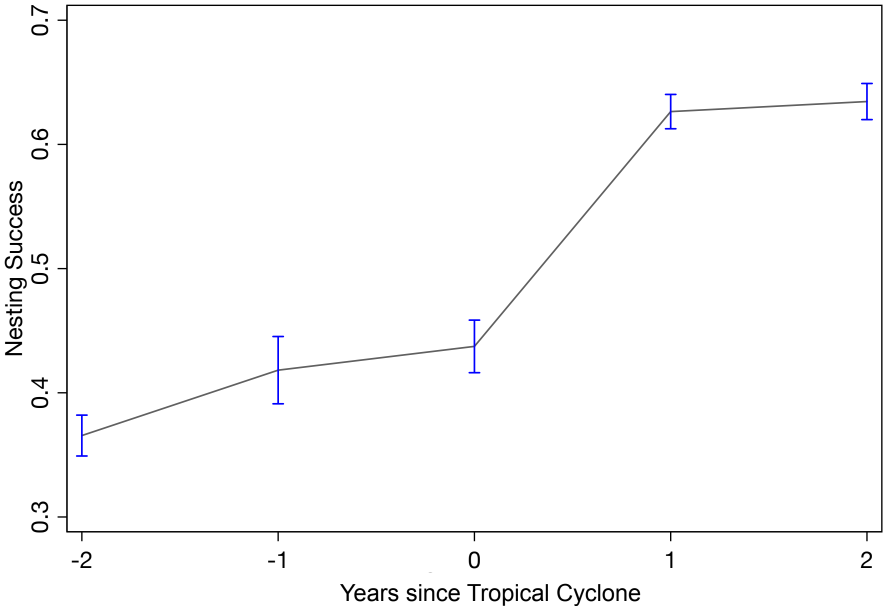

Figure 5. Nesting Success before and after the occurrence of a tropical cyclone

CHAPTER IV

HIGH LEVELS OF POPULATION GENETIC DIFFERENTIATION IN THE AMERICAN CROCODILE (*CROCODYLUS ACUTUS*)

Abstract

The American crocodile (*Crocodylus acutus*) is a widely distributed species across coastal and brackish areas of the Neotropical region of the Americas and the Greater Antilles. Currently available information on patterns of genetic differentiation in *C. acutus* has shown a complex structuring influenced by interspecific interactions (mainly hybridization) and anthropogenic actions (mainly poaching and unintentional translocation of individuals). Most of the work on genetic variation, however, has primarily focused either in a single population concentrated in Southern Florida, or in present hybrid zones with other new world crocodilians. In this study, we used data on mitochondrial DNA control region and 12 nuclear polymorphic microsatellite loci to assess the degree of population structure between and among localities in South America, North America, Central America and the Greater Antilles. We used traditional genetic differentiation indices, Bayesian clustering and multivariate methods to create a more comprehensive picture of the genetic relationships within the species across its range. All analyses for both mtDNA and nuclear markers show evidence of strong population genetic structure in the American crocodile, with unique populations in each of the sampling localities. Our results reinforced previous findings showing the greatest degree of genetic differentiation between the continental *C. acutus* and the Greater Antillean *C. acutus.* We report three new haplotypes unique to Venezuela, considerably less distant from Central and North American haplotypes than Greater Antillean haplotypes. Our overall evidence suggests that Cuban and Jamaican *C. acutus* share a mtDNA haplotype but currently represent at least two different genetic populations when using nuclear, faster evolving, microsatellite markers. Our findings offer the first evidence of genetic differentiation among the populations of Greater Antillean *C. acutus* and provide important information for the regional planning and conservation of the species.

Introduction

The American crocodile is widely distributed along the coastal swamps, estuarine rivers, and lakes of the Neotropical region of the Americas and Insular Caribbean [1]. It is primarily a coastal crocodilian inhabiting brackish water coastal habitats such as the saltwater sections of rivers, coastal lagoons, and mangrove swamps [2]. The species also occurs in freshwater areas located well inland [3-5]. This wide ranging species overlaps its geographic range with three other new world crocodilians: the Cuban crocodile (*Crocodylus rhombifer*) in Zapata, Cuba [6], the Morelet's crocodile (*Crocodylus moreletii*) in the Yucatan Peninsula, Mexico [7] and Belize [8], and the Orinoco crocodile *(Crocodylus intermedius)* in Venezuela [9]. It hybridizes in the wild with *C. moreletii* [10,11] and *C. rhombifer* [6,12]. The ability to tolerate and thrive in highly saline environments [13,14] allowed *C. acutus* to occupy a unique ecological niche and extend geographically more than any other new world crocodilian.

Like many crocodilians, populations of the American crocodile have declined as a result of intense hunting pressure, which has been ongoing since the early 1800s. The species was listed in the Convention on International Trade in Endangered Species of Flora and Fauna (CITES) at the Convention's inception in 1975 [15]. Since then, a combination of sustainable farming [15], habitat protection [16] and conservation and monitoring programs [17] have aided the recovery of the species. However, this recovery has not been uniform throughout the range of *C. acutus* and new conservation challenges have arisen. These new threats have been driven by changes in water regimes and water quality of coastal wetlands, illegal hunting [18], human-mediated movements of animals across the range [19], and habitat loss and fragmentation [16]. The species is categorized as vulnerable by the IUCN [20] and listed in CITES Appendix I [21], which prohibits trade, except for the Cuban population which was transferred to Appendix II in 2007 [2].

An important challenge in the conservation of any widespread species is to identify unique clusters that need to be considered independent management units. These clusters are usually defined using information from many sources including: habitat types within the range of the species, local conservation status, threats, enforcement and legislation [16,22], and the patterns of genetic variation found within and among subpopulations of a species. For *C. acutus,* most of the work on genetic variation has been focused either in a single population concentrated in Southern Florida [19], or in present hybrid zones with *C. rhombifer* [6,23] and *C. moreletti.* Even though genetic differentiation has been used widely as a tool for delineating local management units, including work done on some species of *Crocodylus* [24], there is still a need to incorporate intraspecific genetic information into conservation planning for many species of crocodilians, including the American crocodile.

Information currently available on patterns of genetic differentiation in *C. acutus* has shown a complex structuring influenced by interspecific interactions (mainly hybridization) and anthropogenic actions (mainly poaching and unintentional translocation of individuals). Analysis of variation in the control region of mitochondrial DNA (mtDNA) revealed a minimum of 11 haplotypes in the American crocodile [19,23]. Nine of these are present in populations of Central and North America [19], and two are exclusive to the Greater Antilles [23]. South American *C. acutus*, however, has not been incorporated in these analyses, and the relationship of Antillean crocodiles with South American lineages is still unclear. Hybridization with other crocodilian species, and human-mediated migration (assisted migration) of animals across the range may affect the distribution and frequency of reported haplotypes [19].

Studies of the population genetic structure within, between and among populations based on contemporaneous genetic markers such as microsatellites have been limited. One of the few studies reported significant patterns of population substructure in Southern Florida caused by admixture of native crocodiles with those from Latin America and the Greater Antilles brought to the region by humans [19].

The diagnosis of conservation management units based on molecular data has proven more effective when combining different types of genetic markers [25,26]. The hypervariable control region of the mitochondrial DNA has been one of the most informative and widely used markers to uncover population subdivision [25,27]. In addition, nuclear polymorphic microsatellite markers have proven useful to detect subtle patterns of population structure [28,29] and to accurately assign samples to populations of origin [24]. In the American crocodile, the use of mtDNA markers alone for population differentiation has been demonstrated to be useful for detecting higher hierarchies of structuring, but not for resolving more subtle patters of population subdivision [19]. Hence, for accurate analysis of genetic population structure in *C. acutus* a combination of markers, as well as a diverse suite of methodological approaches, will be required.

To better understand patterns of genetic differentiation in the American crocodile we used data on mitochondrial and nuclear DNA to assess the degree of population structure between and among localities in South America, North America, Central America and the Greater Antilles. We incorporated previously unsampled areas (two protected areas in Jamaica, and one site in Venezuela) into a broader study including populations that had already been studied, to create a more comprehensive picture of the genetic relationships within the species across its range. Our work aims to this understanding of genetic structure to inform conservation efforts and aid the recovery of the species.

