

# Multiple paternity and mating patterns in the American alligator, *Alligator mississippiensis*

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## Abstract

Eggs were sampled from 22 wild American alligator nests from the Rockefeller Wildlife Refuge in south-west Louisiana, along with the females guarding the nests. Three nests were sampled in 1995 and 19 were sampled in 1997. Females and offspring from all clutches were genotyped using five polymorphic microsatellite loci and the three nests from 1995 were also genotyped using one allozyme locus. Genotypes of the hatchlings were consistent with the guarding females being the mothers of their respective clutches. Multiple paternity was found in seven of the 22 clutches with one being fathered by three males, and the remaining six clutches having genotypes consistent with two males per clutch. Paternal contributions of multiply sired clutches were skewed. Some males sired hatchlings of more than one of the 22 clutches either as one of two sires of a multiple paternity clutch, as the sole sire of two different clutches, or as the sole sire of one clutch and one of two sires of a multiply sired clutch. There was no significant difference between females that had multiple paternity clutches and those that had singly sired clutches with respect to female total length ( $P = 0.844$ ) and clutch size ( $P = 0.861$ ). Also, there was no significant correlation between genetic relatedness of nesting females and pairwise nest distances ( $r^2 = 0.003$ ,  $F_{1,208} = 0.623$ ,  $P = 0.431$ ), indicating that females in this sample that nested close to one another were no more related than any two nesting females chosen at random. Eleven mutations were detected among hatchlings at the five loci over the 22 clutches. Most of these mutations (eight of 11) occurred at Am $\mu$ -17, the only compound microsatellite locus of the five used in this study, corresponding to a mutation rate of  $1.7 \times 10^{-3}$ . Finally, most of the mutations (82%) were homoplasious, i.e., mutating to an allelic state already present in this Louisiana population.

**Keywords:** alligator, mating systems, microsatellites, multiple paternity, mutation rate, population genetics

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## Introduction

Many molecular techniques currently available allow insight into areas of reproductive dynamics and patterns of gene flow that have been unattainable previously. Recent genetic studies of mating systems have contrasted sharply with prior hypotheses regarding mating behaviour and reproductive output. For example, most bird species were thought to form monogamous pair bonds that produce

singly sired clutches exclusively (Lack 1968). It is now well recognized that extra-pair copulations in 'monogamous' bird species often result in offspring sired by males other than the attendant male (Gowaty & Karlin 1984; Birkhead *et al.* 1987; Westneat *et al.* 1990). While traditional ethological studies have yielded invaluable insight into behavioural patterns in animal populations, molecular markers such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and mini- and microsatellites have advanced our ability to identify individuals in populations (Parker *et al.* 1998). High-resolution genetic markers can provide detailed information about mating systems such as how many and

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which males are fathering offspring, whether or not females mate with the same males consistently (i.e. exhibit mate choice and fidelity), whether certain males dominate breeding, the breeding range of territorial males, and the extent of inbreeding in populations. Such information illustrates the reproductive dynamics of local populations providing a much needed link between population genetics and ecology (Awise 1996; Sugg *et al.* 1996).

One species that has received a great deal of attention in the past 30 years due to its primitive archosaurian position, its conservation status, its value as a renewable resource, and its potential as a biomonitor is the American alligator, *Alligator mississippiensis*. Several aspects of alligator biology have been investigated including nesting ecology (Joanen 1969; Woodward *et al.* 1984; Joanen & McNease 1989; Rhodes & Lang 1995, 1996), movement patterns (Chabreck 1965; Joanen & McNease 1970, 1972; McNease & Joanen 1974; Brisbin *et al.* 1992), metabolism (Coulson & Hernandez 1983), captive rearing (Joanen & McNease 1976; Elsey *et al.* 1994), physiology and endocrinology (Lance 1989; Guillette *et al.* 1996), and toxicology (Brisbin 1989; Crain *et al.* 1998; Guillette *et al.* 1999). Additionally, several studies have evaluated mating behaviour and reproduction (Garrick & Lang 1977; Lance 1989; Vliet 1989; Taylor *et al.* 1991).

Observations of crocodylian courtship, both in wild and captive settings, have revealed complex patterns of behaviour. Both males and females in many crocodylian species exhibit a number of stereotypical mating behaviours including bellowing and other distinct vocalizations, head slapping, and snout and head rubbing (Garrick & Lang 1977; Joanen & McNease 1989; Vliet 1989). Movement patterns of reproductive alligators in south-west Louisiana have been well-documented by Joanen & McNease (1970, 1972) who found that females typically remain in small, isolated ponds in the marsh interior, but move into deeper water during the April–May courtship and mating period. As a general rule, dominance hierarchies are common among males of most crocodylian species with large, aggressive males controlling access to mates and resources (Lang 1989). Females in captivity move freely between the territories of rival males, and while the dominant male is often the preferred partner, a female may engage in courtship and mating with subordinates as well (Garrick & Lang 1977). The peak of mating in American alligators occurs one month prior to nesting and, similar to Nile crocodiles, probably either coincides with ovulation or ends just prior to ovulation (Joanen & McNease 1976; Kofron 1990). Interestingly, one major difference among crocodylian species with regard to their mating system can be attributed to differences in climate. The two alligator species, *A. mississippiensis* and *A. sinensis*, both live in temperate climates forcing breeding to occur within a restricted time frame. Other crocodylian species inhabit tropical areas which can result in extended mating seasons (Magnusson *et al.* 1989).

Because mating occurs in the water and often involves groups of males and females which are difficult to differentiate, clear observation is rarely possible (Lang 1989). Even if a female is observed to be mounted by more than one male, it is unclear whether multiple males successfully copulate and inseminate her, resulting in fertilized eggs. Such observations have led to the supposition that female alligators may produce clutches sired by multiple males.

Multiple paternity, the occurrence of offspring within a single clutch being fathered by more than one male, is a mating strategy known to be utilized by a variety of taxa, including horseshoe crabs, spiders, black bears and birds (Brockmann *et al.* 1994; Schenk & Kovacs 1995; Kaster & Jakob 1997; Møller & Tegelström 1997). Among the reptilians, three orders have documented accounts of multiple paternity – snakes (Zweifel & Dessauer 1983; Schwartz *et al.* 1989; Höggren & Tegelström 1996), lizards (Abell 1997; Gullberg *et al.* 1997), and terrestrial and marine turtles (Harry & Briscoe 1988; Galbraith 1993; FitzSimmons 1998; Kichler *et al.* 1999). Multiple paternity is suspected as a reproductive strategy in crocodylians (Kofron 1990), but until now has not been demonstrated.

