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Polygamous farmed Nile crocodiles (*Crocodylus niloticus*): Foetal membranes tell the story

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Multiple paternity in crocodilian broods would increase effective population size and slow down the loss of genetic variation due to inbreeding and random genetic drift in real populations. Multiple paternity may also explain variation among offspring of the same brood with respect to characteristics of commercial interest to crocodile farmers. Foetal membranes may provide a non-invasive source of DNA from which to determine the genotypes of Nile crocodile (*Crocodylus niloticus*) hatchlings. The aims of this study were to determine the effectiveness of using the foetal membranes remaining inside the hatched eggs to determine the genotypes of Nile crocodile hatchlings, and to determine whether a brood (the hatchlings from a clutch of eggs) from a communal breeding pond on a commercial farm may have more than one sire. DNA profiles were determined on 4–6 (mean 4.4) foetal membrane specimens (FMSs) from each of 25 broods from the same breeding pond on a commercial Nile crocodile farm. Eleven microsatellite loci were used. DNA amplification occurred at all 11 loci in 95 of the 110 genotyped individuals, at 1–10 loci in 13 and at no locus in two. Three to 20 alleles were found per locus. Single-locus assessment showed that 13 broods had at least two sires. A multilocus programme (Colony) inferred that 19 broods had at least two sires, with polyandry and polygyny being common. Further research is necessary to determine the utility of foetal membranes as a source of DNA from nests in the wild and, using more FMSs per brood, to more precisely determine the extent of polyandry and polygyny in farmed and wild Nile crocodiles.

Keywords: crocodile, egg, hatchling, foetal membrane, DNA, sire

Poligame Nylkrokodille (*Crocodylus niloticus*) op 'n krokodilplaas: Vrugvliese vertel die storie: Meer as een vaar in 'n krokodilbroeisel verhoog die effektiewe populasiegrootte en lei tot 'n stadiger verlies van genetiese variasie as gevolg van inteling en lukraak genetiese swerwing. Meer as een vaar kan ook die variasie met betrekking tot eienskappe wat van kommersiële belang is tussen krokodille uit dieselfde broeisel verklaar. Vrugvliese kan 'n nie-ingrypende bron van DNS verskaf waarmee die genotipe van Nylkrokodilbroeilinge (*Crocodylus niloticus*) bepaal kan word. Die doel van hierdie studie was om vas te stel hoe doeltreffend die genotipe van Nylkrokodilbroeilinge uit die vrugvliese wat in uitgebroeide eiers agterbly bepaal kan word en of 'n broeisel uit 'n kommunale teeldam op 'n kommersiële plaas meer as een vaar kan hê. Elf mikrosatellietloki is gebruik om die DNS-profiele van 4–6 (gemiddeld 4.4) vrugvliesmonsters (VVMe) van elk van 25 broeisels uit dieselfde teeldam op 'n kommersiële Nylkrokodilplaas te bepaal. DNS het op al 11 loki in 95 van die 110 individue vermeerder, op 1–10 loki in 13 en op geen lokus nie in twee. Drie tot 20 allele is per lokus gevind. Afsonderlike beoordeling van loki het getoon dat 13 broeisels minstens twee vaars gehad het. Met 'n multilokusprogram (Colony) is afgelei dat 19 broeisels minstens twee vaars gehad het, en dat poliandrie en poliginie algemeen was. Verdere navorsing is nodig om die nuttigheid van vrugvliese as 'n bron van DNS vir nesse uit die natuur te bepaal en om, deur meer VVMe per broeisel te gebruik, die mate van poliandrie en poliginie op Nylkrokodilplase en in die natuur meer presies te bepaal.

Sleutelwoorde: Krokodille, eier, broeiling, vrugvliese, DNS, vaar.

Introduction

A Nile crocodile (*Crocodylus niloticus*) female lays one clutch of eggs per year during the spring or early summer (Huchzermeyer, 2003), although she may fail to lay any in some years (Kofron, 1990). A Nile crocodile female lays a variable number of eggs (average about 40) (Khosa et al., 2012) in a single hole (nest) that she digs in sandy soil near the water (Kofron, 1989). Once her whole clutch of eggs has been laid, the female covers the nest with soil (Kofron, 1989). Only when hatching occurs about three months later (Hutton, 1987) does the female open the nest, collect the brood (in this paper defined as all the hatchlings from the clutch) and transfer them to the water (Combrink et al., 2016). On commercial farms, workers excavate each nest and remove the clutch of eggs from it as soon as possible after they were laid. The eggs are then placed horizontally, next to one another, in one or, in the case of large clutches, two polystyrene boxes. The boxes are then covered with lids and incubated until the eggs in them hatch.