Materials and Methods

Sample collection

As part of crocodilian conservation and monitoring programs, we collected tissue samples of 218 *C. acutus* at five localities including the Everglades National Park, Belize, Venezuela, Southeastern Cuba, and Jamaica (Fig 1). Samples were removed from the dorsal section of the base of the tail and stored at -20 °C in 95 % ethanol until DNA extraction was performed. Samples from all sites except Venezuela were transported to the American Museum of Natural History (CITES export permits C0001733 and JM2320, and USFWS permit 1018-0093) for laboratory analyses including extraction of DNA, sequencing of mtDNA control region, and amplification of 11 microsatellite loci. Samples from Venezuela were transported to the Museo Nacional de Ciencias Naturales (CITES permit 1395/VE9120190) for laboratory analyses encompassing extraction of DNA and sequencing of mtDNA control region. We were not able to generate microsatellite data for the Venezuela samples.

We complemented our mtDNA control region dataset with additional sequences from Mexico, Costa Rica, and Southwestern Cuba, obtained in Genbank (Table 1).

This project was carried out in collaboration with the Wildlife Conservation Society, the American Museum of Natural History, the Fort Lauderdale Research and Education Center at University of Florida, Facultad de Biología de la Universidad de la Habana, the Department of Life Sciences at University of the

West Indies, Museo de Historia Natural La Salle in Venezuela, the University of Uppsala, Sweden, and Museo Nacional de Ciencias Naturales (CSIC) in Madrid, Spain.

DNA extraction, sequencing and genotyping

We extracted DNA from fresh scale tissue using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following manufacturer recommendations. The final DNA concentration of each sample was measured using a NanoDrop 2000c. A fragment of 525 base pairs (bp) of mitochondrial control region was amplified with two primers (drL15459 and CR2HA [1]). Polymerase chain reaction (PCR) was performed at a final volume of 25µl with 1.25 unit of AmpliTaq Gold (Applied Biosystems, Foster City, California, USA), 1x PCR Buffer, 0.2mM each dNTP, 0.2 µM each primer, 1mM $MgCl_2$ and 50ng/µl DNA. An initial 2-minute denaturation cycle at 94°C was followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 1min, and elongation at 72°C for 7 minutes. Amplicons were purified and concentrated via ethanol precipitation. Double-stranded DNA was sequenced with the dideoxy chain termination method using an ABI 3730 automated sequencer.

To genotype all samples, we used a panel of 11 microsatellite markers previously developed for *Crocodylus*, including C391, Cj16, Cj18, Cj35, Cj127, Cj119, Cj131, CUJ131, Cu5123 [30], Cj20, Cj109 [31],. PCR reactions were prepared at a final volume of 12.5µl with 1.0 unit of AmpliTaq Gold (Applied Biosystems, Foster City, California, USA), 1x PCR Buffer, 0.4mM each dNTP, 0.1 µM fluorescently labeled forward primer, 0.1 µM reverse primer, 3.5mM

$MgCl_2$ and 50ng/µl DNA. Microsatellites were amplified in single PCRs for 33 cycles and three different annealing temperatures (51 ºC for Cj35 and Cj127, 62 ºC for Cj16, Cj20, and Cj109, and 58 °C for all the rest). An initial 2-minute denaturation cycle at 94°C was followed by 33 cycles of denaturation at 94°C for 30 seconds, a different annealing temperature for each microsatellite at 51-62°C for 1minute, elongation at 72°C for 45 seconds and a final elongation step at 72°C for 5 minutes. We visualized PCR products using an ABI 3730 automated sequencer. Genotypes were identified using GeneMapper v5.0 software (Applied Biosystems, Foster City, California, USA). Allelic sizes were scored against the size standard GS500 LIZ. All laboratory work was carried out at the Sackler Institute for Comparative Genomics at the American Museum of Natural History, New York, USA, except for the processing of *C. acutus* samples from Venezuela, which was done at the Museo Nacional de Ciencias Naturales in Spain.

Haplotypic and genotypic variation

We aligned mtDNA sequences in GENIOUS 8.1.7 (Biomatters, Ltd., San Francisco, CA, USA) using the Clustal algorithm and default settings. From the 525 bp mtDNA control region fragment, we selected a 458 bp consensus region containing about 95% of the variation within our samples. We compared the mtDNA sequences obtained with those downloaded from Genbank corresponding to Mexico (AY538614, AY568311-12 [10]), Costa Rica (GU064561-65 [19]), and Southwestern Cuba (EU034586 [12]; KM577701 [23]). We then matched sequences to previously described haplotypes by Rodriguez *et al* [19] using DnaSP v5.10.1 software [32]. We used Rodriguez *et al* [19] haplotypes definitions and nomenclature for our analysis. Haplotypic diversity (H_d) [33], nucleotide diversity (π) [34,35], and the mean number of pairwise differences among sequences (K) [34-36] were calculated in Arlequin 3.5 [37,38] and DnaSP. We used TCS software to construct statistical parsimony haplotype networks [39] in order to depict a intraspecific genealogy for our mtDNA sequences. We then built a median-Joining network [40] to depict patterns of genetic variation among haplotypes using the NETWORK software (www.fluxus-engineering.com).

We used Arlequin to estimate the number of alleles, observed (H_O) and expected heterozygosities (H_E) in our microsatellite marker set. Conformity to Hardy-Weinberg (HW) expectations for each locus and genotypic linkage disequilibrium (LD) between pairs of loci were tested in GENEPOP 4.3 [41]. Significance levels ($p \leq 0.05$) for departure from HW and LD were corrected for multiple comparisons with a sequential Bonferroni correction [42]. Contamination and primer-site mutations may result in the incorrect assignment of microsatellite genotypes (genotyping errors). Additionally, DNA degradation, low DNA concentration, and primer-site mutations can potentially cause non-amplified alleles (null alleles), which can lead to erroneous interpretations of the data. Potential genotyping inconsistencies including the presence of null alleles, large allele dropout, scoring of stutter peaks and typographic errors, were assessed in Micro-checker [43].

Analysis of population structure

For the complete dataset (n=258 individuals), we used mtDNA sequence data to test for population structure across the nine localities where *C. acutus* was sampled by the authors or where other sequences were readily available: Everglades National Park (EVNP), Yucatan, Mexico (MX), Turneffe Atoll, Belize (BZ), Costa Rica (CR), Tacarigua Lagoon National Park, coastal Venezuela (VE), Zapata Swamp National Park, Cuba (ZAP_CU), Wildlife Refuge Monte Cabaniguán, Cuba (WRMC_CU), Black River Lower Morass, Jamaica (BRLM_JM), and Portland Bight Protected Area, Jamaica (PBPA_JM) (Fig 1). The partitioning of genetic variation among putative populations was assessed through a nested analysis of molecular variance (AMOVA) implemented in Arlequin. We computed genetic distances in Arlequin, including pairwise F_{ST} (haplotype frequencies only) and Φ_{ST} statistics (pairwise differences between haplotypes). The significance of values (Fst and ΦST) was tested using 10000 nonparametric random permutations of the data. A Chi-square analysis was conducted in DnaSP to test for significant differences in haplotype frequency distributions among sampling localities.

We obtained and analyzed microsatellite data for a subset of the data, including the populations of EVNP, BZ, WRMC_CU, BRLM_JM, and PBPA_JM (total n=218). In these five putative populations, we assessed spatial structure though the AMOVA analysis, and estimated pairwise F_{ST} statistics in Arlequin, using Wier and Cockerham's [44] estimator, which assumes an infinite allele model of mutation [44]. We further evaluated the degree of partitioning among putative populations using spatially explicit and non-explicit Bayesian clustering methods. The package Geneland 3.1.4 [45,46] implemented in R 3.2.3 [47] uses an algorithm based on a spatial model which assigns individuals into a number of genetic clusters (*K*) making use of genotypes as well as spatial coordinates of sampled individuals. As is common to all non-explicit clustering methods, Geneland weights information of individual's location in the search for the most likely *K* instead of assuming that all clustering solutions are equally possible. STRUCTURE 2.3.4 [48-50] uses the number of populations (*K*) as a fixed parameter to estimate the log-likelihood of the data for the pre-defined *K* values, and assigns memberships for all individuals in the total sample set. Both software programs infer unknown parameters through Markov Chain Monte-Carlo (MCMC) computations, assume Hardy-Weinberg equilibrium with linkage equilibrium between loci, and do not require an *a priori* definition of putative populations. STRUCTURE, however, allows for the incorporation of sampling location priors. Location priors allow STRUCTURE to assign individuals into genetically similar clusters taking into account *a priori* the geographic origin of each individual.