Several genetic techniques are currently available for use in investigating relatedness of individuals in wild populations (Parker *et al.* 1998). One of these methods uses microsatellites, short tandem arrays of DNA sequences with basic repeat units of 1–5 base pairs (Queller *et al.* 1993). Because Glenn *et al.* (1998) have described polymorphic microsatellite loci in American alligators, high-resolution assays can now be performed to answer questions about genetic relatedness in this crocodylian. This study focuses on 22 wild clutches of American alligators from Rockefeller Wildlife Refuge (RWR), Louisiana and the females guarding the nests to confirm maternity of the attendant female and to look for evidence of multiple paternity. Additionally, mutation rates for the microsatellites used in this study, incidence of null alleles, and degree of homoplasy are determined.

## Materials and methods

### Samples

Sampling for this study was conducted on portions of RWR, a 32 000 hectare coastal marsh located in south-western Louisiana (Fig. 1). The refuge boundaries and predominant vegetation have been described previously (Joanen 1969).

Alligator nests were located by helicopter and nests marked by PVC pipes. The positions of nests were plotted on aerial maps to facilitate egg collections by ground crews. Nests were checked by a four-person crew and were selected for this study when the female alligator was present at the nest and aggressive enough for capture to be

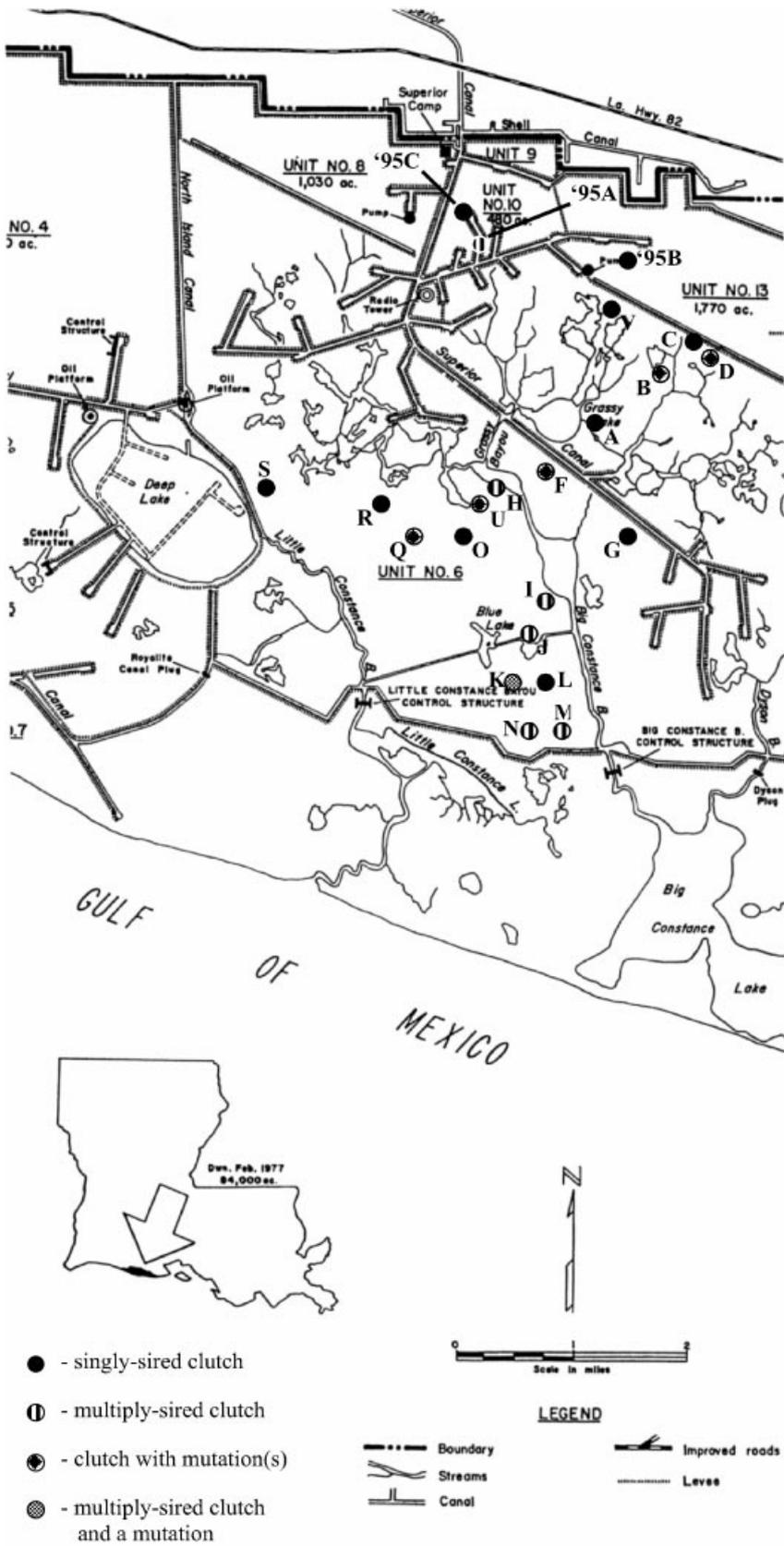


Fig. 1 Distribution of American alligator nests at Rockefeller Wildlife Refuge, Louisiana. Singly sired nests, nests with multiple paternity and nests having hatchlings with mutations are indicated.

possible. (Efforts focused on nests with attending females so that a potential maternal genetic profile could be obtained for comparison with offspring profiles. For the purposes of this study, there was no a priori reason to suspect that such sampling would bias any of our results.) Females in nest defence were captured by a self-locking cable snare mounted on the end of a 3–4 m wooden pole. The snare was placed over the female's head as she approached the nest. She was then pulled to an elevated, smooth location near the nest and a second snare was used to secure the jaws. A burlap sack was thrown over the female's eyes and the jaws secured with heavy rubber bands. A 15–20 mL blood sample was collected in a heparinized syringe from the supravertebral branch of the internal jugular (Olson *et al.* 1975) and kept on ice until returned to the laboratory. A muscle sample was obtained by removing two adjacent tail scutes with a small piece of tail musculature, permanently marking the animal. Each alligator was measured to the nearest half inch and two web tags (National Band and Tag Co., Newport, KY) were placed between the toes for individual identification. Each alligator was released unharmed at the nest site.

Nests were identified by location and by attending female. Eggs were collected from each nest and incubated at RWR's field laboratory as previously described (Joanen & McNease 1987). Clutches were incubated in separate containers (mean number of eggs/clutch = 38.0, range 19–51). Fertile eggs were incubated throughout the summer and within a few days after hatching (hatch rate = 86%) a 0.5–1.0 mL blood sample was obtained from each hatching. The average number of eggs per clutch that was genotyped was 29.2 (range = 14–40).

