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Discovery of Facultative Parthenogenesis in a New World Crocodile

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Ethics

Does your article include research that required ethical approval or permits?:
This article does not present research with ethical considerations

Statement (if applicable):
This study utilized skin shed from the mother and tissue extracted from the stillborn fetus. As such, as no animals were harmed no institutional IACUC approval was required.

Data

It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:
Yes

Statement (if applicable):
Raw data used in this manuscript can be found online in the Dryad data repository at https://doi.org/10.5061/dryad.7sqv9s4x1. Whole genome sequencing was performed by Novogene (Sacramento, CA) on an Illumina platform (NovaSeq 6000 PE150). Raw sequences were mapped by Novogene to the Saltwater crocodile, Crocodylus porosus, reference genome with SNPs identified by Novogene using the following the command in SAMtools: mpileup -m 2 -F 0.002 -d 10. Raw data files are uploaded in .vcf, .txt, .csv, and STRUCTURE file formats. A full description of how the raw data was analyzed can be found in the Genomic Parthenogenesis Assessment section of the online supplemental material.

Conflict of interest

I/We declare we have no competing interests

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DISCOVERY OF FACULTATIVE PARTHENOGENESIS IN A NEW WORLD CROCODILE

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Abstract

Over the past two decades, there has been an astounding growth in the documentation of vertebrate facultative parthenogenesis (FP). This unusual reproductive mode has been documented in birds, non-avian reptiles—specifically lizards and snakes—, and elasmobranch fishes. Part of this growth among vertebrate taxa is attributable to awareness of the phenomenon itself and advances in molecular genetics/genomics and bioinformatics, and as such our understanding has developed considerably. Nonetheless, questions remain as to its occurrence outside of these vertebrate lineages, most notably in Chelonia (turtles) and Crocodylia (crocodiles, alligators, and gharials). The latter group is particularly interesting because unlike all previously documented cases of FP in vertebrates, crocodilians lack sex chromosomes and sex determination is controlled by temperature. Here, using whole-genome sequencing data, we provide the first evidence of FP in a crocodilian, the American Crocodile, *Crocodylus acutus*. The data support terminal fusion automixis as the reproductive mechanism; a finding which suggests a common evolutionary origin of FP across reptiles, crocodilians, and birds. With FP now documented in the two main branches of extant archosaurs, this discovery offers tantalizing insights into the possible reproductive capabilities of the extinct archosaurian relatives of crocodilians and birds, notably members of Pterosauria and Dinosauria.

Key Words

Facultative parthenogenesis; Terminal fusion automixis; Crocodylia; Temperature-dependent sex determination; Archosaur; Dinosauria.

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Introduction

Once considered rare, the ability of sexually reproducing species to generate offspring without genetic contributions of males, termed facultative parthenogenesis (hereafter, FP), has been documented across multiple vertebrate lineages, including both avian [1] and non-avian reptiles (specifically snakes and lizards) [2], and elasmobranch fishes, with notable examples in sharks, rays, and sawfish [3-5]. The widespread phylogenetic distribution and increasing frequency with which FP is reported suggests that the occurrence of this unusual reproductive strategy is less sporadic than previously suggested [6]. In vertebrates, common FP characteristics include: (i) strict occurrence in lineages lacking genomic imprinting, a developmental mechanism believed to be restricted to the main mammalian lineages (Eutheria, Marsupialia) [7] , (ii) the sex of parthenogens is constrained by the species' sex chromosome system, where XX females produce only female (XX) parthenogens [1,3,7] and ZW females produce only male (ZZ) parthenogens [1,8-13], (iii) the capacity to switch between sexual and parthenogenetic modes of reproduction [14], (iv) the ability to produce both sexual and parthenogenetic offspring within single reproductive events [15; Booth pers. observ.], (v) the capacity for consecutive parthenogenetic events [5,13,16,17], and (vi) the reproductive competence of adult parthenogens, with both sexual [10, Booth pers. observ.] and secondary parthenogenetic events documented [19, Booth, pers. observ.]. Furthermore, viable parthenogens have been reported in wild populations [4,20]. Accordingly, these commonalities among taxa suggest that the phyletic distribution and evolutionary significance of FP have been underestimated.

Documentation of FP in vertebrates are increasing for two primary reasons. First, the contribution of specimens with detailed records from zoological institutions and private keepers predominate in studies, including samples otherwise difficult to obtain (e.g., California Condor [21]; Komodo Dragon [12]; King Cobra [22]). Second, the advent of modern molecular analysis, particularly of genome-scale data, has enabled FP to be robustly disentangled from the phenomenon of long-term female sperm storage (LTSS) [23,24]. With records of LTSS exceeding six years [24], it is the main competing hypothesis for the production of offspring after prolonged periods of isolation from potential mates [23,24].