For Geneland, we determined *K* across 10 independent runs with 1,000,000 MCMC iterations and allowing *K* to vary from one to eight. We used the correlated allele model and set the maximum rate of the Poisson process at 218 (the number of individuals, as suggested by [46]), the maximum number of nuclei in the Poisson-Voronoi tessellation at 654 (three times the number of individuals as suggested by [46]), and the uncertainty of spatial coordinates of collection at 20 meters. In our study, the uncertainty of spatial coordinates accounted for the recording error. We then inferred the most likely number of clusters as the modal *K* with the highest posterior probability. We ran STRUCTURE analysis without sampling location priors, through 20 independent runs for *K* = 1–8. We set a burn-in period of 100,000 and 1.000.000 MCMC iterations to identify the genetic clusters. We assumed an admixture model with correlated allele frequencies, which assumes that individuals have mixed ancestry. We determined the optimal value of *K* according to the Δ*K* method [51] using STRUCTURE HARVESTER 0.6.94 online application [52]. We then performed consensus analyses for the average scores for the inferred *K* value in CLUMPP 1.1.2 software [53] and used STRUCTURE PLOT software [54] to generate the STRUCTURE output. Lastly, we used a third method to complement Bayesian inference analyses. We carried out a Principal Component Analysis (PCA [55]) using the Adegenet package [56] (function dapc) implemented in R 3.2.3 [47]. PCA is a multivariate analysis that clusters samples based on the variance in allele frequencies to identify the main principal components (PCs) that account for the largest amount of variability in the data that explain the main patterns [57].

Results

Genetic diversity

We identified a total of ten haplotypes in our genetic analysis of mtDNA control region of *C. acutus* across the Americas and Greater Antilles (Table 2). Four of these *C. acutus* haplotypes had not been previously described, and the other six matched previously identified haplotypes [10,19]. Only a single haplotype, β, described by Cedeño-Vazquez *et al* [10] was observed in the Antillean population of *C. acutus*. In our mtDNA control region sequences from the Everglades, the haplotype Ca1 was most common. In the Everglades NP, we also found two individuals with Antillean haplotype β and one with a newly reported haplotype (Ca10). American crocodiles sampled at Turneffe Atoll, Belize, exhibited the two more common haplotypes for the species in Central and North America (Ca1 and Ca2). We found three haplotypes for South American *C. acutus* (Ca11, Ca12, and Ca13).

Greater Antillean *C. acutus* populations from Jamaica (BRLM and PBPA) and southeastern Cuba (WRMC) showed the lowest values for every estimate of mtDNA control region genetic diversity (Table 2). Maternal lineages sampled from these populations seemed to be identical in terms of molecular distances, with the same mtDNA haplotype (β) and zero values for pairwise differences among sequences and nucleotide diversity. Likewise, Costa Rica had zero values for all genetic diversity estimators with only one haplotype sampled to date. However, the available sample of only five individuals may not represent the true number of haplotypes found in the population. Mexico exhibited the highest value of haplotypic diversity, followed by Venezuela, Zapata and the Everglades. Molecular distances, however, were relatively similar among Mexican samples, and higher for Zapata, the Everglades, and Venezuela.

We detected strong patterns of haplotype distribution across geographic locations. The statistical parsimony haplotype network (Fig 2) reveals differences in haplotype identity between the Americas and the Greater Antilles. Cuba and

Jamaica comprised a cluster separated from all other populations. Haplotypes present in *C. acutus* from the Everglades, Central America and Venezuela are closer related than those in the Antilles.

Analysis of microsatellite data for a subset of populations comprising Everglades, Belize, Southwestern Cuba (WRMC) and Jamaica (BRLM and PBPA) did not find evidence for LD and rejected deviations from HW in all pairwise tests ($p < 0.05$). We found no significant differences between the expected heterozygosity under HW and that observed in the data, for any of the putative populations (Table 3). Cj109 and Cj127 were the loci with highest allele numbers, and CUJ131 and CUD68 showed the lowest allele numbers.

Population structure

For mtDNA sequence data, the among-groups component of the AMOVA analysis was significant when both the haplotype frequencies as well as the molecular distances were considered (F_{ST}: 0.9623, $p<0.0001$; $\Phi_{ST}=0.75422$, $p<0.001$). Similarly, the χ^2 showed significant differentiation among populations ($\chi^2 = 659.027$, $p<0.001$, df=72). Pairwise comparisons showed significant structure for all pairs of populations except Mexico and Belize, Mexico and Costa Rica, and population pairs belonging to the Greater Antilles (Cuba and Jamaica) (Table 4). Greater Antillean *C. acutus* populations exhibited strong differentiation when compared with all other populations, with fixation indices ranging from 0.4514 to 1 (F_{ST}). We found significant structure between same pairs of populations when performing the exact test of populating differentiation (Table 4).

For microsatellite data the among-groups component of the AMOVA analysis was significant (F_{ST}: 0.2046, $p<0.0001$) (Table 4). In contrast to the data from mtDNA control region analysis, pairwise comparisons revealed population structure between Antillean populations of Cuba and Jamaica. As in the mtDNA analysis, all remaining comparisons between population pairs were significant.

Spatially explicit Bayesian clustering analysis implemented in Geneland yielded a modal number of four populations. All 10 independent runs converged in the same number of populations (Table 5). The comparison of posterior probability of assignment of individuals to populations allocated each individual to the population to which it was geographically sampled. Non-spatial Bayesian clustering implemented in STRUCTURE with no prior distribution specified revealed the subdivision of our samples in three populations (K=3) as the most likely configuration (Delta *K* 950.33). STRUCTURE clustered together individuals from the Everglades and Belize, with individuals from Cuba and Jamaica as separate clusters. The second best configuration assigned individuals into each of four populations (Delta *K* 147.37) (Fig 4). STRUCTURE clustered individuals into Everglades, Belize, Cuba and Jamaica, with a degree of admixture of individuals in all populations.

Our principal component analysis (Fig. 4) suggested four genetically distinct groups: (1) Everglades, (2) Belize (3) Cuba, and (4) Jamaica (PBPA and BRLM). This grouping was based predominantly on the first principal component (PC1), which explained 19.23 % of the variance in allele frequencies among samples, and was augmented by PC2 (12.50 %) and, at a lesser extent by PC3

(5.76%). Overall F_{ST} was 0.102 (SE 0.038) and significantly greater than zero ($p < 0.001$).

Discussion

All analyses for both mtDNA and nuclear markers show evidence of population genetic structure in the American crocodile, with unique populations in North America, Central America, South America, and the Greater Antilles. In accord to other studies [6,19], our results suggest that the greatest degree of genetic differentiation occurs between the continental *C. acutus* and the Greater Antillean *C. acutus.* The suite of genetic differentiation estimators used in our mtDNA sequence analysis indicated that this differentiation is strong, reaching pairwise comparisons estimator values (F_{ST} and Φ_{ST}) as high as one between Antillean and continental populations. The same estimators found a lack of differentiation between *C. acutus* populations in the Yucatan Peninsula and Costa Rica, suggesting deep matrilineal phylogenetic divergences between Central American populations and their conspecifics in the Insular Caribbean.

We report four newly discovered haplotypes: three in Venezuela, and one in the Everglades. Adding South American sampling locations into haplotype designation and analysis fills an existing gap in the understanding of *C. acutus* intraspecific population genealogies. Our results show that Venezuelan haplotypes are considerably less distant from Central (2-3 substitutions apart) and North American haplotypes (2-4 substitutions apart) than Greater Antillean haplotypes (14-20 substitutions apart). A recent study by Bloor *et al* [58] using mtDNA cytochrome b and cytochrome oxidase I gene sequences in captive *C.*

acutus from Colombia revealed two distinct lineages: one closely related to Central American haplotypes and a second one unique to Colombian *C. acutus*. These results overlap with our findings and comparative research should be done to better understand haplotype relationships within South American *C. acutus*

In addition, 99% of sampled Greater Antillean *C. acutus* share one haplotype (β). Because haplotype β is closer to Cuban crocodile haplotype α than to any of the reported in American crocodile haplotypes from the Americas [6,23], mitochondrial capture may have occurred during an ancient hybridization event between these Greater Antillean *C. acutus* and *C. rhombifer* species [19]. An alternative scenario postulates multiple colonization events of Cuba by the ancestors, first of *C. rhombifer* and later on of *C. acutus*, with sustained periods of hybridization and dispersion across the island [23]. In fact, hybridization between Cuban and American crocodiles in Cuba seems to have taken place both historically and in recent times [6]. Regardless of potential evolutionary explanations for haplotype origins, the geographic distribution of *C. acutus* haplotypes suggests that strong genetic structuring revealed from mtDNA analysis would be principally shaped by differences in haplotype identity, followed by differences in haplotype frequencies.