For the three 1995 nests, the polymorphic allozyme locus LDH2 was evaluated using methods described previously (Gartside *et al.* 1977). For the microsatellite analyses, DNA was extracted from red blood cells using either standard phenol/chloroform extraction (Maniatis *et al.* 1982) or a protocol modified from Carter & Milton (1993) utilizing guanidinium thiocyanate (Davis *et al.* 2001; or see <http://gator.biol.sc.edu/lisa>). DNA quality and quantity were estimated by electrophoresis through a 1% agarose gel and visualized by ethidium bromide staining and UV transillumination.

#### *Preliminary Screening*

One microsatellite locus previously described by Glenn *et al.* (1998), Am $\mu$ -17, was used in a preliminary screening of at least 24 individuals per clutch (or all individuals if there were fewer than 24) to discover which nests showed evidence of multiple paternity. This locus was chosen for its high degree of polymorphism. Explicit protocols used previously to obtain and score microsatellites (Glenn 1997;

also available on the Internet at <http://gator.biol.sc.edu/Msats/Protocols.html>) were followed with slight modifications. Briefly, one primer was labelled with Cy-5 (Pharmacia) or IRD-800 (Li-Cor), depending upon the detection instrument used (see below). Polymerase chain reaction (PCR) amplifications with Cy-5 had final concentrations of: 250  $\mu$ g/mL BSA, 150  $\mu$ M of each dNTP, 2 mM MgCl<sub>2</sub>, 1.0 unit of Promega *Taq* DNA polymerase (with appropriate buffer from the supplier), and 0.5  $\mu$ M of each primer. Reactions using IRD-800 were the same except no BSA was added and the final primer concentrations were 0.5  $\mu$ M for the unlabelled primer a, 0.5  $\mu$ M for unlabelled primer b, and 0.2  $\mu$ M of the labelled primer b. Amplicons, i.e. PCR products, were generated from genomic DNA using a Techne Genius thermal cycler with the following parameters: 95 °C for 2 min 45 s, followed by 32 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 45 s. Fragments were then denatured and separated on either 6% polyacrylamide or Long-ranger (FMC Bioproducts) gels. Amplicons of all individuals within a clutch were run together on the same gel with those of the putative mother and a known size standard. Following electrophoresis, Cy-5 labelled fragments were visualized using a Molecular Dynamics Storm scanner. IRD-800 labelled fragments were analysed using a Li-Cor 4000 L instrument. Within-clutch genotypes were assigned by visual inspection, parental genotypes determined, and unexpected alleles noted. Unexpected alleles, or those that did not conform to Mendelian expectations of allele frequency within a clutch, were considered to be either evidence for multiple paternity, mutations or cross-contamination. For those clutches that showed no unexpected alleles in the preliminary screening, the same individuals per clutch were re-assessed using four additional microsatellite loci – Am $\mu$ -6, Am $\mu$ -8, Am $\mu$ -15, and Am $\mu$ -18 (Glenn *et al.* 1998). In cases where clutches showed unexpected alleles, all individuals in the clutch were genotyped using the same five loci.

#### *Multiplex microsatellite amplification and detection*

One primer from each of the five loci was tagged with a fluorescent label (Davis *et al.* 2001) for detection on an ABI Prism 377 automated DNA sequencer (Perkin Elmer, Applied Biosystems, Inc.). Each sample contained approximately 25 ng of PCR product to which 3  $\mu$ L Dextran/formamide loading buffer and 0.65  $\mu$ L CXR fluorescent ladder (Promega Corp., Madison WI) were added. After denaturing the samples at 95 °C for 5 min, 1.2  $\mu$ L of this cocktail was loaded into the wells of a 0.2-mm thick 4.5% polyacrylamide gel (12 or 36 cm well-to-read length) and the amplicons separated for 1.5–2.0 h. GENESCAN and GENOTYPER programs (Perkin Elmer, Applied Biosystems Inc.) were used to identify and quantify microsatellite peaks.

### Statistical analyses

Estimates of allele frequencies for the RWR alligator population were taken from Davis *et al.* (2001) for the microsatellite loci and Gartside *et al.* (1977) for LDH2. Previous studies have shown these microsatellite loci to be unlinked (Glenn *et al.* 1998; Davis *et al.* 2001), but no tests have been done with these microsatellite loci and LDH2. Thus, LDH2 genotypes were added to 18 alligators genotyped in Glenn *et al.* (1998), and tested using the default setting within the Hardy–Weinberg probability and genotypic disequilibrium tests of GENEPOP 3.1c (Raymond & Rousset 1995). Loci were found to conform to Hardy–Weinberg expectations of panmixia except for Am $\mu$ -6 and Am $\mu$ -17 which showed an excess of homozygous genotypes. Am $\mu$ -17, which is known to have a low incidence of null (nonamplifying) alleles in this population, was included in this study due to its high polymorphism and because null alleles would be apparent in analyses of parent/offspring genotypes. Null allele frequencies were estimated with CERVUS 1.0 (Marshall *et al.* 1998). Additionally, genetic distances were calculated between all pairs of females and between all pairs of males using Kinship (Queller & Goodnight 1989). This relatedness measure is based on a sum of squared allele sizes and incorporates the estimated allele frequencies of the population of interest. Further, SAS 6.12 (SAS Institute, Cary, NC) was used to calculate the correlation between female genetic relatedness and pairwise nest distances.

The probability of identical genotypes among individuals (s) was calculated from FitzSimmons (1998, equation 3; cf. Hanotte *et al.* 1991). Each hatchling genotype was compared to the genotype of the female guarding the nest to confirm maternity. To provide a comparison with previous studies and an indication of the probability of detecting multiple paternity, exclusion probabilities for individual hatchlings when one parent is known (d) was calculated according to FitzSimmons (1998, equation 1; cf. Westneat *et al.* 1987). Multilocus probabilities for d were calculated according to FitzSimmons (1998, equation 2; cf. Chakraborty *et al.* 1974), which assumes independence of loci. BROOD (DeWoody *et al.* 2000) was used to calculate the number of hatchlings ( $n$ ) needed to detect all parental alleles within a clutch. We attempted to use additional software programs to provide estimates of the number of fathers contributing to the individual clutches. Because our data violate the assumptions of GAMETES, HAPLOTYPES (DeWoody *et al.* 2000), CERVUS 1.0 (Marshall *et al.* 1998), and LAMP3 (Kichler *et al.* 1999), making estimates from these programs impossible or nonsensical for some clutches, we used the most conservative estimate of the number of males contributing to clutches (cf. DeWoody *et al.* 2000). Candidate males genotypes were determined by inspection. For clutch 1995A, the number of hatchlings per putative male

genotype were assigned using CERVUS 1.0 (Marshall *et al.* 1998).