Nonetheless, questions remain as to the occurrence of FP outside of squamates, birds, and elasmobranchs. For example, FP has not been documented in Chelonia (turtles) or Crocodylia (crocodiles, alligators, and gharials). The latter group is particularly interesting as, unlike all previously documented cases of FP in vertebrates, all crocodilians lack sex chromosomes, and sex determination is controlled by temperature, a mechanism termed temperature-dependent sex determination (TSD) [25]. Furthermore, crocodilians are members of a larger monophyletic lineage (Archosauria), a crown group which at its base splits into two groups, one (Pseudosuchia) comprising crocodilians and their extinct relatives and the other (Avemetatarsalia) that contains birds and their extinct relatives, including pterosaurs and non-avian dinosaurs. Here, leveraging whole-genome sequencing and bioinformatic analysis, we provide the first evidence of FP in a crocodilian, the American Crocodile, *Crocodylus acutus*.

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Methods

The American Crocodile (Figure 1A) is a large and wide-ranging apex predator. Owing to its saltwater tolerance, the species occurs from the southern U.S. (Florida) to Northern South America (Colombian, Venezuela, and Peru). It is IUCN listed as vulnerable and is at risk of extinction in the wild (http://www.iucnredlist.org/species/5659/212805700). On 17 January 2018, a clutch of 14 eggs was discovered in the enclosure of an 18-year-old female American Crocodile housed on public exhibit at Parque Reptilandia, Costa Rica. This female was obtained when it was 2 years old (2002) and maintained in isolation from other crocodilians for its entire life. Of the 14 eggs laid, 7 appeared to be fertile and were artificially incubated. After three months of incubation the eggs had failed to hatch and were opened to assess their contents. The contents of six eggs were not discernable, but one egg was found to contain a fully-formed nonviable fetus that upon dissection was determined via gross gonadal morphology as female (Figure 1B) (Supplemental information). DNA was extracted from a scute shed by the mother and cardiac muscle obtained from the fetus using a Qiagen DNeasy Blood & Tissue kit (Qiagen. Hilden, Germany) was sent to Novogene (Sacramento, CA) for whole genome sequencing on an Illumina platform (NovaSeq 6000 PE150). Raw sequences were mapped by Novogene to the Saltwater crocodile, *Crocodylus porosus*, reference genome with single nucleotide polymorphisms (SNPs) identified by Novogene using the following the command in SAMtools: mpileup -m 2 -F 0.002 -d 10.

Three thinned SNP data sets comprising 191,255 SNPs (thinned to one variant per 10 kb), 78,603 SNPs (thinned to one variant per 25 kb), and 39,746 SNPs (thinned to one variant per 50 kb), were analyzed using ParthenoGenius [26] to test for evidence and mode of parthenogenesis. ParthenoGenius first determines whether paternal alleles are present in the offspring. If the number of maternally homozygous loci for which the offspring has non-identical genotypes to the mother is less than that expected due to genotyping error alone (i.e., the offspring is homozygous at all or nearly all of the mother's homozygous loci), the offspring is identified as a likely parthenogen. ParthenoGenius then scans maternally heterozygous loci to identify those at which the offspring has retained heterozygosity. Based on the proportion of retained heterozygosity and taking into consideration the estimated per-base error rate, the parthenogenetic mode is identified as either gametic duplication or automixis (Supplemental

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Information). Automictic parthenogenesis can be subdivided into terminal fusion, where the terminal meiotic products fuse resulting in near genome-wide homozygosity [22], and central fusion, where parthenogens exhibit extensive genome-wide heterozygosity at a comparable level to that of the mother [27]. The latter is not known to occur in vertebrates [28].

Results

The offspring was found to have identical genotypes to the mother at > 99.9% of her homozygous loci, demonstrating a lack of paternal alleles. Genome-wide maternal heterozygosity was 0.349, compared to 0.045 for the offspring, demonstrating a significant reduction in heterozygosity in the offspring when compared to the mother. Regardless of the data set evaluated, the offspring retained approximately 3% of the maternal heterozygosity (Table 1), supporting facultative parthenogenesis through terminal fusion automixis as the mode of reproduction. Note that in instances of central fusion, retained heterozygosity is expected to exceed 33% of that of the mother, whereas terminal fusion will be less than 33% [29]. Due to the lack of a chromosome-scale *C. acutus* genome, it is not possible at this time to identify the actual genomic regions that retain heterozygosity with any accuracy.

Discussion

Our results provide both novel and substantive evidence of facultative parthenogenesis in an extant archosaur, the American Crocodile. Crocodilians diverged from other archosaurs ~240 million years ago [30]. Following this split, Pterosauria and Dinosauria underwent substantive diversification, eventually giving rise to modern birds circa 65 mya [30,31], the only extant theropod dinosaur lineage (Figure 2). As such, we now have evidence of FP in the two main branches of extant archosaurians: crocodilians (Pseudosuchia) and birds (Avemetatarsalia). Furthermore, the most-recent common ancestor of squamates (snakes, lizards, and amphisbaenids), lineages for which FP has been widely documented in both snakes and lizards [1], and crocodilians, diverged ~267.0 – 312.3 mya [32], suggesting that FP may be a primitive condition. Comparable to FP studies of birds, snakes, and lizards, the evidence presented here supports terminal fusion automixis as the parthenogenetic developmental mechanism [1,22]. Accordingly, at this time, these commonalities do not suggest independent evolutionary origins of this trait across these lineages, but instead a trait likely possessed by a distant common

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ancestor of these lineages. While beyond the scope of the current study, it is clear that more work is required to fully test the evolutionary distribution and dynamics of FP across deeper evolutionary time, allowing for a more comprehensive understanding of the ancestral states and rates of FP across vertebrate lineages.