The new haplotype found in the Everglades seems to be closer to South and Central American haplotypes. In addition, we found another individual in the Everglades with *C. acutus* Greater Antillean haplotype β. Rodriguez *et al* [19] reported the presence of several haplotypes from Latin America and the

Caribbean within Southern Florida. The study attributed the genetic structuring in Southern Florida to be the result of admixture of local haplotypes with those of released foreign and captive American crocodiles. In addition, authors reported only one haplotype (Ca1) collected in areas representing the core nesting population of *C. acutus* in the Everglades National Park. In this context, the two haplotypes found within our sampled *C. acutus* at the EVNP could most likely be introduced or admixed individuals. As we collected samples within the Everglades National Park, these findings are worrisome and suggest that individuals with foreign haplotypes are being released, and/or moved from areas nearby, where non-local haplotypes to the Everglades have been previously reported [19].

In addition to the clustered populations of Everglades and Belize, our analysis of microsatellite data uncovered patterns of genetic subdivision between the Greater Antillean populations of Cuba and Jamaica. The most likely clustering of individuals according to STRUCTURE, however, revealed only three populations, grouping individuals from the Everglades and Belize together, probably as a result of genetic similarity or lack of resolution for subtler structure. STRUCTURE second most likely configuration of four populations coincided with our results from spatially explicit Bayesian clustering analysis (Geneland) and multivariate analysis (PCA). Spatial explicit models applied to other studies have detected biologically meaningful clusters where STRUCTURE failed to detect any population subdivision [59,60], and may be more accurate for populations exhibiting some degree of IBD [61]. Our overall evidence suggests that Cuban and Jamaican *C. acutus* share mtDNA haplotype β but currently represent at least two different genetic populations when using nuclear, faster evolving, microsatellite markers.

Our findings offer the first evidence of genetic differentiation among the populations of Greater Antillean *C. acutus* and provide important information for the regional planning and conservation of the species. We propose the incorporation of independent conservation management units for Cuban and Jamaican *C. acutus* based on our genetic findings of population subdivision between both sampling localities [16,62]. Because our sampling in Jamaican localities was biased, with only 16 samples collected at the Black River Lower Morass versus 66 at the Portland Bright Protected Area, we strongly suggest that further sampling is needed to further clarify potential patterns of subtle structuring between American crocodiles at these two locations.

We present new and relevant information to understanding *C. acutus* evolutionary history and reveal patterns of genetic partitioning relevant to its conservation. As a widespread species living across a diversity of fresh and brackish water habitat and able to move long distances across salt water, the American crocodile has been often considered “homogenous” across its range. Our findings show that that is not the case: the species has geographically distinct populations exhibiting unique genetic structure. Future research should incorporate comparative analysis for other sampling localities in South America and Central America, and better attempt to understand relationships between continental and Greater Antillean *C. acutus*. We suggest the creation of a regional genetic interface/protocol for researchers across the Americas to design studies that can be comparable and to share data and lessons learned, as the effective conservation of this widely distributed species will require both local and regional approaches.

References

1. Thorbjarnarson J (1989) Ecology of the American Crocodile, *Crocodylus acutus*. In Crocodiles, their Ecology, Management and Conservation IUCN Publications New Series Gland, Switzerland: 228-258.
2. Thorbjarnarson J (2010) American Crocodile *Crocodylus acutus*. Crocodiles Status Survey and Conservation Action Plan. Third Edition ed. Darwin: S.C. Manolis and C. Stevenson. Crocodile Specialist Group. pp. Pp. 46-53.
3. Thorbjarnarson J (1988) The status and ecology of the American crocodile in Haiti. Bulletin of the Florida State Museum of Biological Sciences 33: 1-86.
4. Schubert A, Santana G (1996) Conservation of the American crocodile (*Crocodylus acutus*) in the Dominican Republic. Contributions to Herpetology 12: 425-433.
5. Schubert A (2012) Lagos Enriquillo y Azuei : donde la naturaleza siempre tiene una sorpresa; Espanola EA, editor: LAP LAMBERT Academic Publishing GmbH & Co. KG.
6. Milian-Garcia Y, Venegas-Anaya M, Frias-Soler R, Crawford AJ, Ramos-Targarona R, Rodriguez-Soberon R, et al. (2011) Evolutionary History of Cuban Crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* Inferred From Multilocus Markers. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 315A: 358-375.
7. Machkour-M'Rabet S, Henaut Y, Charruau P, Gevrey M, Winterton P, Legal L (2009) Between introgression events and fragmentation, islands are the

last refuge for the American crocodile in Caribbean Mexico. Marine Biology 156: 1321-1333.

8. Platt SG, Thorbjarnarson JB, Price A (2000) Nesting ecology of the American crocodile in the coastal zone of Belize. Copeia 2000: 869-873.

9. Barros T, Jiménez-Oraá M, Heredia HJ, Seijas AE (2010) Artificial incubation of wild-collected eggs of American and Orinoco crocodiles (*Crocodylus acutus* and *C. intermedius*), Guárico and Zulia, Venezuela. Conservation Evidence 7: 111-115.

10. Cedeño-Vazquez JR, Rodriguez D, Calme S, Ross JP, Densmore LD, Thorbjarnarson JB (2008) Hybridization Between *Crocodylus acutus* and *Crocodylus moreletii* in the Yucatan Peninsula: I. Evidence From Mitochondrial DNA and Morphology. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 309A: 661-673.

11. Rodriguez D, Cedeno-Vazquez JR, Forstner MRJ, Densmore LD (2008) Hybridization Between *Crocodylus acutus* and *Crocodylus moreletii* in the Yucatan Peninsula: II. Evidence From Microsatellites. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 309A: 674-686.

12. Weaver JP, Rodriguez D, Venegas-Anaya M, Cedeno-Vazquez JR, Forstner MRJ, Densmore LD (2008) Genetic characterization of captive Cuban crocodiles (*Crocodylus rhombifer*) and evidence of hybridization with the American crocodile (*Crocodylus acutus*). Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 309A: 649-660.

13. Mazzotti FJ, Dunson WA (1984) Adaptations of *Crocodylus acutus* and Alligator for life in saline water. Comparative Biochemistry and Physiology Part A: Physiology 79: 641-646.

14. Wheatley PV, Peckham H, Newsome SD, Koch PL (2011) Estimating marine resource use by the American crocodile Crocodylus acutus in southern Florida, USA. Marine Ecology Progress Series 447: 211-229.

15. Thorbjarnarson J (1999) Crocodile tears and skins: international trade, economic constraints, and limits to the sustainable use of crocodilians. Conservation Biology 13: 465-470.

16. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006) Regional habitat conservation priorities for the American crocodile. Biological Conservation 128: 25-36.

17. Kushlan JA (1988) Conservation and management of the American crocodile. Environmental Management 12: 777-790.

18. Platt SG, Thorbjarnarson JB (2000) Status and conservation of the American crocodile, Crocodylus acutus, in Belize. Biological Conservation 96: 13-20.

19. Rodriguez D, Forstner MR, Moler PE, Wasilewski JA, Cherkiss MS, Densmore III LD (2011) Effect of human-mediated migration and hybridization on the recovery of the American crocodile in Florida (USA). Conservation genetics 12: 449-459.

20. IUCN (2010) IUCN Red List of Threatened Species. Crocodylus acutus. Crocodile Specialist Group.

21. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006) Regional habitat conservation priorities for the American crocodile. Biological Conservation 128: 25-36.

22. Sanderson EW, Redford KH, Chetkiewicz CLB, Medellin RA, Rabinowitz AR, Robinson JG, et al. (2002) Planning to save a species: the jaguar as a model. Conservation Biology 16: 58-72.

23. Milián-García Y, Ramos-Targarona R, Pérez-Fleitas E, Sosa-Rodríguez G, Guerra-Manchena L, Alonso-Tabet M, et al. (2014) Genetic evidence of hybridization between the critically endangered Cuban crocodile and the American crocodile: implications for population history and in situ/ex situ conservation. Heredity.

24. Hekkala ER, Amato G, DeSalle R, Blum MJ (2010) Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (*Crocodylus niloticus*) populations. Conservation Genetics 11: 1435-1443.

25. Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. Molecular Ecology 3: 401-411.