Allele frequencies were calculated for each clutch. Deviations from expectations of Mendelian inheritance were noted. These deviations were classified as being the result of either multiple paternity, mutation, null alleles or genotyping errors (includes mislabelled samples and technical errors). A clutch was considered to exhibit multiple paternity if the following conditions were met: (i) all hatchling genotypes within a locus contained at least one maternal allele that was consistent within the clutch (but see 'Mutations'); (ii) the remaining paternal alleles could not be accounted for by one father due to inconsistencies in Mendelian inheritance; and (iii) hatchlings containing an inconsistent allele in their genotype at one locus had inconsistent alleles in other loci or shared that inconsistent allele with at least one other nest-mate at that locus. In nests with multiple paternity, the most common paternal allele(s) were considered to be from the primary (1°) father. Paternal alleles that were unique within a clutch (i.e. different than those of the 1°) or that produced genotypes that were inconsistent with genotypes expected by the mother and the 1° father, were considered to be alleles of the secondary father (2°). An allele was considered to be a mutation if it was inconsistent with the parental genotypes, occurred in only one hatchling genotype in the clutch, and all the genotypes in the remaining loci were consistent with the other hatchling genotypes in that clutch. Null alleles were detected in clutches by deviations from Mendelian inheritance in the form of excess homozygous genotypes or by an apparent absence of one parental allele in the offspring genotypes. Inconsistent genotypes were attributed to genotyping error if they were inconsistent with all other genotypes within a clutch at two or more loci in only one individual in a clutch. Genotypes from genotyping error were not considered in further analyses.

## Results

### Multiple paternity and mating patterns

A total of 3180 single-locus genotypes were determined for 643 hatchlings and 22 adult female American alligators (complete data set available at <http://gator.biol.sc.edu/lisa>). Most hatchlings had unique multilocus genotypes. Shared genotypes occurring within clutches were uncommon as only 40 of the 643 multilocus genotypes were shared by two individuals within clutches, seven were shared among three individuals within a clutch, and in one case, four individuals within a clutch shared a common multilocus genotype. Thus, the five loci were sufficient to distinguish between most of the hatchlings in this study (92%), even full siblings. Using the RWR allele frequencies from Davis *et al.* (2001), the probability of any two randomly drawn

**Table 1** Summary statistics for five polymorphic microsatellite loci for American alligators from Rockefeller Wildlife Refuge, Louisiana

Locus	Alleles	$H_O$	$H_E$	Probability of shared genotype (s)	Probability of detecting multiple paternity (d)
Ami $\mu$ -6	7	0.63*	0.75	0.101	0.526
Ami $\mu$ -8	9	0.70	0.68	0.159	0.429
Ami $\mu$ -15	5	0.49	0.49	0.299	0.281
Ami $\mu$ -17	16	0.75*	0.86	0.032	0.731
Ami $\mu$ -18	7	0.83	0.80	0.075	0.588
Overall		0.66	0.69	$1.19 \times 10^{-5}$	0.978

\*denotes loci that showed deviation from Hardy–Weinberg equilibrium in a previous study of RWR alligators ( $P < 0.05$  Davis *et al.* 2001).

alligators at RWR sharing the same multilocus genotype (s), across all five loci was one in 100 000 (Table 1). The number of hatchlings necessary to detect all parental alleles for these markers, assuming a maximum of three fathers contributing equally or two fathers with the majority male contributing 80% of the hatchlings, averaged 15.4 and 16.2 (25.4 and 31.4 for 95% confidence), respectively using BROOD. Additional simulations with similar parameters gave similar estimates of the number of hatchlings needed to detect all parental genotypes.

Examination of parental genotypes revealed a number of interesting mating patterns. For all clutches, the offspring genotypes were consistent with the female guarding the nest being the mother of the clutch as each hatchling genotype included one allele found in the genotype of the guarding female at each locus (except in the case of a maternal null allele or a maternal mutation in the hatchling genotype). All 22 females could be differentiated from one another genetically, i.e. each had unique genotypes. Because it is unlikely that a large group of siblings would share a common maternal/paternal genotype at random (cumulative probability approaches 1/s), the guarding females were assigned as known parents. There was no significant difference in total body length between females that had clutches with multiple paternity (MP females) and those that did not (mean = 198.0 cm and 196.4 cm, respectively,  $P = 0.844$ ). There was also no significant difference between the clutch sizes of MP females and non-MP females (mean = 37.7 and 38.1, respectively,  $P = 0.861$ ).

To determine if females that nested near to one another were more closely related genetically rather than randomly, pairwise genetic distances (i.e. estimates of relatedness) were plotted against pairwise nest distances. Overall, there was no significant correlation between genetic relatedness and pairwise nest distances ( $r^2 = 0.003$ ,  $F_{1,208} = 0.623$ ,  $P = 0.431$ ). Among all pairwise comparisons of female genetic distances ( $22 \times 21/2$ ), eight were significant at  $P < 0.05$  and two were significant at  $P < 0.001$  (A/B and R/F). Since 11 comparisons would be expected to occur at random at  $\alpha = 0.05$  given the number of comparisons

involved, only those with  $P$ -values less than 0.001 were considered to be significant. Additionally, females A and B shared eight pairs of alleles across a possible 10. While females R and F only shared five out of 10 possible pairs of alleles, they shared one rare allele at locus Ami $\mu$ -8. Thus, although some of the females in this study were probably related, females which nested close to one another were no more closely related than any two females chosen at random.

Based on previous studies of RWR allele frequencies (Davis *et al.* 2001), the index  $d$ , indicating detection of multiple paternity using all 5 microsatellite loci, was almost 98% (Table 1). Seven of the 22 clutches (31.8%) showed evidence of multiple paternity, having offspring genotypes inconsistent with one father (Tables 2 & 3). Genotypes from one clutch (1995A) that exhibited multiple paternity suggested that three males (1°, 2° and 3°) contributed to that clutch with the males siring 19, 10 and nine hatchlings (Table 2). The remaining six clutches demonstrating multiple paternity could be accounted for by two fathers per clutch. The number of diagnostic loci for each individual fathered by a 2° father ranged from 2 to 5, and the number of individuals per clutch fathered by a 2° father was four, four, eight, three, three and seven for clutches H, I, J, K, M, and N, respectively (Table 3). Two individuals from clutch 1995A were removed from further analyses due to genotyping error.