Building on previous studies, the data presented here advance our understanding of the distribution of FP in vertebrates, particularly in that all previous studies relate to species whose sex is genetically determined (i.e., XX|XY or ZZ|ZW). In crocodilians, sex chromosomes are absent and the sex of the offspring is thus temperature-dependent [25]. *Crocodylus acutus* exhibits a Female-Male-Female TSD pattern, with temperatures below 30ºC and above 33ºC producing 100% females, and temperatures around 31.5ºC producing a majority of males [25,33]. Here, eggs were incubated at 29-30ºC and the fully formed yet stillborn fetus was female. Temperature-dependent sex determination is an ancestral trait in crocodilians, being present also in turtle, lizard and tuatara lineages. Within turtles, recent genomic analyses have revealed that many genes associated with calcium signaling pathways and neuroactive ligand-receptor interactions are associated with TSD [32]. If these genes are also involved in the sex determination process in crocodiles, it appears that the genomic reduction inherent with terminal fusion automixis has not affected the expected offspring sex; larger sample sizes are required for confirmation of this finding.

While it is disappointing that the crocodile parthenogen produced here failed to hatch, it is not uncommon to see non-viable fetuses and developmental abnormalities within litters or clutches of parthenogens [1,22,34], and long-term failure to thrive even for individuals born outwardly healthy [35]. For example, following a nine-year study of FP in turkeys, it was found that on average 3.3% of parthenogenetic eggs hatch [34]. That study followed the fate of 2084 parthenogenetic eggs, with 68 hatching. Furthermore, parthenogens have attained adulthood in a variety of species studied, including Turkeys, *Meleagris gallopavo* [10], California Condors, *Gymnogyps californianus* [21, Boa constrictors, *Boa imperator* [16], and the whitespotted bambooshark *Chiloscyllium plagiosum* [19]. Studies following the fitness of parthenogens are lacking, however substantial negative costs associated with the trait have been demonstrated in sharks [35]. While fitness costs are evident, it has been shown that turkey parthenogens and both Boa Constrictors, *Boa imperator*, and Royal Pythons, *Python regius*, that survive to adulthood are reproductively competent [34, Booth, pers. observ.]. As such, the failure of this crocodile

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parthenogen to hatch should not be viewed as an indicator that all crocodile parthenogens will be non-viable.

The evidence presented here provides the first documentation of parthenogenesis in the reptilian lineage Crocodylia. Its discovery was dependent on the attention provided by those that maintain the animal in captivity. For instance, while it is not uncommon for captive reptiles to lay clutches of eggs, given the period of isolation from mates, these would normally be considered non-viable and discarded. These findings therefore suggest that eggs should be assessed for potential viability when males are absent. Furthermore, given that FP can occur in the presence of potential mates [4,9,20], instances of FP in crocodilians may be missed when reproduction occurs in females co-habited with males. The discovery of FP in several snake species was reliant on the presence of color or pattern variants, where offspring exhibited variants inconsistent with sexual reproduction by the putative parents [9,16]. Due to the rarity of widespread color/pattern variants in crocodilians, such anomalies indicative of FP would be missed, and hence parthenogens produced by females cohabiting with males would be overlooked without genomic testing. Moreover, it has been hypothesized that FP may be more common in low-density populations, such as those on the verge of extinction [4]. Accordingly, genomic studies investigating wild populations should assess for the potential presence of cryptic parthenogens [4,20].

With terminal fusion automictic FP documented in squamates, birds, and now crocodilians, this new evidence offers tantalizing insights into the possible reproductive capabilities of extinct archosaurian relatives of crocodilians, notably the Pterosauria and Dinosauria [36,37].

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Figure legends

Figure 1. (A) Adult American Crocodile, *Crocodylus acutus*. Photo courtesy of Q. Dwyer**. (B)** Stillborn fetus of American Crocodile, *Crocodylus acutus*, Parthenogen. Photo courtesy of Q. Dwyer.

Figure 2. A simplified phylogeny for the crown group Reptilia with major clades depicted. Highlighted lineages have records of facultative parthenogenesis via terminal fusion automixis, with some exemplar species reflecting phylogenetic spread where possible. Note that the divergence time scale is not linear.