26. Schlötterer C, Pemberton J (1998) The use of microsatellites for genetic analysis of natural populations—a critical review. Molecular approaches to ecology and evolution: Springer. pp. 71-86.

27. Clarke CR, Karl SA, Horn RL, Bernard AM, Lea JS, Hazin FH, et al. (2015) Global mitochondrial DNA phylogeography and population structure of the silky shark, *Carcharhinus falciformis*. Marine Biology 162: 945-955.

28. Goldstein DB, Roemer GW, Smith DA, Reich DE, Bergman A, Wayne RK (1999) The use of microsatellite variation to infer population structure and demographic history in a natural model system. Genetics 151: 797-801.

29. Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. Molecular ecology 11: 155-165.

30. FitzSimmons N, Tanksley S, Forstner M, Louis E, Daglish R, Gratten J, et al. Microsatellite markers for *Crocodylus*: new genetic tools for population genetics, mating system studies and forensics; 2001. Surrey Beatty & Sons. pp. 51-57.

31. Dever JA, Densmore LD (2001) Microsatellites in Morelet's crocodile (*Crocodylus moreletii*) and their utility in addressing crocodilian population genetics questions. Journal of Herpetology 35: 541-544.

32. Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.

33. Nei M (1987) Molecular evolutionary genetics: Columbia university press.

34. Nei M (1975) Molecular population genetics and evolution: North-Holland Publishing Company.

35. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of molecular evolution 16: 111-120.

36. Tajima F (1983) Evolutionary relationship of DNA sequences in finite populations. Genetics 105: 437-460.

37. Schneider S, Roessli D, Excoffier L (2000) Arlequin: a software for population genetics data analysis. User manual ver 2: 2496-2497.

38. Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources 10: 564-567.

39. Posada D, Crandall KA (2001) Intraspecific gene genealogies: trees grafting into networks. Trends in Ecology & Evolution 16: 37-45.

40. Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Molecular biology and evolution 16: 37-48.

41. Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Molecular Ecology Resources 8: 103-106.

42. Rice WR (1989) Analyzing tables of statistical tests. Evolution: 223-225.

43. Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535-538.

44. Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. evolution: 1358-1370.

45. Guillot G, Santos F, Estoup A (2008) Analysing georeferenced population genetics data with Geneland: a new algorithm to deal with null alleles and a friendly graphical user interface. Bioinformatics 24: 1406-1407.

46. Guillot G, Mortier F, Estoup A (2005) GENELAND: a computer package for landscape genetics. Molecular ecology notes 5: 712-715.

47. Team RC (2015) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

48. Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

49. Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164: 1567-1587.

50. Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular ecology notes 7: 574-578.

51. Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14: 2611-2620.

52. Earl DA (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation genetics resources 4: 359-361.

53. Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23: 1801-1806.

54. Ramasamy RK, Ramasamy S, Bindroo BB, Naik VG (2014) STRUCTURE PLOT: a program for drawing elegant STRUCTURE bar plots in user friendly interface. SpringerPlus 3: 431.

55. Cavalli-Sforza LL, Menozzi P, Piazza A (1994) The history and geography of human genes: Princeton university press.

56. Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24: 1403-1405.

57. Reich D, Price AL, Patterson N (2008) Principal component analysis of genetic data. Nature genetics 40: 491-492.

58. Bloor P, Ibáñez C, Viloria‐Lagares TA (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.

59. Lavandero B, Miranda M, Ramirez C, Fuentes-Contreras E (2009) Landscape composition modulates population genetic structure of *Eriosoma lanigerum* (Hausmann) on Malus domestica Borkh in central Chile. Bulletin of entomological research 99: 97-105.

60. Barr KR, Lindsay DL, Athrey G, Lance RF, Hayden TJ, Tweddale SA, et al. (2008) Population structure in an endangered songbird: maintenance of genetic differentiation despite high vagility and significant population recovery. Molecular Ecology 17: 3628-3639.

61. Renner SC, Suarez‐Rubio M, Wiesner KR, Drögemüller C, Gockel S, Kalko EK, et al. (2015) Using multiple landscape genetic approaches to test the validity of genetic clusters in a species characterized by an isolation‐by‐distance pattern. Biological Journal of the Linnean Society.

62. Alonso Tabet M, Rodriguez Soberón R (2012) *Crocodylus acutus*. In: Gonzalez Alonso H, Rodriguez Schettino L, Rodriguez A, Mancina C, Ramos Garcia I, editors. Libro Rojo de los Vertebrados de Cuba. Cuba: Instituto de Ecologia y Sistematica, Editorial Academia.

Table 1. Geographic distribution of the American crocodile samples used in this study including information on published data for samples presented in previous studies, and type of molecular markers used for population genetic analyses

Locality	**n**	**Authors**	**mtDNA**	**Microsatellites**
Everglades National Park, Florida, US	38	this study	Yes	yes
Quintana Roo, Mexico	19	Ray *et al* 2004; Cedeño-Vazquez *et al* 2008	Yes	no
Turneffe Atoll, Belize	55	this study	Yes	yes
Costa Rica	5	Rodriguez *et al.* 2011	Yes	no
Tacarigua Lagoon National Park, coastal Venezuela	11	this study	Yes	no
Zapata Swamp National Park, Cuba	5	Milian *et al.* 2015	Yes	no
Wildlife Refuge Monte Cabaniguán, Cuba	43	this study	Yes	yes
Black River Lower Morass, Jamaica	16	this study	Yes	yes
Portland Bight Protected Area, Jamaica	66	this study	Yes	yes
Total	258		258	218

Table 2. Genetic diversity indices for mtDNA data, where n is the sample size, H(n) is the number of haplotypes, H_d is the haplotypic diversity, K is the mean number of pairwise differences among sequences, and π is the nucleotide diversity

Population	n	H(n)	Haplotypes	H_d	π	K
EVNP	38	3	Ca1 (35), β (2), Ca10 (1)	0.1520 (0.0770)	0.0059 (0.0051)	2.3560 (0.9099)
MEX	19	4	Ca1 (7), Ca2 (10), Ca3 (1), Ca5 (1)	0.6140 (0.0750)	0.0016 (0.0003)	0.7250 (0.5186)
BE	55	2	Ca1 (36), Ca2 (19),	0.4610 (0.0410)	0.0010 (0.0001)	0.4610 (0.3987)
CR	5	1	Ca2 (5)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)
VE	11	3	Ca11 (5), Ca12 (5), Ca13 (1)	0.5450 (0.0720)	0.0036 (0.0005)	1.6360 (0.8093)
WRMC_CU	43	1	β (43)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)
BRLM_JA	16	1	β (16)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)
PBPA_JA	66	1	β (66)	0.0000 (0.0000)	0.0000 (0.0000)	0.0000 (0.0000)
Zapata_CU	5	2	C (1), β (4)	0.4000 (0.2370)	0.0061 (0.0036)	2.8000 (1.1832)
Total	258	10	Ca1 (78), Ca2 (34), Ca3 (1), Ca5 (1), Ca10 (1), Ca11 (5), Ca12 (5), Ca13 (1), β (127), C (1)	0.634 (0.020)	0.0229 (0.0002)	10.4670 (3.5160)

Standard errors are presented in parenthesis

Table 3. Genetic diversity indices of the microsatellite data, where n is the sample size, (H_O) is the observed heterozygosity, (H_E) is the expected heterozygosity, and numbers in each box are the number of alleles per marker per putative population

	n	Mean # alleles	H_O	H_E	Cj16	Cj18	Cj20	Cj35	Cj109	Cj119	Cj127	Cj131	CU5123	CUJ131	CUD68
EVNP	38	6.091 (3.270)	0.5615 (0.2207)	0.6289 (0.1630)	7	3	6	2	10	7	13	3	7	5	4
BE	55	6.364 (2.942)	0.4970 (0.2526)	0.5027 (0.2082)	12	7	8	5	5	5	11	3	6	3	5
WRMC_CU	43	7.000 (3.162)	0.4939 (0.2246)	0.5339 (0.1840)	7	9	8	7	15	9	8	4	5	4	5
BRLM_JA	16	7.273 (1.954)	0.4550 (0.2398)	0.5465 (0.2131)	7	8	11	7	7	7	8	7	9	6	3
PBPA_JA	66	3.909 (1.868)	0.4545 (0.1862)	0.5446 (0.1695)	4	4	7	2	6	2	7	2	5	2	3
Total	218			Total	37	31	40	23	43	30	47	19	32	20	20