Paternal genotypes indicate that some males mate successfully with more than one female (Fig. 2). Given the spatial location of nests (Fig. 1), some of these results might have been predicted. For example, of the clutches that were singly sired, clutches C and D, which are in close proximity to one another, have the same paternal genotypes at all five loci (Table 2). Similarly, clutches O and Q share common paternal genotypes at all loci as well. Quite interestingly, of the six 1997 clutches that were fathered by two males, clutches M and N appear to share both the same 1° and 2° fathers while the sole sire of clutch L was also the 2° sire of clutch K. Kinship (Queller & Goodnight 1989) was used to test the genetic relatedness of all males based on their inferred genotypes. Significant relationships were found at

**Table 2** Grouping of genotypes for American alligator clutch 1995A. Parental genotypes are in bold. Multilocus genotypes were such that hatchlings formed a minimum of three groups with one mother and three fathers

ID*	LDH2	Amiμ-6	Amiμ-8	Amiμ-15	Amiμ-17	Amiμ-18
Mom	<b>a/a</b>	<b>122/128</b>	<b>132/132</b>	<b>159/159</b>	<b>253/265</b>	<b>180/188</b>
1° Dad	<b>b/b</b>	<b>122/128</b>	<b>132/136</b>	<b>159/159</b>	<b>259/261</b>	<b>180/188</b>
A2	a/b	128/128	132/136	159/159	261/265	180/188
A3	a/b	128/128	132/136	159/159	253/261	188/188
A4	a/b	122/128	132/132	159/159	261/265	180/188
A5	a/b	122/128	132/136	159/159	253/261	188/188
A10/32	a/b	122/122	132/136	159/159	253/261	180/180
A11	a/b	128/128	132/136	159/159	253/259	180/180
A15	a/b	122/128	132/132	159/159	253/259	180/188
A16	a/b	128/128	132/132	159/159	253/261	188/188
A17	a/b	128/128	132/132	159/159	253/261	180/188
A24	a/b	122/122	132/132	159/159	259/265	180/180
A26	a/b	122/122	132/132	159/159	253/261	180/188
A27/39	a/b	122/128	132/136	159/159	253/259	180/180
A28	a/b	128/128	132/136	159/159	253/259	188/188
A29	a/b	122/128	132/132	159/159	259/265	180/180
A30	a/b	122/122	132/136	159/159	259/265	180/180
A34	a/b	128/128	132/136	159/159	253/259	180/188
A35	a/b	122/128	132/136	159/159	261/265	180/180
2° Dad	<b>a/a</b>	<b>132/132</b>	<b>136/138</b>	<b>159/161</b>	<b>225/273</b>	<b>164/188</b>
A1	a/a	128/132	132/136	159/159	253/273	164/180
A6	a/a	122/132	132/136	159/161	253/273	188/188
A8	a/a	122/132	132/138	159/161	225/265	180/188
A9	a/a	128/132	132/138	159/159	225/265	164/180
A13	a/a	122/132	132/138	159/161	225/253	188/188
A14	a/a	122/132	132/136	159/161	225/253	164/180
A18	a/a	122/132	132/138	159/159	253/273	180/188
A21	a/a	122/132	132/138	159/159	225/265	164/180
A25	a/a	122/132	132/138	159/161	253/273	164/180
A33	a/a	128/132	132/138	159/159	253/273	164/180
3° Dad	<b>a/b</b>	<b>122/122 or 128</b>	<b>134/152</b>	<b>159/161</b>	<b>265/281</b>	<b>172/192</b>
A7	a/b	122/122	132/152	159/159	265/265	172/180
A12	a/b	122/128	132/134	159/159	265/281	188/192
A19	a/b	122/128	132/134	159/161	265/281	172/188
A20	a/b	122/128	132/152	159/161	253/265	180/192
A22	a/a	122/122	132/134	159/159	265/281	172/188
A23	a/b	122/128	132/152	159/159	265/281	188/192
A31	a/a	122/122	132/152	159/161	253/281	172/180
A37	a/a	122/128	132/134	159/161	265/265	180/192
A40	a/b	122/128	132/152	159/161	265/281	172/180

\*Hatchling identifications.

the level of  $P < 0.001$  for all pairs of males with the same genotypes across all five loci except for the 1° father of M and 1° father of N ( $P < 0.01$ ) due to common alleles in that genotype.

*Mutations and homoplasy*

Eleven mutations were found in these clutches (Table 4) overall. Two mutations were found in locus Amiμ-6, one in Amiμ-8, and eight in Amiμ-17. This corresponds to an

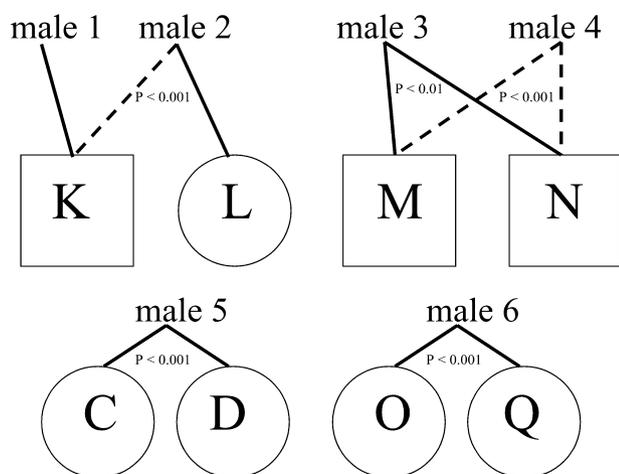
average mutation rate of  $1.73 \times 10^{-3}$  mutations/locus/generation (or  $5.8 \times 10^{-4}$  without locus Amiμ-17), which is consistent with the mutation rates found for microsatellites in other vertebrate species (Weber & Wong 1993; Craighead *et al.* 1995; FitzSimmons 1998). Taking into account the possible, yet improbable, case where all of the paternal mutations could be the result of multiple paternity, a calculation of mutation rate based solely on maternal mutations was performed. This rate was  $1.57 \times 10^{-3}$  based on five mutations in 3180 genotypes. It should be noted

**Table 3** Genotypes of guarding females (moms), primary (1° dad) and secondary (2° dad) fathers of multiple paternity nests and hatchlings of the 2° dads. Underlined alleles are paternal alleles. Bold type indicates alleles that are diagnostic for the 2° dad. Question marks indicate that another undetected allele might be present in the genotype of the 2° dad given that the 2° dad fathers few offspring