Upon hatching, the Nile crocodile foetus breaks through the shell membrane and the shell at one pole of the egg (Nöthling et al., 2019a), leaving the foetal membranes attached to the inside of the egg.

In fertilised crocodilian eggs, the foetal membranes – which include the chorion, allantois, amnion and yolk sac – as well as the embryo are derived from the zygote through mitosis (McGeady et al., 2017). The foetal membranes, therefore, have the same genotype as the embryo and, eventually, the hatchling that hatches from the egg. Therefore, any foetal membranes remaining inside the egg shells provide a potential source of DNA by which to non-invasively determine the genotypes of the hatchlings. Foetal membranes have not yet been used to determine the genotype of crocodilian hatchlings.

Eggs in a box often hatch in short succession. Upon hatching, hatchlings crawl around in the box, potentially contaminating the outer surface of other eggs with their DNA.

Provided the foetal membranes inside the egg shells remain uncontaminated, they may be collected after all hatchlings have been removed from the box and used to determine the genotypes of the hatchlings.

Multiple paternity of broods is generally beneficial. It increases effective population size (Sugg and Chesser, 1994), thereby slowing down the loss of genetic variation in a real population due to inbreeding and random genetic drift (Hartl, 2000). A female with a multisire brood may have an advantage because her brood is likely to be more adaptable to a variety of habitats (Davis et al., 2001). Multiple paternity of crocodile broods may have specific significance to crocodile farmers. Nöthling et al. (2019b) have shown that, for a given egg size, hatchling mass varies within Nile crocodile broods. It is of interest to know whether this variation within broods may be due to genetic differences brought about by different sires. The same holds for other characteristics of commercial interest to crocodile farmers that may be found to vary within broods.

Multiple paternity of a brood occurs in the American alligator (*Alligator mississippiensis*) (Davis et al., 2001; Lance et al., 2009), the broad-snouted caiman (*Caiman latirostris*) (Amavet et al., 2008), Morelet's crocodile (*Crocodylus moreletii*) (McVay et al., 2008), the Orinoco crocodile (*Crocodylus intermedius*) (Lafferriere et al., 2016) and the black caiman (*Melanosuchus niger*) (Muniz et al., 2011). Multiple paternity has not yet been described in Nile crocodile broods.

As early as 1981 Miesfeld et al. described a tandem block of 17 TG dinucleotides in an interspersed region between the human δ and β globin genes, that also occurs throughout the human genome. A few years later, Litt and Luty (1989) used PCR to identify a hypervariable (TG)n microsatellite in the human actin gene, and demonstrated that it is inherited by codominant mendelian inheritance. Litt and Luty saw the potential to use microsatellites for genetic linkage studies. Rassmann et al. (1991) described the use of flanking primers and PCR to isolate microsatellites from any eukaryotic DNA and its use for DNA fingerprinting. Schlötterer and Tautz (1992) showed that mutation in microsatellites occurs due to slippage during DNA replication and that this is the cause of the high degree of polymorphism in microsatellites. Fitzsimmons et al. (2000) reported that microsatellites are not as common in crocodiles as in other taxa. Yet, Miles et al. (2009) assessed 82 microsatellites, previously isolated for the saltwater crocodile (*C. porosus*) for cross-amplification in 18 non-target crocodilian species, among which was *C. niloticus*. The 82 microsatellites were selected from 253 STRs from whole-genome scanning due to their relatively even map distribution and high polymorphic information content in *C. porosus*. All 11 loci used in the current study were among the 82 tested by Miles et al. and confirmed to have amplified and yield 5–9 alleles among eight Nile crocodile specimens.

The aims of this study on the Nile crocodile were to determine whether the foetal membranes in recently-hatched eggs provide DNA that amplifies successfully and whether a brood from a communal breeding pond on a commercial Nile crocodile farm may have more than one father.