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Table legends

Table 1. Summary of statistical analyses of three SNP data sets for the mother American crocodile and her parthenogen offspring discussed in this study. The SNP data sets were thinned to one variant per 10,000 base pairs (=thin 10kb), 25,000 bp (=thin 25kb), and 50,000 bp (=thin 50kb), respectively. SNPs = total number of SNP loci in the data set that were genotyped in both mother and offspring. Initial per-base error = conservative estimate of per-base genotyping error rate . Maternal homozygous = number of SNP loci in the data set for which the mother was homozygous. Expected discordant = number of offspring genotypes at maternal homozygous loci expected to differ from maternal genotype based on initial per-base error rate. Observed discordant = number of offspring genotypes at maternal homozygous loci that were observed to differ from the maternal genotype. Updated per-base error = per-base genotyping error rate assumed for heterozygosity scan analysis based on observed discordance between maternal and offspring genotypes at maternal homozygous loci. Maternal heterozygous = Number of SNP loci for which the mother was heterozygous. Expected offspring heterozygous = Number of offspring genotypes at maternal heterozygous loci that were expected to be heterozygous based on updated per-base genotyping error rate alone, assuming a null hypothesis of gametic duplication. Observed offspring heterozygous = number of observed heterozygous offspring genotypes at maternal heterozygous loci.

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Abstract

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Introduction

Once considered rare, the ability of sexually reproducing species to generate offspring without genetic contributions of males, termed facultative parthenogenesis (hereafter, FP), has been documented across multiple vertebrate lineages, including both avian [1] and non-avian reptiles (specifically snakes and lizards) [2], and elasmobranch fishes, with notable examples in sharks, rays, and sawfish [3-5]. The widespread phylogenetic distribution and increasing frequency with which FP is reported suggests that the occurrence of this unusual reproductive strategy is less sporadic than previously suggested [6]. In vertebrates, common FP characteristics include: (i) strict occurrence in lineages lacking genomic imprinting, a developmental mechanism believed to be restricted to the main mammalian lineages (Eutheria, Marsupialia) [7] , (ii) the sex of parthenogens is constrained by the species' sex chromosome system, where XX females produce only female (XX) parthenogens [1,3,7] and ZW females produce only male (ZZ) parthenogens [1,8-13], (iii) the capacity to switch between sexual and parthenogenetic modes of reproduction [14], (iv) the ability to produce both sexual and parthenogenetic offspring within single reproductive events [15; Booth pers. observ.], (v) the capacity for consecutive parthenogenetic events [5,13,16,17], and (vi) the reproductive competence of adult parthenogens, with both sexual [10, Booth pers. observ.] and secondary parthenogenetic events documented [19, Booth, pers. observ.]. Furthermore, viable parthenogens have been reported in wild populations [4,20]. Accordingly, these commonalities among taxa suggest ~~that~~ that the phyletic distribution and evolutionary significance of FP have been underestimated.

Documentation of FP in vertebrates are increasing for two primary reasons. First, the contribution of specimens with detailed records from zoological institutions and private keepers predominate in studies, including samples otherwise difficult to obtain (e.g., California Condor [21]; Komodo Dragon [12]; King Cobra [22]). Second, the advent of modern molecular analysis, particularly of genome-scale data, has enabled FP to be robustly disentangled from the phenomenon of long-term female sperm storage (LTSS) [23,24]. With records of LTSS exceeding six years [24], it is the main competing hypothesis for the production of offspring after prolonged periods of isolation from potential mates [23,24].

Nonetheless, questions remain as to the occurrence of FP outside of squamates, birds, and elasmobranchs. For example, FP has not been documented in Chelonia (turtles) or Crocodylia (crocodiles, alligators, and gharials). The latter group is particularly interesting as, unlike all previously documented cases of FP in vertebrates, all crocodilians lack sex chromosomes, and sex determination is controlled by temperature, a mechanism termed temperature-dependent sex determination (T~~D~~SD) [25]. Furthermore, crocodilians are members of a larger monophyletic lineage (Archosauria), a crown group which at its base splits into two groups, one (Pseudosuchia) comprising crocodilians and their extinct relatives and the other (Avemetatarsalia) that contains birds and their extinct relatives, including pterosaurs and non-avian dinosaurs. Here, leveraging whole-genome sequencing and bioinformatic analysis, we provide the first evidence of FP in a crocodilian, the American Crocodile, *Crocodylus acutus*.