Standard deviations for the mean number of alleles, observed, and expected heterozygosities

Table 4. Pairwise estimates of F_{ST} and Φ_{ST} between *C. acutus* sampling localities based on mitochondrial data

	EVNP	BZ	MX	CR	VE	WRMC_CU	BRLM_JM	PBPA_JM	ZAP_CU
EVNP	-	**0.2106**	**0.4833**	**0.8689**	**0.7089**	**0.9244**	**0.8891**	**0.9405**	**0.7996**
BZ	**0.0907***	-	0.0783	**0.4709**	**0.4841**	**0.7479**	**0.6781**	**0.7866**	**0.5527**
MX	**0.2775***	0.0397	-	0.1920	**0.3763**	**0.7922**	**0.6732**	**0.8410**	**0.4531**
CR	**0.9239***	**0.4242***	0.1667	-	**0.5845**	**1.0000**	**1.0000**	**1.0000**	**0.8000**
VE	**0.6057***	**0.4515***	**0.3748***	**0.6818***	-	**0.8519**	**0.7309**	**0.8927**	**0.4514**
WRMC_CU	**0.8713***	**0.7697***	**0.6930***	**1.0000***	**0.6818***	-	0.0000	0.0000	0.5094
BRLM_JM	**0.8713***	**0.7697***	**0.6930***	**1.0000***	**0.6818***	0.0000	-	0.0000	0.2558
PBPA_JM	**0.8713***	**0.7697***	**0.6930***	**1.0000***	**0.6818***	0.0000	0.0000	-	0.6179
ZAP_CU	**0.6818***	**0.5697***	**0.4930***	**0.8000***	**0.4818***	0.0000	0.0000	0.0000	-

Pairwise F_{ST} and Φ_{ST} values are above and below the diagonal, respectively. Statistically significant values for pairwise comparisons with $p<0.05$ are highlighted in bold. Asterisks (*) below the diagonal indicate significant values ($p<0.05$) for the exact test of population differentiation

Table 5. Pairwise estimates of F_{ST} between *C. acutus* sampling localities based on microsatellite data

	EVNP	BZ	WRMC_CU	PBPA_JM	BRLM_JM
EVNP	-	**0.1240**	**0.2163**	**0.1797**	**0.1733**
BZ		-	**0.3035**	**0.3214**	**0.2704**
WRMC_CU			**-**	**0.1251**	**0.2363**
PBPA_JM				-	0.0195
BRLM_JM					-

Statistically significant values for pairwise comparisons with p<0.05 are highlighted in bold.

Table 6. Multiple runs for inferring the number of populations of American crocodiles using genetic and geographic information obtained in GENELAND software.

Run	Modal number	% of modal number	Mean of probability density
1	4	38.9	-4795.73
2	4	40.4	-4834.58
3	4	41.2	-4838.88
4	4	38.6	-4676.18
5	4	38.2	-4827.42
6	4	37.5	-4848.96
7	4	41.1	-4984.79
8	4	40.2	-4834.83
9	4	41.8	-5000.75
10	**4**	**39.8**	**-4663.37**

Highest average posterior probability in bold

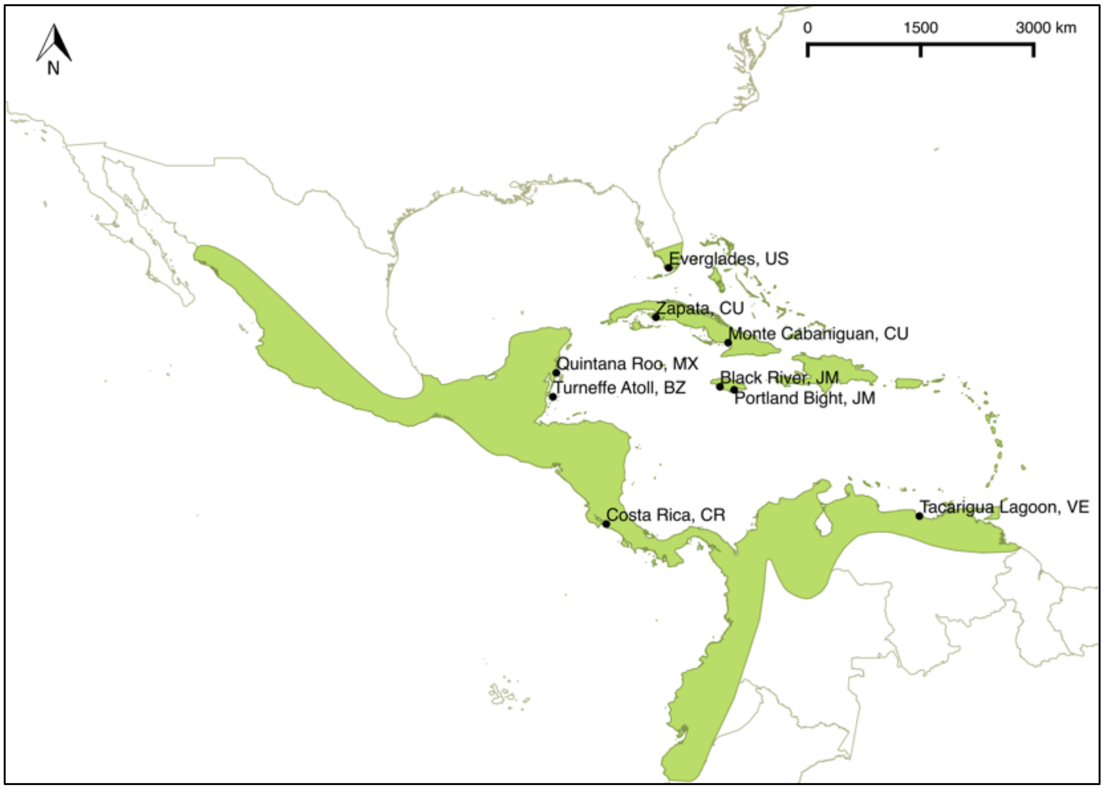

Figure 1. Sampling localities (black dots) and distribution range of the American crocodile (green)

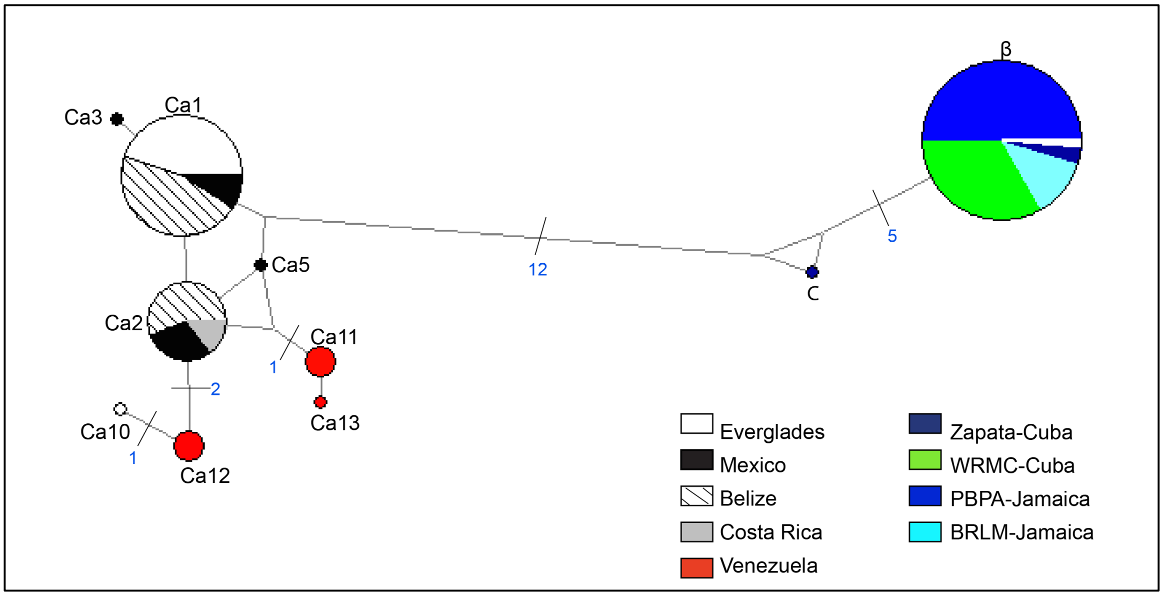

Figure 2. Statistical parsimony haplotype network depicting spatial patterns of genetic diversity of the mtDNA control region of American crocodile across sampling localities; haplotype colors signify their geographic location and circle size is proportional to haplotype frequency. Hatch marks and blue numbers below indicate additional steps to a single mutation separating haplotypes