Materials and methods

Method of sample collection and labelling

Specimens were collected during January 2013 from the only breeding pond on a commercial Nile crocodile farm during a single hatching season. Le Croc Estate is situated 19 km north-west of the town of Brits, North West Province, South Africa (25 ° 29′ 37′′ S, 27 ° 40′ 50′′ E, altitude 1028 m). The pond is approximately rectangular, 100 m × 57 m with an area of approximately 5650 m^2 and a basking area of approximately 5 m in width around the pond and, on the outside thereof, approximately 180 nesting areas of 4 m ×

1.8 m, each surrounded on three sides with a brick wall, with the open end facing the water. In addition, there is a sandy nesting area of approximately 2050 m^2 on the south-western side of the pond, where many females nest. Every morning, once the eggs were removed from the nests laid during the previous night, the soil was raked to facilitate identification of new nests the following morning. At most 17 nests were laid during one night. The number of females in the pool during 2012, when the eggs were laid, is unknown but they yielded 207 clutches of 8 to 77 (mean 32, SD 9.1) eggs of which 166 yielded broods of one to 47 (mean 18.5, SD 10.5) hatchlings. The origin and relatedness of the breeding animals is unknown, except that the farmer had introduced an additional 10 adult males from another farm that were presumably unrelated to the animals that had already been on the farm. The ratio between males and females was approximately 1:10.

A foetal membrane specimen (FMS, or FMSs in the plural) was taken from each hatched egg in each of 25 polystyrene incubation boxes. Each box contained eggs of a different clutch and from one clutch only. For most boxes of which the eggs hatched during the night, the specimens were collected during the next day. For most boxes of which the eggs hatched during the day, specimens were collected during that same day. For some boxes, the specimens were only taken 1–4 days after hatching.

Eggs were incubated horizontally and hatched in that orientation. Hatchlings from other eggs crawling over a hatched egg in the box posed a risk of contaminating its foetal membranes with their DNA. We kept the hatched egg in the horizontal position to reduce the risk of foreign material from entering the egg via the broken pole while collecting the FMS. After hatching, the resilient shell membrane, which lies on the inside of the shell and tears during hatching (Ferguson, 1982) often returned to a position covering the broken pole, which may have reduced the risk of contaminating content of the hatched egg. If the shell membrane covered the broken pole we deflected it or trimmed it away with a pair of scissors to create an opening through which to collect the FMS using a cytobrush (Craig Brush, Cell Path Services, Johannesburg, South Africa). The cytobrushes were individually packed. The stem of the brush was marked with the numbers of the brood and the FMS. The brush was air-dried and then returned into its sachet. The sachet was then sealed, marked with the numbers of the brood and the FMS and stored in a freezer at –18 °C for about 17 months until the DNA was extracted and amplified, and the genotype determined. A set of four or five (and in one case six) FMSs from each of the 25 broods were genotyped.

DNA extraction

DNA was extracted from the cytobrush by cutting the brush into an Eppendorf tube and adding 500 µL of lysis buffer from the PrepFiler™ Automated Forensic DNA Extraction Kit (ThermoFisher) on a heated shaker (Vortemp 56, Labnet) for 60 min at 70 ºC. This was followed by an automated extraction process on a Kingfisher 96 Magnetic Particle Processor (ThermoFisher) according to the Prepfiler™ V2 protocol (supplied by Applied Biosystems) that included DNA binding for 10 min followed by 3 washes using 300 µL Prepfiler™ Wash Solution per wash, 5 min drying at room temperature and elution into 75 µL of elution buffer.

PCR and electrophoresis

Apart from the DNA extractions of the 110 FMSs, PCR and genotyping was also done on a control sample. The control sample was from a Nile crocodile that had amplified successfully previously and for which the genotype was known.

The PCR was performed on a GeneAmp PCR System 9700 Thermal Cycler (Life Technologies) using KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems) and 4 µL of primer mix in a 10 µL reaction volume. Primer concentrations were 0.1 nmol/L for all STR markers used in multiplex panel 1, 0.06 nmol/L for STR marker CpP4311 and 0.08 nmol/L for all other STR markers used in multiplex panel 2 (Table 1). Cycling conditions included 3 min at 95 °C, 30 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s followed by an extension step at 72 °C for 10 minutes. PCR product (0.5 µL) was loaded with 10 µL Hi-Di™ Formamide (ThermoFisher) and 0.25 µL GeneScan™ 500 LIZ™ dye size standard (ThermoFisher) and run on a 3500xl

TABLE 1: STR (short tandem repeat) markers were co-amplified in two multiplex PCR panels (1 and 2) based on the dye label and fragment size.