Clutch	Amiμ-6	Amiμ-8	Amiμ-15	Amiμ-17	Amiμ-18
MomH	122 128	132 136	149 157	253 277	164 192
1° dadH	122 128	132 152	149 157	269 269	188 192
2° dadH	124 124	134 132 or 136	157 149 or 157	261 265	172 172 or?
H7	122 <u>124</u>	132 136	157 <u>157</u>	<b>261</b> 277	164 <u>172</u>
H18	122 <u>124</u>	132 136	149 157	<b>261</b> 277	164 <u>172</u>
H21	<u>124</u> 128	132 136	157 <u>157</u>	253 <b>265</b>	<u>172</u> 192
H25	122 <u>124</u>	<u>134</u> 136	149 157	253 <b>261</b>	164 <u>172</u>
MomI	124 128	132 136	157 161	265 273	164 180
1° dadI	124 130	132 132	149 157	265 273	164 172
2° dadI	122 122 or?	136 132 or 136	157 159	237 261	172 180
I9	<u>122</u> 128	132 136	157 <b>159</b>	<b>237</b> 273	<u>172</u> 180
I15	<u>122</u> 124	136 <b>136</b>	<b>159</b> 161	<b>237</b> 273	164 <u>172</u>
I19	<u>122</u> 128	132 136	<u>157</u> 161	<b>261</b> 265	180 <b>180</b>
I21	<u>122</u> 128	132 136	157 <u>157</u>	<b>261</b> 273	164 <b>180</b>
MomJ	122 130	132 148	153 157	265 269	172 192
1° dadJ	122 122	136 136	159 161	265 265	164 164
2° dadJ	122 128	136 148	157 159	237 281	164 172
J1	<b>128</b> 130	148 <b>148</b>	153 <u>157</u>	<b>237</b> 269	<u>172</u> <u>172</u>
J9	122 <u>122</u>	132 <u>148</u>	157 <u>159</u>	<b>237</b> 269	<u>164</u> 192
J10	122 <b>128</b>	148 <u>148</u>	157 <u>157</u>	265 <b>281</b>	<u>172</u> <u>172</u>
J12	122 <u>122</u>	148 <u>148</u>	157 <u>159</u>	<b>237</b> 269	<u>172</u> <u>172</u>
J14	122 130	132 <u>148</u>	153 <u>159</u>	269 <b>281</b>	<u>164</u> 192
J24	122 <b>128</b>	148 <u>148</u>	157 <u>157</u>	<b>237</b> 265	<u>164</u> 192
J30	122 <u>122</u>	<b>136</b> 148	157 <u>159</u>	<b>237</b> 265	<u>172</u> <u>172</u>
J31	<b>128</b> 130	132 <u>148</u>	157 <u>157</u>	265 <b>281</b>	<u>164</u> 172
MomK	124 130	136 152	149 157	269 269	172 188
1° dadK	122 124	134 144	157 161	265 277	164 180
2° dadK	122 122 or?	132 132 or?	157 161	265 265 or?	164 192
K17	<u>122</u> 130	<b>132</b> 152	149 <u>157</u>	265 269	188 <b>192</b>
K19	<u>122</u> 124	<b>132</b> 136	157 <u>157</u>	265 269	<b>164</b> 188
K26	<u>122</u> 124	<b>132</b> 152	149 <u>161</u>	265 269	<b>164</b> 172
MomM	128 134	136 152	149 157	265 265	172 188
1° dadM	122 124	132 136	157 157	265 265	164 172
2° dadM	122 122 or?	136 136 or?	161 161 or?	265 265 or?	164 192
M1	<u>122</u> 128	136 <u>136</u>	149 <b>161</b>	265 <u>265</u>	<u>164</u> 172
M9	<u>122</u> 134	136 <u>136</u>	149 <b>161</b>	265 <u>265</u>	<u>164</u> 172
M17	<u>122</u> 128	136 <u>136</u>	157 <b>161</b>	265 <u>265</u>	188 <b>192</b>
MomN	128 134	132 136	157 157	265 269	172 180
1° dadN	122 124	132 136	157 157	265 265	164 172
2° dadN	122 122	132 132 or 136	161 161	265 265	164 192
N2	<u>122</u> 128	132 136	157 <b>161</b>	265 <u>265</u>	<u>164</u> 172
N3	<u>122</u> 128	132 136	157 <b>161</b>	265 269	180 <b>192</b>
N31	<u>122</u> 128	132 136	157 <b>161</b>	265 <u>265</u>	<u>164</u> 180
N32	<u>122</u> 134	132 136	157 <b>161</b>	000 000	164 180
N37	<u>122</u> 128	132 136	157 <b>161</b>	265 <u>265</u>	172 000
N38	<u>122</u> 134	132 <u>132</u>	157 <b>161</b>	265 269	<u>164</u> 172
N40	<u>122</u> 128	132 <u>132</u>	157 <b>161</b>	265 <u>265</u>	180 <b>192</b>

that Amiμ-17 was the only compound microsatellite locus used in this study consisting of two dinucleotide and two tetranucleotide motifs (Glenn *et al.* 1998) and, concomitantly, was also the most polymorphic. The stepwise mutation

model for microsatellites assumes that mutations occur most often in a single, stepwise fashion with either the addition or subtraction of one repeat motif per mutational event (Goldstein & Pollock 1997). Therefore, seven of the



**Fig. 2** Schematic showing the paternal relationships between selected American alligator nests from Rockefeller Wildlife Refuge, Louisiana. Squares denote clutches fathered by multiple males. Circles denote singly sired clutches. Bold lines indicate the primary male and dashed lines indicate the secondary male. For each pair of paternal comparisons with *P*-values, the paternal genotypes are identical across all five loci. *P*-values indicate confidence that the two identical genotypes are the same male. For the relationship between primary male M and primary male N (male 3), the *P*-value is < 0.01 due to common alleles in the multilocus genotype.

mutations can be most easily explained by the addition of one repeat motif, with a dinucleotide being the repeat motif in Am $\mu$ -6 and Am $\mu$ -8 and a tetranucleotide being the repeat motif in Am $\mu$ -17 (Table 4). The mutation in individual 19 of clutch O could have involved either the gain or loss of one repeat unit. Three other mutations involved multiple repeat motifs with either a loss of two, loss of three, or a gain of three repeat motifs in Q21, F1, and

Q10, respectively. Five of the mutations can most easily be explained as arising from a maternal allele, four from a paternal allele, and two could have arisen from either parent (i.e. parents shared a common allele from which the mutant allele could have been derived). Sequencing of these mutant alleles and their corresponding maternal (from the mother's sample) and paternal (from siblings sharing that allele) alleles is required to further elucidate their mutational modes.