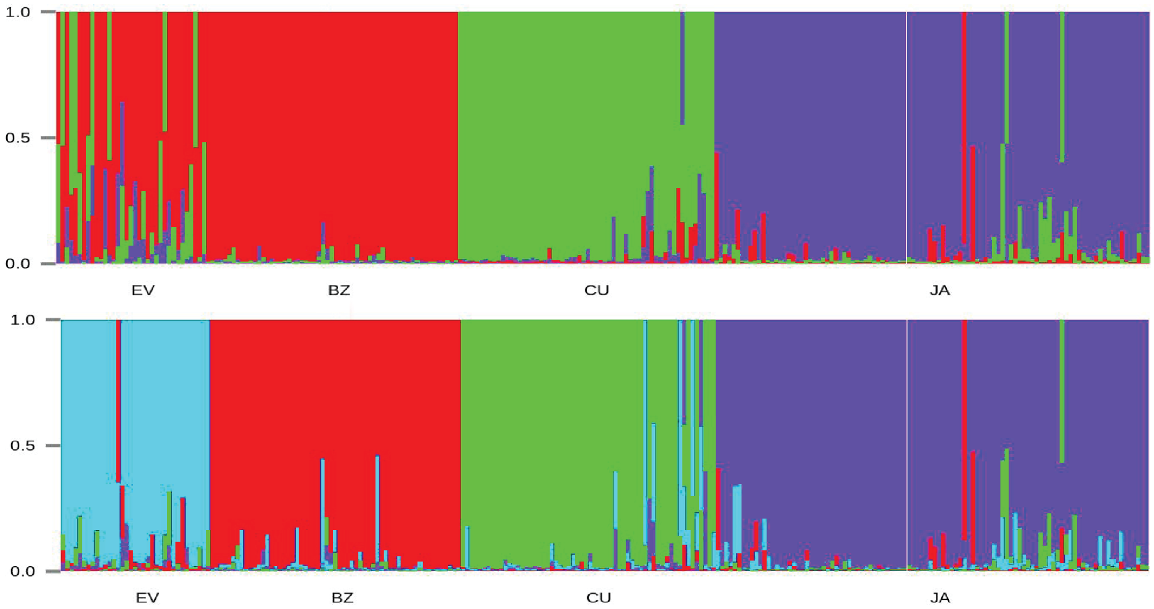

Figure 3. Assignment of American crocodiles to each of 3 (above) or 4 (below) genetic populations based on Bayesian cluster analysis implemented in STRUCTURE; horizontal labels show sampling localities.

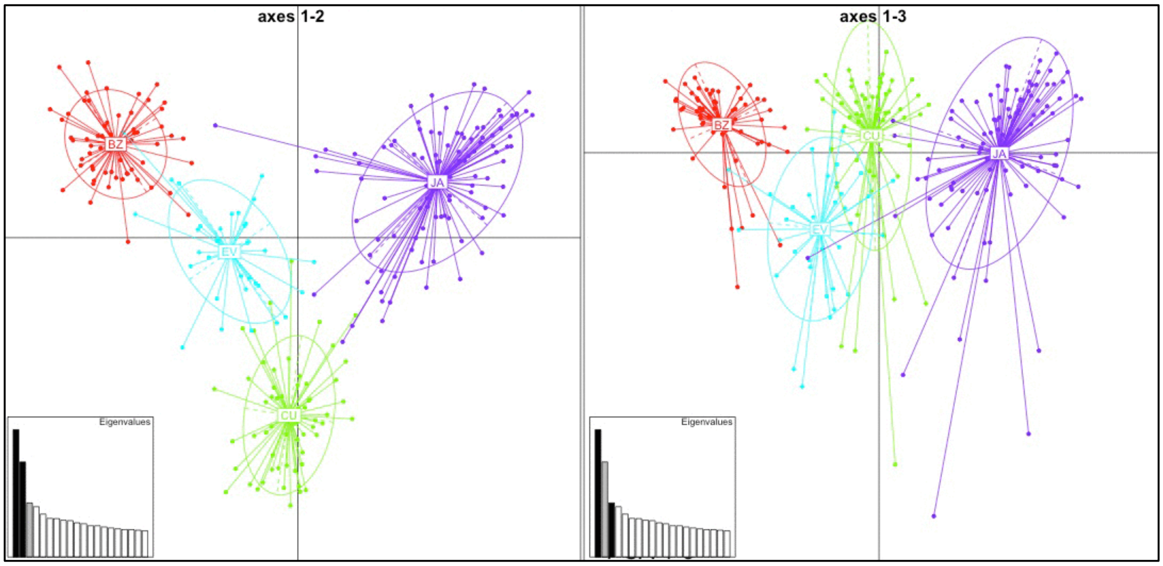

Figure 4. PCA plots for first (PC1 and PC2; left) and second (PC1 and PC3; right) pairs of principal components derived from allele frequencies at twelve polymorphic microsatellite loci; the proportion of the variation attributable to each component is indicated for the axes; different colors correspond to sampling localities of Everglades (EV; light blue), Belize (BZ; red), Cuba (CU; green) and Jamaica (JM; purple).

CONCLUSION

Interpretation of Ecological and Genetic Information in a Conservation Context

Throughout this research, I have incorporated genetic and ecological information, and employed a combination of methodological approaches, to gain a better understanding of crocodilian mating systems and phylogeography, as well as the influence of environmental factors in their reproductive success. The results of this research advance our understanding of the ecology and evolution of *Crocodylus,* and provide critical information useful for the conservation of the species studied. My work addresses questions for a number of different crocodilian species, across diverse geographies and time frames. It assesses the mating system of the Orinoco crocodile in a relict population in the Llanos of Venezuela, models the effects of tropical cyclones in the reproductive success of the American crocodile in the coastal wetlands of southeastern Cuba, and unravels patterns of population genetic subdivision in the American crocodile across the Americas and Insular Caribbean.

Chapter II of my dissertation shows, for the first time, a polygamous mating system for *C. intermedius* in a reintroduced population at the El Frio Biological Station in the Llanos of Venezuela. By reconstructing parental genotypes, I identified a minimum of 16 females and 14 males contributing to the reproductive output at this site. The paternal contribution of males to the clutches studied was biased towards certain males, with six males fathering 90% of the total offspring, and three of those six males fathering more than 70% of the total offspring. Understanding this complex reproductive system is critical for conservation planning and shows the importance of studying the mating system and breeding strategies of reintroduced populations if we want to evaluate the success of these efforts and create adaptive management programs that ensure effective species management [1]. For instance, the occurrence of multiple paternity may accelerate the recovery and resilience of reintroduced populations of *C. intermedius* by increasing genetic diversity among siblings, potentially increasing effective population size [2], and overall genetic diversity of the population [3]. However, the high skew in reproductive success, and the dominance of certain males, could potentially have the opposite effect by reducing the number of breeding males and increasing the variance in their contribution to the gene pool of successive generations. In this chapter, I report heterozygosity values similar to those of other crocodilian populations in the wild [4-6]. This suggests that the genetic composition of the founders, and/or rapid population growth after individuals were reintroduced, may have quickly compensated any potential founder effect, despite biases in the contribution of the different males.

Before the first reintroductions of *C. intermediu*s, the species had been depleted from the El Frío Biological Station and surrounding areas [7] within the historical range of the species [8,9]. Reintroduction efforts in 1990, in combination with a suite of species management interventions resulted in a successful population recovery with increasing nesting numbers with at least 93 nests observed between 1996 and 2007 [7]. Nesting numbers observed at the EFBS are similar to those previously reported at other sites where the species is still found in Venezuela [10-13]. Hence, in the context of the few remaining natural populations of *C. intermedius* in Venezuela, the EFBS reproductive output makes it a very important population for the recovery of the species.