STR ID	Dye label	Multi-plex	Minimum allele size	Maximum allele size	Forward primer sequence	Reverse primer sequence
CpDi06	FAM	1	236	274	CAGTCGGGCGTCATCATGTTGGGCACTTTGAAC	GTTTAAGAAAAATGGTGGAAAAC
CpP801	FAM	1	159	195	CAGTCGGGCGTCATCATTGGCATTAGATTGGTAGAC	GTTTCTATGCCAAAGCTACAAC
cpP4308	FAM	1	111	114	CAGTCGGGCGTCATCACATATGTAAATTTGGAATGA	GTTTGATTGAGCCATCCTTAAC
CpDi28	NED	1	135	142	CAGTCGGGCGTCATCACTATGCACTCCCTGATTTAAG	GTTTCCCACTCACGAATCTAAAG
CpDi21	PET	1	177	192	CAGTCGGGCGTCATCAAAACAGTTGGCTCTGTG	GTTTATACTTCCTGTGGCATCAT
CpP309	PET	1	225	306	GTTTAATACCTGGCATGTGTTCTTC	CAGTCGGGCGTCATCACATCAGGTTGGCATTTCA
CpP4006	PET	1	99	120	CAGTCGGGCGTCATCAAGTGAGATTTGGGTATATTT	GTTTCATTTCCTTACCATGATAG
CpP4311	NED	2	211	241	CAGTCGGGCGTCATCAGGCTGCTCTGTGTTTG	GTTTGGGTTTAGCATCATGT
CpDi42	PET	2	114	124	GTTTTTCAGTTTATTTGCCAAAG	CAGTCGGGCGTCATCAGATTGGGGAGGGAAGT
CpP218	PET	2	164	187	GTTTGGCATTTGAATTATTAACT	CAGTCGGGCGTCATCACTGGCAAATCACTTCTG
CpP314	VIC	2	260	279	GTTTGAAATGCCACTAATACACACA	CAGTCGGGCGTCATCACCAATTCTTCAGGTCCTTAT

Genetic Analyzer (ThermoFisher). Data was analysed using STRand software (University of California, Davis). Table 1 provides the detail on the short tandem repeat markers (microsatellite markers) and the multiplex panels.

Using a purpose-written programme to assess the genotypes at a locus in order to identify broods with more than one father

The simplest method of identifying broods with more than one father is the single locus minimum method, where the number of paternal alleles at the locus with most paternal alleles in a brood is divided by two and rounded up to give the minimum number of sires in the brood (Jones, 2005). We assessed the genotypes of a locus over all FMSs in a set to determine whether the alleles indicated that the set of FMSs had at least three parents and, if it did, whether the alleles allowed for a common parent, which was assumed to be the mother. A programme was written in Python to facilitate the assessment of the 4–6 (mean 4.36, SD 0.56) FMS genotypes in each set at each of 11 loci, for a total of 109 FMSs. For brevity we named the programme Al3p1c, standing for "at least 3 parents with one in common". Similar to the single locus minimum method described by Jones (2005), Al3p1c is also a single locus method. The Python script of the programme appears in the supplement file "Crocodile_Al3p1c.py". Although 110 FMSs were genotyped, one FMS had identical genotypes at all 11 loci to those of another FMS from the same brood (see Results) and was excluded from the analysis with Al3p1c as well as with Gerud2.0 and Colony, as described below.

Using Gerud2.0 to determine the minimum number of fathers in a brood

Gerud2.0 considers multiple loci (Jones, 2005). Gerud2.0 first constructs all possible multilocus maternal genotypes in the set and then all possible multilocus paternal genotypes based on the non-maternal alleles. If a single multilocus paternal genotype in combination with a maternal genotype could explain all genotypes of the set, Gerud2.0 declares that the set has one father only or, if two multilocus paternal genotypes are required, it declares that the set has a minimum of two fathers and so on. Gerud2.0 was used to determine the minimum number of fathers in each set of FMSs. Gerud2.0 requires that the genotype is known at all loci used. Following Bretman and Tregenza (2005), only the three most informative loci (CpDi06, CpDi42 and CpP801) were then used. All FMSs in a set with a genotype at each of these loci were used to determine the minimum number of fathers in the set.