Based on the alleles and allele frequencies characterized in Davis *et al.* (2001), considerable homoplasy was detected in all three loci where mutations were found. Three mutant alleles were found in fairly high frequency in the previous RWR sample (Davis *et al.* 2001) – 124 and 132 in Am $\mu$ -6 with frequencies of 0.19 and 0.10, respectively, and 273 in Am $\mu$ -17 with a frequency of 0.09. Four other mutant alleles were found in frequencies of less than 0.05. Only two of the 11 mutant alleles had not been found previously in the RWR population – 263 and 271 in Am $\mu$ -17. Additionally, two paternal alleles, 225 from clutch U and 249 from clutch F, were novel alleles in this population.

*Null alleles*

The presence of null, or nonamplifying, alleles can dramatically alter the interpretation of genetic structure in populations causing an underestimation of variation (Pemberton *et al.* 1995). Genotypes from 53 randomly sampled alligators from RWR (including the 22 females from this study) were analysed using CERVUS (Marshall *et al.* 1998) to calculate null allele frequencies in this population. These frequencies were estimated to be 0.07, 0, 0, 0.06, and 0 for Am $\mu$ -6, Am $\mu$ -8, Am $\mu$ -15, Am $\mu$ -17 and Am $\mu$ -18, respectively. Null alleles were detected in clutches by the excess of homozygous genotypes and inconsistencies of hatchling genotypes with those of their

**Table 4** Mutations in hatchling American alligator genotypes. Mutant alleles are in bold. Underlined alleles are those parental alleles from which the mutation is most likely derived based on the stepwise mutation model. In B15 the mutant allele could have arisen from either 269 parental allele. The mutant allele in O19 could have arisen from the maternal 233 allele or the paternal 241 allele. Seven of the 11 mutations represent a gain of one repeat motif from the parental allele

Locus	Clutch/hatch.#	Hatchling genotype	Maternal genotype	Paternal genotype
Am $\mu$ -6	D23	<b>124</b> 128	<u>122</u> 128	128 132
Am $\mu$ -6	K1	122 <b>132</b>	124 <u>130</u>	122 124
Am $\mu$ -8	Q21	132 <b>142</b>	132 136	132 <u>146</u>
Am $\mu$ -17	B7	<b>263</b> 265	<u>259</u> 269	265 269
Am $\mu$ -17	B15	269 <b>273</b>	259 <u>269</u>	265 <u>269</u>
Am $\mu$ -17	D29	237 <b>271</b>	237 237	265 <u>267</u>
Am $\mu$ -17	F1	<b>229</b> 249	<u>241</u> null	249 273
Am $\mu$ -17	O19	<b>237</b> 265	<u>233</u> 265	<u>241</u> 265
Am $\mu$ -17	Q10	<b>261</b> 265	<u>245</u> 281	241 265
Am $\mu$ -17	U10	<b>229</b> null	273 null	<u>225</u> 259
Am $\mu$ -17	1995C	233 <b>241</b>	233 257	<u>237</u> 249

parents. Four of the 22 clutches were found to have null alleles at *Ami $\mu$ -17*, three which were maternal in origin, corresponding to a realized null frequency of 0.09. These values are similar to those reported in snakes and humans (Callen *et al.* 1993; Gibbs *et al.* 1997). No null alleles were observed in any of the clutches at locus *Ami $\mu$ -6*. Given that 22 clutches were analysed and null alleles were discovered in four of them for *Ami $\mu$ -17*, it is unlikely that *Ami $\mu$ -6* has null alleles at an appreciable frequency.

## Discussion and Conclusions

These genetic data support the longstanding, but previously undemonstrated suspicion, that male alligators successfully mate with more than one female and that females can produce clutches fathered by more than one male. While these data show that some males in 1997 contributed to two clutches, it is possible that they sired more offspring in other nests as only 19 of some 300 nests available in the RWR study area were sampled. Given that one out of three nests in 1995 and six out of 19 nests in 1997 were multiply sired, this estimate of 32% multiple paternity may be close to the true frequency in this population per year. This is a much higher incidence of multiple paternity than found by FitzSimmons (1998) in green sea turtles and lower than reported for Kemp's ridley sea turtles (Kichler *et al.* 1999), but similar to both in that one male fathers the majority of the offspring within such clutches.

A number of possible scenarios could account for the finding that some females produce multiply sired clutches while the others do not (Table 5). For singly sired clutches (IA) some females may mate either only once, which is unlikely given behavioural observations, or multiple times with the same male. (IB) It is also possible that females mate with multiple males but the male whose copulation is best timed with ovulation has sperm precedence and fertilizes the eggs. (IC) Alternately, sperm competition in the reproductive tract of females (Parker 1970; Adkinsregan 1995; Luiselli 1995; Stockley 1997) or sperm selection for a particular male's sperm (Olsson *et al.* 1996; Wirtz 1997) might

also account for singly sired clutches by females who have multiply mated. (IIA) In multiple paternity clutches, some females may mate with one male preferentially, but allow additional copulations with one or more other males producing clutches sired mostly by the dominant male. An alternate outcome of this scenario could be that most of the offspring are sired by a subordinate male due to sperm depletion in the dominant male (Hoelzel *et al.* 1999). (IIB) Some females mate indiscriminately with several males and the ones whose copulations are best timed with her ovulation have sperm precedence and fertilize the eggs. (IIC) Also, sperm competition and sperm selection could determine which males sire offspring in multiply sired clutches when females mate multiply.