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Methods

The American Crocodile (Figure 1A) is a large and wide-ranging apex predator. Owing to its saltwater tolerance, the species occurs from the southern U.S. (Florida) to Northern South America (Colombian, Venezuela, and Peru). It is IUCN listed as vulnerable and is at risk of extinction in the wild (http://www.iucnredlist.org/species/5659/212805700). On 17 January 2018, a clutch of 14 eggs was discovered in the enclosure of an 18-year-old female American Crocodile housed on public exhibit at Parque Reptilandia, Costa Rica. This female was obtained when it was 2 years old (2002) and maintained in isolation from other crocodilians for its entire life. Of the 14 eggs laid, 7 appeared to be fertile and were artificially incubated. After three months of incubation the eggs had failed to hatch and were opened to assess their contents. The contents of six eggs were not discernable, but one egg was found to contain a fully-formed nonviable fetus that upon dissection was determined via gross gonadal morphology as ~~to be~~ female (Figure 1B) (Supplemental information). DNA was extracted from a scute shed by the mother ~~the mother~~ and cardiac muscle obtained from the fetus using a Qiagen DNeasy Blood & Tissue kit (Qiagen. Hilden, Germany) was sent to Novogene (Sacramento, CA) for whole genome sequencing on an Illumina platform (NovaSeq 6000 PE150). Raw sequences were mapped by Novogene to the Saltwater crocodile, *Crocodylus porosus*, reference genome with single nucleotide polymorphisms (SNPs) identified by Novogene using the following the command in SAMtools: mpileup -m 2 -F 0.002 -d 10.

Three thinned SNP data sets comprising 191,255 SNPs (thinned to one variant per 10 kb), 78,603 SNPs (thinned to one variant per 25 kb), and 39,746 SNPs (thinned to one variant per 50 kb), were analyzed using ParthenoGenius [26] to test for evidence and mode of parthenogenesis. ParthenoGenius first determines whether paternal alleles are present in the offspring. If the number of maternally homozygous loci for which the offspring has non-identical genotypes to the mother is less than that expected due to genotyping error alone (i.e., the offspring is homozygous at all or nearly all of the mother's homozygous loci), the offspring is identified as a likely parthenogen. ParthenoGenius then scans maternally heterozygous loci to identify those at which the offspring has retained heterozygosity. Based on the proportion of retained heterozygosity and taking into consideration the estimated per-base error rate, the parthenogenetic mode is identified as either gametic duplication or automixis (Supplemental

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Information). Automictic parthenogenesis can be subdivided into terminal fusion, where the terminal meiotic products fuse resulting in near genome-wide homozygosity [22], and central fusion, where parthenogens exhibit extensive genome-wide heterozygosity at a comparable level to that of the mother [27]. The latter is not known to occur in vertebrates [28].

Results

The offspring was found to have identical genotypes to the mother at > 99.9% of her homozygous loci, demonstrating a lack of paternal alleles. Genome-wide maternal heterozygosity was 0.349, compared to 0.045 for the offspring, demonstrating a significant reduction in heterozygosity in the offspring when compared to the mother. Regardless of the data set evaluated, the offspring retained approximately 3% of the maternal heterozygosity (Table 1), supporting facultative parthenogenesis through terminal fusion automixis as the mode of reproduction. Note that in instances of central fusion, retained heterozygosity is expected to exceed 33% of that of the mother, whereas terminal fusion will be less than 33% [29]. Due to the lack of a chromosome-scale *C. acutus* genome, it is not possible at this time to identify the actual genomic regions that retain heterozygosity with any accuracy.

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Discussion

Our results provide both novel and substantive evidence of facultative parthenogenesis in an extant archosaur, the American Crocodile. Crocodilians diverged from other archosaurs ~240 million years ago [30]. Following this split, Pterosauria and Dinosauria underwent substantive diversification, eventually giving rise to modern birds circa 65 mya [30,31], the only extant theropod dinosaur lineage (Figure 2). As such, we now have evidence of FP in the two main branches of extant archosaurians: crocodilians (Pseudosuchia) and birds (Avemetatarsalia). Furthermore, the most-recent common ancestor of squamates (snakes, lizards, and amphisbaenids), lineages for which FP has been widely documented in both snakes and lizards [1], and crocodilians, diverged ~267.0 – 312.3 mya [32], suggesting that FP may be a primitive condition. Comparable to FP studies of birds, snakes, and lizards, the evidence presented here supports terminal fusion automixis as the parthenogenetic developmental mechanism [1,22]. Accordingly, at this time, these commonalities do not suggest independent evolutionary origins of this trait across these lineages, but instead a trait likely possessed by a distant common ancestor of these lineages. While beyond the scope of the current study, it is clear that more work is required to fully test the evolutionary distribution and dynamics of FP across deeper evolutionary time, allowing for a more comprehensive understanding of the ancestral states and rates of FP across vertebrate lineages.

Building on previous studies, the data presented here advance our understanding of the distribution of FP in vertebrates, particularly in that all previous studies relate to species whose sex is genetically determined (i.e., XX|XY or ZZ|ZW). In crocodilians, sex chromosomes are absent and the sex of the offspring is thus temperature-dependent [25]. *Crocodylus acutus* exhibits a Female-Male-Female TSD pattern, with temperatures ~~Incubation temperatures~~ below 30ºC and above 33ºC ~~at 30ºC~~ produc~~e~~ing 100% females, ~~whereas~~ and temperatures ~~at 34ºC~~around 31.5ºC ~~produce~~ producing a majority of males [25,33]. Here, eggs were incubated at 29-30ºC and the fully formed yet stillborn fetus was ~~confirmed via gross gonadal morphology as~~ female. Temperature-dependent sex determination is an ancestral trait in crocodilians, being present also in ~~both~~ turtle, ~~and~~ lizard and tuatara lineages. Within turtles, recent genomic analyses have revealed that many genes associated with calcium signaling pathways and neuroactive ligand-receptor interactions are associated with T~~D~~SD [32]. If these genes are also involved in the sex determination process in crocodiles, it appears that the genomic reduction inherent with terminal fusion automixis has not affected the expected offspring sex; larger sample sizes are required for confirmation of this finding.