Using Colony to infer the parental genotypes and estimate the number fathers per set

Colony uses a computationally efficient likelihood-based method to infer sibship and parentage among individuals from their multilocus marker genotypes (Wang, 2004; Wang, 2012). Colony (Wang, 2004; Wang, 2012) may be downloaded from http://www.zsl.org/science/software/colony. We used Colony 2.0.6.5 to estimate the number of fathers in a set of FMSs from each of 24 broods. Colony requires DNA to have amplified in at least half the loci in each individual (Colony user guide). At least three FMSs per set are required to assess whether the set may have had two or more fathers. In 101 of the 110 FMSs DNA amplified in seven or more loci (in no FMS did DNA amplify at six loci). After excluding the FMS that was identical to another and the only two FMSs of set 132 in which DNA had amplified in at least seven loci, the remaining 98 FMSs were used. These 98 FMSs consisted of a set of 3–6 FMSs (mean 4.1, SD 0.78) from each of 24 broods. We used six models in which we assumed that inbreeding did not occur and that the mothers and fathers were polygamous. The 24 maternal sib-groups were included in the models.

In the first model (model 1) we assumed a genotype typing error rate of 0.1 and an allelic dropout rate of 0.1, with unknown allele frequencies (Sefc et al., 2008). The outcome of this model yielded estimated allelic dropout and genotyping error rates for each locus, which were used in some of the following models.

We then ran a set of four models (models 2–5), with allele frequencies set as unknown. We ran models 2–5 three times each, using a different seed for the random number generator for each replicate. In model 2 we used the genotyping error rates and allelic dropout rates for each locus as estimated with model 1. In model 3 we used the estimated genotyping error rates and an allelic dropout rate for each locus that was set equal to the fraction of the 98 genotypes that were homozygous at the locus. (Here we assumed that all genotypes appearing as homozygous had one allele that failed to amplify.) In model 4 we used the estimated genotyping error rates and an allelic dropout rate of zero at each locus. (All genotypes that appeared homozygous were deemed true homozygotes.) In model 5 the allelic dropout rates were set to zero and the genotyping error rates at a very low level of 0.0001 for each locus.

As shown under Results (Table 3) all six models inferred fewer mothers than the 24 mothers there actually were. The first and third replicate of model 5 both inferred a number of mothers that was closest to the real number of mothers. The first replicate of model 5, however, inferred one fewer fathers than the third run of model 5 and was used as the model inferring the likely number of sires per set of FMSs.

Based on the outcome of models one to five, we also ran a sixth (model MinCol5). Model MinCol5 was the same as model 5, except that the allele frequencies were set according to the Minimum Colony method as described by Sefc et al. (2008) and Sefc and Koblmüller (2009) in order to minimise the number of inferred fathers. The frequency of each allele at each locus among the 98 FMSs was set to 0.0001, and a non-existing allele was added to each locus for which the frequency was set such that the frequencies for each locus added up to one.

When inbreeding is selected as mating system Colony accounts for deviations from Hardy-Weinberg equilibrium (Colony user guide). In order to account for the effects of deviation from Hardy-Weinberg equilibrium on the inference of paternity, we ran three replicates of a seventh model (named model 5ib), using the same analysis parameters as those of model 5, except that inbreeding was selected as mating system. We used a paired *t*-test to compare the mean number of fathers per FMS set inferred by model 5ib over its three runs to the mean number inferred over the three runs of model 5.

Allele frequency analysis and exclusion probability

The mothers of the 24 broods and the 24 fathers inferred by Colony in the first replicate of model 5 were used as a random sample from the population of crocodiles in the pool and an allele frequency analysis was done on their inferred genotypes, using Cervus 3.0.7 (Kalinowski et al., 2007). Where the first replicate of model 5 inferred that two (it was never more than two) sets of FMSs had the same mother we allocated the second set to a different mother, with the second mother having the same genotypes as the first. In this way each of the 24 sets had its own mother, according to the biological reality. The inferred fathers assigned to these 24 sets of FMSs were kept as Colony assigned them. Jamieson and Taylor (1997) reported an equation by which the allele frequencies are used to calculate the probability of excluding a random male that is unrelated to the true father from being the father of an offspring if only the genotype of the offspring is known. For brevity we henceforth refer to this probability as the probability of exclusion or PE. Cervus uses this equation by Jamieson and Taylor and reports the probability of not excluding a random male that is unrelated to the father if only the genotype of the FMS is known, denoting it as NE-1P. We calculated the probability of excluding a random male that is unrelated to the true father of a full-sib group from being the father of the group for full-sib groups from one to five, using Equation (6) in Wang (2007). For brevity we henceforth refer to these probabilities of exclusion as PE*n*, where *n* is the number of full-sibs in the groups that were genotyped.