In Chapter III I show that tropical cyclones have a detrimental effect on *C. acutus* nesting success with string impacts on nests located along the shores and mangrove swamps of southeastern Cuba. A dataset of 21 years of information on nesting success at the Wildlife Refuge Monte Cabaniguán, Cuba, allowed me to investigate the impact of tropical cyclones during the nesting season, and to assess the impact in the years following the extreme climatic disturbance. These types of long-term datasets are extremely rare for crocodilians and of great value for conservation. Results indicate negative effects when tropical cyclones occur within a nesting season and positive effects on nesting success in successive nesting seasons. Predicted nesting success before and after the occurrence of a tropical cyclone confirmed a positive increase in nesting success for the first nesting season after the tropical cyclone event and, to a lesser degree, for the second season following a cyclone. Tropical cyclones may benefit crocodile nesting in subsequent years by moving sediments across the landscape, which can improve existing nesting sites or create new ones [14]. In addition, for all years where cyclones occurred during the nesting season, nesting success in the coastal areas was zero whereas hatchlings still emerged in nesting sites located farther from the coastline. These results suggest that mangroves offer the first barrier to buffer heavy storms associated with tropical cyclones, and that sheltered nesting sites can produce surviving hatchlings even in years of severe cyclones. Mangroves may additionally offer shelter for juvenile and adult crocodiles: in 21 years of data only one event of adult mortality during a cyclone event was recorded. Findings of this chapter are particularly relevant as Cuba's economic development is expected to accelerate, especially in the areas of tourism, fisheries, and hydrocarbon exploration, placing growing pressures on the archipelago's coastal mangroves.

Climate forecasting indicates that human induced climate change will cause an increase in the intensity and frequency of tropical cyclones at the global scale [15-17], with substantial increases in the precipitation rate within 100 kilometers of the cyclone center [15]. The Caribbean region is expected to be amongst the most severely affected by climate change over the course of the next century [18,19]. Higher-intensity tropical cyclones can be devastating when they strike during the nesting season and the positive impact in following years may be offset by increased frequency of cyclones. In archipelagos and islands, population persistence is tightly linked to nesting success and adult survival, as immigration is unlikely and demographically irrelevant. As plans for coastal adaptation in the context of climate change are developed in Cuba, results from this chapter strongly suggest that persistence of these crocodilians will rely on incorporating crocodilians and their habitats in such planning.

Chapter IV reports evidence of strong population genetic structure in the American crocodile, with unique populations in North America, Central America, South America, and the Greater Antilles. In accordance with other studies [20,21], the greatest degree of genetic differentiation occurs between the continental *C. acutus* and the Greater Antillean *C. acutus.* In addition, this chapter reports three newly discovered haplotypes for Venezuela. Adding South American sampling locations into haplotype designation and analysis fills a gap in the understanding of *C. acutus* intraspecific population genealogies. Results show that Venezuelan haplotypes are considerably less distant from Central and North American haplotypes than Greater Antillean haplotypes, reinforcing hypotheses on higher divergence between continental and Greater Antillean *C. acutus*.

Research results of Chapter IV also reveal patterns of genetic subdivision between the Greater Antillean populations of Cuba and Jamaica. These findings offer the first evidence of genetic differentiation among the populations of Greater Antillean *C. acutus* and provide important information for the regional planning and conservation of the species. Based on these results, the designation of Cuban and Jamaican *C. acutus* as independent conservation management units would advance regional conservation efforts [22,23].

In conclusion, research findings of my dissertation are the product of combining ecological data collected in the field, genetic data generated in the lab, and the use of a suite of classic and inference-based methodological approaches to gain a better understanding of the behavior and evolution of crocodilians. The dissertation presents the first genetic research on *C. intermedius,* shows the importance of coastal mangrove conservation for the persistence of *C. acutus* in

Cuba, and depicts phylogeographic linkages among distinct *C. acutus* populations across the Americas and Greater Antilles. The outcomes of this research provide science-based information to influence decision-making processes for the conservation of endangered crocodilians and their habitats across the study areas.

Literature

1. Schmidt F, Franke F, Shirley M, Vliet K, Villanova V (2015) The importance of genetic research in zoo breeding programmes for threatened species: the African dwarf crocodiles (genus *Osteolaemus*) as a case study. International Zoo Yearbook 49: 125-136.
2. Sugg DW, Chesser RK (1994) Effective population sizes with multiple paternity. Genetics 137: 1147-1155.
3. Rafajlović M, Eriksson A, Rimark A, Hintz-Saltin S, Charrier G, Panova M, et al. (2013) The effect of multiple paternity on genetic diversity of small populations during and after colonisation. PloS One 8: e75587.
4. Dever JA, Strauss RE, Rainwater TR, McMurry ST, Densmore III LD, Wood R (2002) Genetic diversity, population subdivision, and gene flow in Morelet's crocodile (*Crocodylus moreletii*) from Belize, Central America. Copeia 2002: 1078-1091.
5. Farias IP, Silveira RD, de Thoisy B, Monjeló LA, Thorbjarnarson J, Hrbek T (2004) Genetic diversity and population structure of Amazonian crocodilians. Animal Conservation 7: 265-272.
6. Milián‑García Y, Venegas‑Anaya M, Frias‑Soler R, Crawford AJ, Ramos‑Targarona R, Rodríguez‑Soberón R, et al. (2011) Evolutionary history of Cuban crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* inferred from multilocus markers. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 315: 358-375.

7. Antelo R (2008) Biología del Cocodrilo o Caimán del Orinoco (*Crocodylus intermedius*) en la Estación Biológica El Frío, Estado Apure (Venezuela): Tesis Doctoral. Universidad Autónoma de Madrid, Spain.

8. Gumilla J (1745) El Orinoco Ilustrado y Defendido, Historia Natural, Civil, y Geographica de este Gran Rio: Manuel Fernandez, editor.

9. Thorbjarnarson JB (1992) Recent investigations of the status and distribution of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. Biological Conservation 62: 179-188.

10. Thorbjarnarson JB, Hernández G (1993) Reproductive ecology of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. II. Reproductive and social behavior. Journal of Herpetology: 371-379.

11. Barros T, Jiménez-Oraá M, Heredia HJ, Seijas AE (2010) Artificial incubation of wild-collected eggs of American and Orinoco crocodiles (*Crocodylus acutus* and *C. intermedius*), Guárico and Zulia, Venezuela. Conservation Evidence 7: 111-115.

12. Hernández O, Boede E, Amauci J (2014) Evaluation of the Orinoco crocodile nesting (*Crocodylus intermedius*) in Capanaparo River, Santos Luzardo National Park, Apure State, Venezuela Boletín de la Academia de las Ciencias Físicas, Matemáticas y Naturales 2: 53-59.

13. Espinosa-Blanco AS, Seijas AE, Hernández O (2013) Egg collectIon as a conservation tool of Orinoco crocodiIle (*Crocodylus intermedius*) in the Cojedes river system, Venezuela. Interciencia 38: 358-363.

14. Charruau P, Thorbjarnarson JB, Henaut Y (2010) Tropical cyclones and reproductive ecology of *Crocodylus acutus* Cuvier, 1807 (Reptilia: Crocodilia: Crocodylidae) on a Caribbean atoll in Mexico. Journal of Natural History 44: 741-761.
15. Knutson TR, McBride JL, Chan J, Emanuel K, Holland G, Landsea C, et al. (2010) Tropical cyclones and climate change. Nature Geoscience 3: 157-163.
16. Pachauri RK, Allen M, Barros V, Broome J, Cramer W, Christ R, et al. (2014) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.
17. Emanuel K (2005) Increasing destructiveness of tropical cyclones over the past 30 years. Nature 436: 686-688.
18. Rhiney K (2015) Geographies of Caribbean Vulnerability in a Changing Climate: Issues and Trends. Geography Compass 9: 97-114.
19. IPCC (2014) Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; Barros VR, C.B. Field, D.J. Dokken, M.D. Mastrandrea, K.J. Mach, T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea, and L.L. White, editor. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press. 688 p.

20. Milian-Garcia Y, Venegas-Anaya M, Frias-Soler R, Crawford AJ, Ramos-Targarona R, Rodriguez-Soberon R, et al. (2011) Evolutionary History of Cuban Crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* Inferred From Multilocus Markers. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 315A: 358-375.

21. Rodriguez D, Forstner MR, Moler PE, Wasilewski JA, Cherkiss MS, Densmore III LD (2011) Effect of human-mediated migration and hybridization on the recovery of the American crocodile in Florida (USA). Conservation genetics 12: 449-459.

22. Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, et al. (2006) Regional habitat conservation priorities for the American crocodile. Biological Conservation 128: 25-36.

23. Alonso Tabet M, Rodriguez Soberón R (2012) *Crocodylus acutus*. In: Gonzalez Alonso H, Rodriguez Schettino L, Rodriguez A, Mancina C, Ramos Garcia I, editors. Libro Rojo de los Vertebrados de Cuba. Cuba: Instituto de Ecologia y Sistematica, Editorial Academia.