While it is disappointing that the crocodile parthenogen produced here failed to hatch, it is not uncommon to see non-viable fetuses and developmental abnormalities within litters or clutches of parthenogens [1,22,34], and long-term failure to thrive even for individuals born outwardly healthy [35]. For example, following a nine-year study of FP in turkeys, it was found that on average 3.3% of parthenogenetic eggs hatch [34]. That study followed the fate of 2084 parthenogenetic eggs, with 68 hatching. Furthermore, parthenogens have attained adulthood in a variety of species studied, including Turkeys, *Meleagris gallopavo* [10], California Condors, *Gymnogyps californianus* [21]~~, Smalltooth Sawfish, *Pristis pectinata* [4], and Zebra Sharks, *Stegostoma tigrinum* [35]~~, Boa constrictors, *Boa imperator* [16], and the whitespotted bambooshark *Chiloscyllium plagiosum* [19]. Studies following the fitness of parthenogens are lacking, however substantial negative costs associated with the trait have been demonstrated in sharks [35]. While fitness costs are evident, it has been shown that turkey parthenogens and both

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Boa Constrictors, *Boa imperator*, and Royal Pythons, *Python regius*, that survive to adulthood are reproductively competent [34, Booth, pers. observ.]. As such, the failure of this crocodile parthenogen to hatch should not be viewed as an indicator that all crocodile parthenogens will be non-viable.

The evidence presented here provides the first documentation of parthenogenesis in the reptilian lineage Crocodylia. Its discovery was dependent on the attention provided by those that maintain the animal in captivity. For instance, while it is not uncommon for captive reptiles to lay clutches of eggs, given the period of isolation from mates, these would normally be considered non-viable and discarded. These findings therefore suggest that eggs should be assessed for potential viability when males are absent. Furthermore, given that FP can occur in the presence of potential mates [4,9,20], instances of FP in crocodilians may be missed when reproduction occurs in females co-habited with males. The discovery of FP in several snake species was reliant on the presence of color or pattern variants, where offspring exhibited variants inconsistent with sexual reproduction by the putative parents [9,16]. Due to the rarity of widespread color/pattern variants in crocodilians, such anomalies indicative of FP would be missed, and hence parthenogens produced by females cohabiting with males would be overlooked without genomic testing. Moreover, it has been hypothesized that FP may be more common in low-density populations, such as those on the verge of extinction [4]. Accordingly, genomic studies investigating wild populations should assess for the potential presence of cryptic parthenogens [4,20].

With terminal fusion automictic FP documented in squamates, birds, and now crocodilians, this new evidence offers tantalizing insights into the possible reproductive capabilities of extinct archosaurian relatives of crocodilians, notably the Pterosauria and Dinosauria [36,37].

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~~Supplemental Information~~

~~Additional information including experimental procedures, supplemental results, figures and acknowledgments can be found with this article in the online Supplemental Information.~~

~~Data Availability~~

~~Raw data used in this manuscript can be found online in the Dryad data repository at~~

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Figure legends

Figure 1. (A) Adult American Crocodile, *Crocodylus acutus*. Photo courtesy of Q. Dwyer**. (B)** Stillborn fetus of American Crocodile, *Crocodylus acutus*, Parthenogen. Photo courtesy of Q. Dwyer.

Figure 2. A simplified phylogeny for the crown group Reptilia with major clades depicted. Highlighted lineages have records of facultative parthenogenesis via terminal fusion automixis, with some exemplar species reflecting phylogenetic spread where possible. Note that the divergence time scale is not linear.

Table legends

Table 1. Summary of statistical analyses of three SNP data sets for the mother American crocodile and her parthenogen offspring discussed in this study. The SNP data sets were thinned to one variant per 10,000 base pairs (=thin 10kb), 25,000 bp (=thin 25kb), and 50,000 bp (=thin 50kb), respectively. SNPs = total number of SNP loci in the data set that were genotyped in both mother and offspring. Initial per-base error = conservative estimate of per-base genotyping error rate . Maternal homozygous = number of SNP loci in the data set for which the mother was homozygous. Expected discordant = number of offspring genotypes at maternal homozygous loci expected to differ from maternal genotype based on initial per-base error rate. Observed discordant = number of offspring genotypes at maternal homozygous loci that were observed to differ from the maternal genotype. Updated per-base error = per-base genotyping error rate assumed for heterozygosity scan analysis based on observed discordance between maternal and offspring genotypes at maternal homozygous loci. Maternal heterozygous = Number of SNP loci for which the mother was heterozygous. Expected offspring heterozygous = Number of offspring

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genotypes at maternal heterozygous loci that were expected to be heterozygous based on updated per-base genotyping error rate alone, assuming a null hypothesis of gametic duplication. Observed offspring heterozygous = number of observed heterozygous offspring genotypes at maternal heterozygous loci.