Results

Frequency of amplification failure

The supplementary file named "Crocodile_genotypes.csv" shows the genotypes at all loci of the 110 FMSs.

The control sample yielded a complete genotype at each locus, confirming that the PCR, electrophoresis and genotyping performed as expected. DNA failed to amplify in 91 (7.5%) of the 1210 possible genotypes. Amplification failed at all loci in two FMSs, at 9–10 loci in three, at 7–8 loci in three, at six loci in one, at four loci in one, at 2–3 loci in two and at one locus in three of the FMSs. Amplification occurred at all loci in the remaining 95 (86.4%) of the FMSs. Once it has hatched, a hatchling may crawl over other eggs that have hatched, thereby posing a risk of contaminating the DNA of the FMSs of such eggs. Such contamination may cause an FMS to yield more than two alleles at a locus. The number of hatchlings in the 25 broods from which the FMS sets were genotyped varied from seven to 39 (mean 23). Yet, no FMS yielded more than two alleles at any locus, suggesting that the DNA of no FMS was contaminated by that of another individual.

Having used only five polymorphic microsatellite loci, Davis et al. (2001) identified American alligator hatchlings from the same brood with the same multilocus genotype. In the current study, the genotypes of FMS 21 and FMS 23 of brood 098 were identical on all loci.

Sets of FMSs with more than one sire identified using the purpose-written programme Al3p1c

Al3p1c identified approximately half of the 25 sets of FMSs as having more than one father. Only two of the 11 loci (CpP309 and CpP4006) showed no indication that any set had more than two parents. The nine loci where FMSs from at least one set yielded at least three paternal alleles appear in Table 2. Considering apparently homozygous loci as truly homozygous and rare alleles as true alleles and not due to error, 13 of the 25 sets of FMSs had at least one locus (1–5, mean 2.0, SD 1.2) where the FMSs showed the presence of at least two fathers (Table 2). From this follows that the minimum number of full-sib groups determined with Al3p1c was 38.

Allelic dropout and spurious rare alleles may inflate the proportion of FMS sets inferred to have more than one father. Even when apparently homozygous loci were all deemed spurious due to the dropout of one allele and rare alleles were considered erroneous, 11 of the 25 FMS sets had at least one (mean 1.33, SD 0.65) locus indicating that the brood had at least two fathers.

The minimum number of fathers in a brood as determined with Gerud2.0

Initially, Gerud2.0 indicated that 13 FMS sets had one father and 11 had at least two. Among the 13 FMS sets with one father was FMS set 079 for which Al3p1c indicated that it had at least two fathers (Table 2). Apart from FMS set 079, the other 12 sets of FMSs with one father according to Gerud2.0 were the same as those determined to have one father using Al3p1c. When loci CpP218 and CpP4308 – which were the loci where Al3p1c indicated that three paternal alleles were present in FMS set 079 (Table 2) – were included in the analysis of FMS set 079 with Gerud2.0, Gerud2.0 also indicated that FMS set 079 had at least two fathers. Apart from FMS set 132 – which was not analysed with Gerud2.0 because it had only two FMSs with genotypes at the three loci used with Gerud2.0 – all 12 sets that Al3p1c identified as having had more than one father (Table 2) also had a minimum of two fathers according to Gerud2.0. Finally, therefore, Gerud2.0 indicated that 12

FMS sets had one father and 12 had at least two. Gerud2.0 indicated that the 24 FMS sets analysed with it contained at least 36 full-sib groups.

Overview of the results obtained with the original six models using Colony

In models 1–5 and model MinCol5, which were all based on a mating system without inbreeding, Colony inferred substantially fewer mothers than the 24 there actually were (Table 3). Colony did not split any of the true 24 maternal sib groups to assign the partial sib groups to different inferred mothers but it assigned two or more whole maternal sib groups to the same inferred mother. This underestimation of the number of mothers was especially severe in model 1 and model MinCol5. The number of mothers inferred with the three replicates of model 5 was closest to the true number of 24 (Table 3). All six models without inbreeding inferred that at least 17 (70%) of the 24 sets of FMSs had more than one father (Table 3).