Female choice likely plays a critical role in narrowing potential offspring genotypes initially through preferential mate choice. Timing of copulation or cryptic female choice via sperm competition/selection may ultimately play the largest role in determining offspring genotypes once a preferred male or males have been selected. Observations of alligator courtship have shown that females move between the territories of rival males and that females often break off courtship (Garrick & Lang 1977). Courtship in crocodylians involves congregations of males and females in breeding areas in which a very complex and prolonged series of behaviours can take hours to days to successfully complete (Vliet 1989). Bellowing by both males and females is an integral part of the courtship and culminates in sonorous choruses. Males display their size and strength by elaborate posturing, head-slapping, and physical interactions with other males. These behaviours might be necessary not only to stimulate ovulation in females (Lance 1989), but also to synchronize the timing of fertilization (Garrick & Lang 1977). These behaviours are reminiscent of avian leks given that females choose a male or males with whom to mate among several males in a breeding territory. But how many males would a female be expected to mate with in a mating system marked by male dominance hierarchies and little to no paternal care? Observations of alligator mating in captivity have shown that females actively pursue

**Table 5** Potential scenarios to explain singly sired and multiply sired clutches of American alligators

I. Single Sire	II. Multiple Sires
(A) Female mates once with one male or with only one male repeatedly.	(A) Biased copulation frequency – female mates with one dominant male preferably, but also has few copulations with other males.
(B) Biased timing/sperm preference – female mates with multiple males, but only one fertilizes the eggs because of timing of copulation with respect to ovulation.	(B) Biased timing/sperm precedence – female mates with multiple males and timing of copulation with respect to ovulation determines which males fertilize the eggs.
(C) Sperm competition/selection – female mates with multiple males and sperm competition or sperm selection determines which male fertilizes the eggs.	(C) Sperm competition/selection – female mates with multiple males and sperm competition or sperm selection determines which males fertilize the eggs.

copulations with dominant males preferentially, but will initiate mating with subordinate males when the dominant male is mating with another female (J.W. Lang, personal communication). Studies of copulation frequency in birds in which males participate in the rearing of young and the potential for extra-pair copulations is high, show that mating frequency is extremely high (Birkhead *et al.* 1987). Yet in bird species in which there is no paternal care of the young by males, in most cases copulation frequency is low and females time acceptance of copulation with ovulation (Birkhead *et al.* 1987). This has been shown in several lekking bird species, a mating system in which sperm competition is rare (Birkhead & Moller 1993).

Males and females obtain fitness advantages in different ways by mating multiply. Mating with several females would enhance the fitness of males, particularly dominant males whose reproductive cost is low after establishment of a territory confers a status advantage. However, as females can produce only a limited number of offspring per season and reproduction carries immense energetic costs, limiting the number of mates or choosing the most fit male or males would seem the better strategy for them (Avisé 1996). Clearly, increased offspring variation and increase in effective population size result from multiple paternity (Sugg & Chesser 1994). There have also been several documented accounts of increased reproductive success with multiple matings by females (Madsen *et al.* 1992; Gray 1997). Additionally, increased offspring variation may be important in situations in which females colonize new territories (but see Judson 1995). It is well established that in many crocodylian species individuals can move long distances both over land and across expanses of fresh and salt water (Ross & Magnusson 1989). Colonization of island habitats far from the mainland by *Crocodylus porosus* have occurred numerous times (Ross & Magnusson 1989). Females carrying offspring of varying genetic composition would seem to have an advantage over females whose offspring are less genetically variable as half-sibling broods represent a wider range of tolerance for new habitats.

One question remaining open is whether multiple paternity is the result of within-season multiple matings or from sperm storage. To date no sperm storage structures have been found in any crocodylian, though Davenport (1995) reported a female caiman in captivity that laid eggs long after being separated from a male. While sperm storage has been well-documented in many snake, lizard and turtle species (Coker 1906; Schuett & Gillingham 1986; Gist & Jones 1989; Galbraith 1993; Villaverde & Zucker 1998; Valenzuela 2000), many of the arguments for the adaptive significance of sperm storage in these taxa do not apply to crocodylians. For example, it has been proposed that female turtles, many of which are relatively solitary, may store sperm for extended periods of time, including over winter.

Sperm stored in the reproductive tract would then serve as 'insurance' in the event that they do not encounter a male the following breeding season (Galbraith 1993). While female alligators may be considered to be solitary nesters, crocodylians often form loosely organized social groups (Lang 1989). It is unlikely that a reproductive female would disperse a great distance from conspecifics (Joanen & McNease 1970), particularly to the extent that she would not encounter a male during the breeding season. Therefore, that 'insurance' would not be necessary. It has also been proposed that in turtles producing multiple clutches in one season, eggs moving down the oviduct may 'sweep' away sperm moving upwards that would have been used to fertilize subsequent clutches (Gist & Jones 1989). Sperm storage would insure against any loss in fertility that would result from this. But crocodylians produce only one clutch per season (Magnusson *et al.* 1989; but see Whitaker & Whitaker 1984) and would not need stored sperm for subsequent clutches in the same year. Another argument for sperm storage in turtles and some snakes is that many species show asynchrony in gonadal cycles between the sexes (Halpert *et al.* 1982; Galbraith 1993). Sperm storage would then serve as a reservoir of viable sperm for fertilization of the eggs when the female ovulates. Studies of American alligator hormonal cycles have demonstrated remarkable within-season synchrony in testosterone production/sperm development of the males and oestrogen production/follicle development in females (Lance 1989). Sperm storage is apparently not necessary, therefore, to counteract asynchrony in gonadal cycles in alligators. Timing of gonadal cycles in American alligators is particularly important given their temperate distribution. Cooler climates allow a much shorter timeframe for reproduction compared to tropical crocodylian species in which courtship can persist for as long as 4–5 months prior to egg laying (Magnusson *et al.* 1989). Interestingly, in a unique situation where American alligators inhabited a reservoir where thermal effluent kept water temperatures unusually high year-round, males were found to produce sperm prematurely (Murphy 1981). It was suggested that the low nesting productivity at this site might have been a result of reproductive asynchrony between the sexes due to the premature reproductive activity of the males.

Crocodylian physiology and behaviour would also support the absence of long-term sperm storage structures. Ovulation occurs after courtship and mating (Garrick & Lang 1977; Kofron 1990) with the most intense mating occurring about one month prior to nesting (Garrick & Lang 1977). While successful copulation cannot be confirmed by observation, studies of alligator behaviour in captivity have documented a number of females mating with multiple males prior to nesting, with each female having her own peak period of receptivity (J.W. Lang, personal communication). Any sperm stored from the previous

mating season would probably be out-numbered and out-competed by that season's matings. While the lack of long-term sperm storage in alligators might seem surprising given that other reptilian taxa utilize this strategy, most arguments for its utilization in those taxa do not apply to alligators. Therefore, multiple paternity in American alligators is most likely due to within season multiple matings rather than long-term sperm storage.

While these data lend insight into several aspects of alligator reproduction, a number of questions remain to be answered. Is multiple paternity a strategy used by alligators in other populations and other crocodylian species? Do females who produce multiply sired clutches in one breeding season do so in subsequent breeding seasons as well? How does timing of copulation and multiple matings by females affect offspring genotypes? Controlled experiments involving varying access of captive males to reproductive females may provide insight, but a number of factors must be considered when attempting such manipulations. Undoubtedly, further investigations of crocodylian mating systems are needed to uncover complex inter-gender interactions as well as genetic and environmental determinants of offspring genotypes.

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