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Data set	SNPs	Initial per-base error	Maternal homozygous	Expected discordant	Observed discordant	Updated per-base error	Maternal heterozygous	Expected offspring heterozygous	Observed offspring heterozygous
thin 10kb	191,255	0.001	186,240	186.24	83	0.00045	5,015	2.23	152 (3.0%)
thin 25kb	78,603	0.001	76,580	76.58	33	0.00043	2,023	0.87	76 (3.8%)
thin 50kb	39,746	0.001	38,720	38.72	12	0.00031	1,026	0.32	34 (3.3%)

Table 1. Summary of statistical analyses of three SNP data sets for the mother American crocodile and her parthenogen offspring discussed in this study. The SNP data sets were thinned to one variant per 10,000 base pairs (=thin 10kb), 25,000 bp (=thin 25kb), and 50,000 bp (=thin 50kb), respectively. SNPs = total number of SNP loci in the data set that were genotyped in both mother and offspring. Initial per-base error = conservative estimate of per-base genotyping error rate. Maternal homozygous = number of SNP loci in the data set for which the mother was homozygous. Expected discordant = number of offspring genotypes at maternal homozygous loci expected to differ from maternal genotype based on initial per-base error rate. Observed discordant = number of offspring genotypes at maternal homozygous loci that were observed to differ from the maternal genotype. Updated per-base error = per-base genotyping error rate assumed for heterozygosity scan analysis based on observed discordance between maternal and offspring genotypes at maternal homozygous loci. Maternal heterozygous = Number of SNP loci for which the mother was heterozygous. Expected offspring heterozygous = Number of offspring genotypes at maternal heterozygous loci that were expected to be heterozygous based on updated per-base genotyping error rate alone, assuming a null hypothesis of gametic duplication. Observed offspring heterozygous = number of observed heterozygous offspring genotypes at maternal heterozygous loci.