The results obtained with model 5ib compared to those of model 5 demonstrate the effect that inbreeding and deviation from Hardy-Weinberg equilibrium may have had on the inference of paternity. Averaged over its three runs, model 5ib inferred 27.3 fathers over all 24 FMS sets and Model 5 inferred 25.3. Model 5ib inferred an average of 2.60 (SD 1.04, n = 24) fathers per FMS set over its three runs, which is unlikely to have differed from the 2.65 (SD 1.06. n = 24) over the three runs of model 5 (P = 0.1). Colony inferred similar numbers of mothers, numbers of full-sib groups and numbers of FMS sets with more than one father using model 5ib than it did using model 5 (Table 3). Except for the results shown in Table 3, all further results refer to those obtained in the first replicate of model 5.

TABLE 2: Loci where the programme Al3p1c indicated that 25 sets of 4–6 foetal membrane specimens (total 109) from each of 25 Nile crocodile (*Crocodylus niloticus*) broods had at least three paternal alleles, showing that the brood had at least two sires

		Loci								
Brood number	**FMS (n)**	**CpDi06**	**CpDi21**	**CpDi28**	**CpDi42**	**CpP218**	**CpP314**	**CpP4308**	**CpP4311**	**CpP801**
053	4[a]	3[b]								
061	5				4	3			3	
079	4					3		3		
084	4	3			4					
094	3			3	3					3
097	6									3
126	4	3				3	3	3		4
127	4				4					4
128	5	3								
132	4									3
138	4	4								
146	4	3								
149	5	4	3		4					

[a] Figures in this column show the number of hatched eggs from which foetal membrane specimens (FMSs) were genotyped. [b] Figures below and to the right of this one show the number of paternal alleles among the FMSs from the brood that were genotyped.

TABLE 3: Numbers of fathers and mothers that Colony inferred in a sample of 98 foetal membrane specimens (FMSs) consisting of a set of 3–6 FMSs from each of 24 Nile crocodile (*Crocodylus niloticus*) broods

Model	**Replicate**	**Number of fathers**	**Sets of FMSs with more than one father**	**Number of mothers**	**Number of full-sib groups**
Model 1	1	19	20	14	59
Model 2	1	23	21	18	60
Model 2	2	23	18	17	57
Model 2	3	23	20	17	63
Model 3	1	27	19	16	57
Model 3	2	22	18	15	51
Model 3	3	26	18	16	56
Model 4	1	24	19	17	62
Model 4	2	25	19	17	60
Model 4	3	23	21	18	61
Model 5	1	24	19	20	65
Model 5	2	27	18	19	62
Model 5	3	25	20	20	62
MinCol5	1	20	17	14	56
MinCol5	2	20	18	15	58
MinCol5	3	19	18	15	57
Model 5ib	1	27	20	20	62
Model 5ib	2	30	21	20	62
Model 5ib	3	25	18	19	60

Results obtained with the first replicate of model 5

Allele frequency analysis and exclusion probability

The genotypes of the 48 parents of the 98 FMSs, as inferred with the first run of model 5, had all the alleles that were present among all 110 genotyped FMSs, except for one allele at locus CpDi21. The results of the frequency analysis done on the alleles of the 48 inferred parents appear in Table 4. Three to 20 (mean 6.8, SD 4.75) alleles were found per locus. The mean observed and expected heterozygosity were 0.54 and 0.64. The observed heterozygosity was lower than expected in all but one of the loci. The genotypes at four loci were unlikely ($P < 0.05$) to have been in Hardy-Weinberg equilibrium. The combined probability of exclusion over all 11 loci for an inferred full-sib group of one individual was 0.963295 and for those consisting of more than one individual were above 0.990 (Table 4). The weighted average for the probability of exclusion over all 65 full-sib groups, according to the size of each full-sib group, was 0.972421.

Broods with more than one inferred father among the genotyped FMSs

Polyandry was common. Only five of the 24 sets of FMSs had one father, whereas two thirds of the sets had three or more (Figure 1a). On average, an FMS set had 2.71 (SD 1.08) fathers. Five of the 19 FMS sets with more than one father had a number of fathers equal to the number of FMSs included in the assessment with Colony, suggesting that more fathers may have been identified had more FMSs been included in those sets. The polyandry resulted in a large number of small full-sib groups (mean 1.51 full sibs per group, SD 0.99, n = 65). Forty five (69%) of the full-sib groups consisted of one offspring only (Figure 1b).