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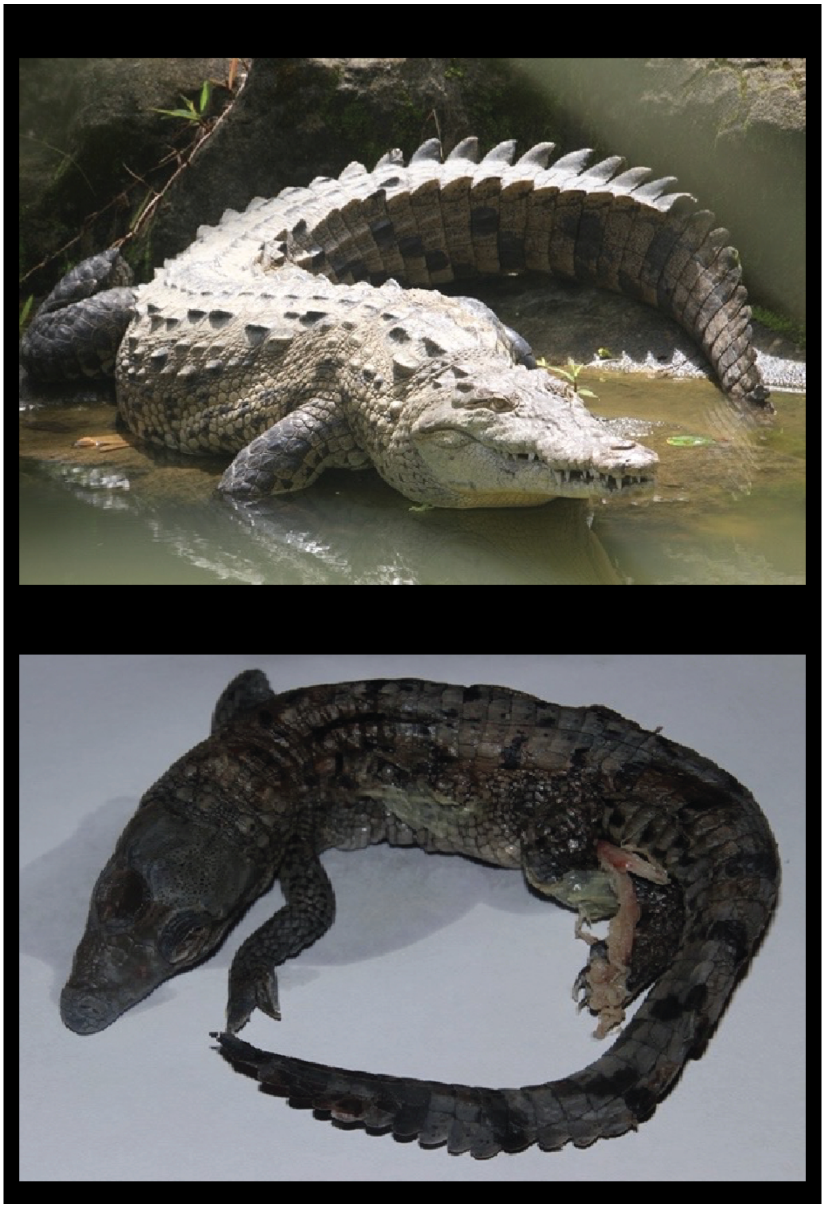
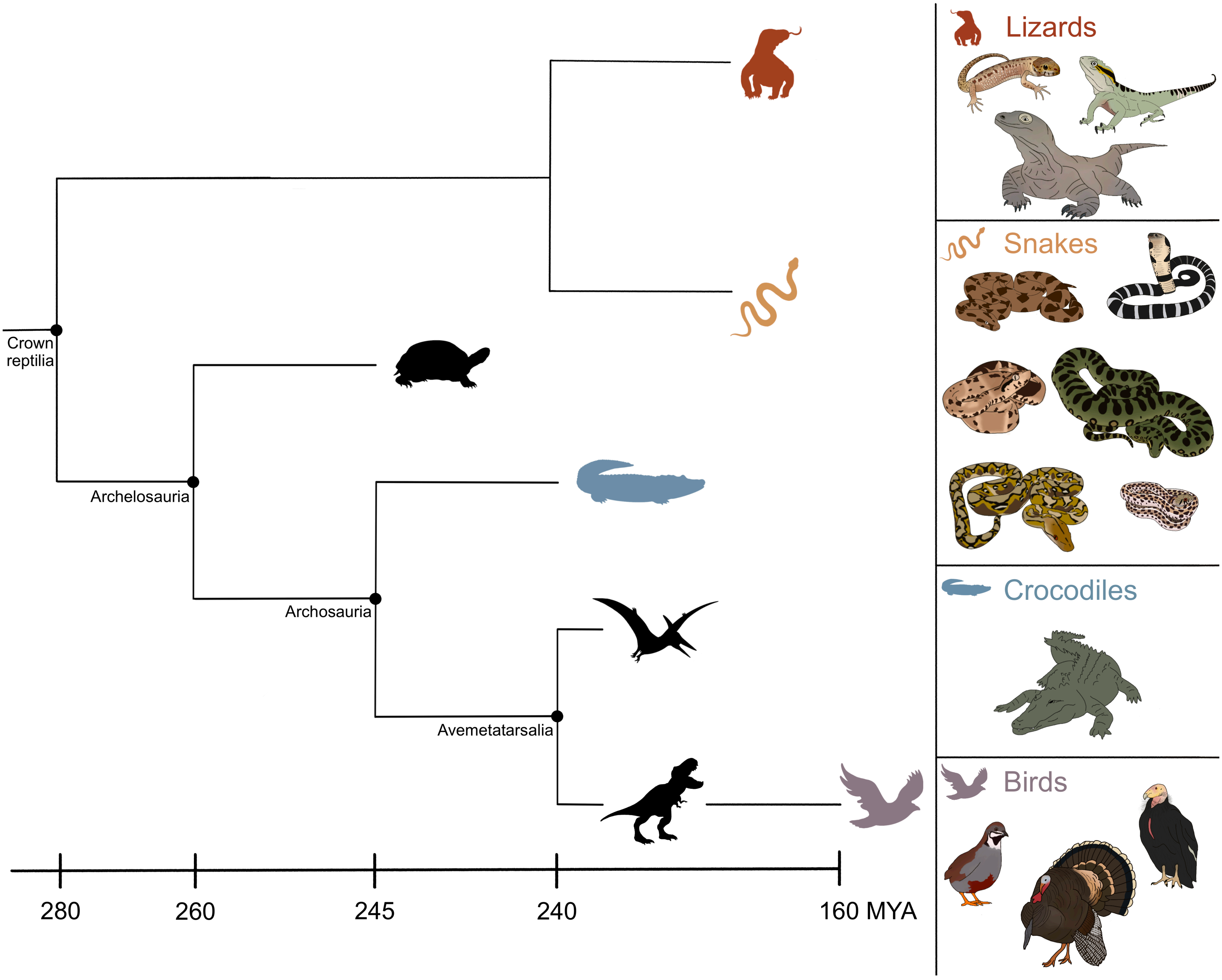


Figure 1. (A) Adult American Crocodile, Crocodylus acutus. Photo courtesy of Q. Dwyer. (B) Stillborn fetus of American Crocodile, Crocodylus acutus, Parthenogen. Photo courtesy of Q. Dwyer.

79x116mm (300 x 300 DPI)


Lizards
Snakes
Crocodiles
Birds
Crown reptilia
Archelosauria
Archosauria
Avemetatarsalia
280
260
245
240
160 MYA