Polygyny was also common. Nineteen of the 24 fathers had offspring in more than one FMS set with a third of the fathers having had offspring in 4–5 FMS sets (Figure 1c). On average, a father had offspring in 2.71 (SD 1.23) FMS sets.

The mating success of fathers varied (Figure 1d). Fathers sired from one to nine offspring each, with a mean of 4.08 (SD 2.50, n = 24 fathers). Seven of the 24 fathers (29%) sired from six to nine offspring each, accounting for 52% of the offspring.

Discussion

The first aim of this study was to determine whether the foetal membranes in recently hatched Nile crocodile (*Crocodylus niloticus*) eggs provide DNA that amplifies successfully. Over all 110 FMSs, 7.5% of the possible 1210 genotypes were not obtained, due to DNA amplification failure. Amplification failure was limited to 15 FMSs, with half to all of the 11 loci failing to amplify in nine of them, suggesting that insufficient quality or quantity of template may have contributed to the amplification failure (Gagneux et al., 1997). Amplification occurred at all loci in 95 (86.4%) of the FMSs, suggesting that foetal membranes provide a useful non-invasive source of DNA in the Nile crocodile. No more than two alleles were found at any locus in an individual, suggesting that it is unlikely that the foetal membranes of one egg was contaminated with DNA from a hatchling that hatched from another egg in the same box.

The second aim of this study was to determine whether a Nile crocodile brood from a communal breeding pond on a commercial farm may have more than one father. Colony inferred that 19 FMS sets had more than one father, which is substantially more than the 12 identified with Al3p1c and Gerud2.0 in the same 24 FMS sets. Our findings agree with those of Sefc et al. (2008) who found that Colony inferred a higher proportion of broods with multiple fathers than the single locus minimum method and Gerud2.0. Nevertheless, even Al3p1c, which is the most conservative method of

TABLE 4: Summary of an allele frequency analysis at each locus in the 48 inferred parents of sets of 3–6 offspring (foetal membrane specimens) from 24 broods from the only breeding pond on a commercial Nile crocodile (*Crocodylus niloticus*) farm

Locus	K[a]	Ho[b]	He[c]	HWE[d]	Fnull[e]	FSG1[f]	FSG2[g]	FSG3[h]	FSG4[i]	FSG5[j]
CpDi06	20	0.708	0.796	0.4004	0.0582	0.453661	0.559054	0.634461	0.678396	0.702336
CpDi21	5	0.417	0.468	0.9321	0.0391	0.109199	0.15306	0.184045	0.203755	0.216128
CpDi28	6	0.688	0.634	0.2275	−0.0671	0.218805	0.297909	0.354885	0.389832	0.410497
CpDi42	10	0.583	0.816	0.001	0.1545	0.453054	0.573843	0.655514	0.702736	0.728744
CpP218	5	0.396	0.524	0.006	0.1557	0.146453	0.200184	0.24249	0.268944	0.284576
CpP309	4	0.417	0.58	0.0330	0.1617	0.166903	0.235184	0.279449	0.306904	0.324057
CpP314	6	0.604	0.663	0.0141	0.0659	0.2486	0.335032	0.39792	0.436164	0.458399
CpP4006	3	0.354	0.504	0.0866	0.1707	0.124242	0.174878	0.209234	0.231016	0.244815
CpP4308	5	0.563	0.659	0.4894	0.0753	0.251728	0.337022	0.400856	0.439602	0.461831
CpP4311	4	0.604	0.628	0.9007	0.0141	0.212153	0.290153	0.34668	0.381446	0.402046
CpP801	7	0.583	0.757	0.1035	0.1310	0.36012	0.47095	0.549214	0.595477	0.621455
All loci combined						0.963295	0.990663	0.996961	0.998569	0.999101

[a] Number of alleles found at each locus. [b] Observed heterozygosity. [c] Expected heterozygosity. [d] The probability (without Bonferroni correction) that a deviation as large or larger of the observed genotype frequencies from those expected under Hardy-Weinberg equilibrium occurred by chance. [e] The estimated frequency of null alleles. [f–j] The probability that a random male in the population that is unrelated to the true father of a full-sib group will be excluded if one ([f]), two ([g]), three ([h]), four ([i]) or five ([j]) members of a full-sib group were genotyped.

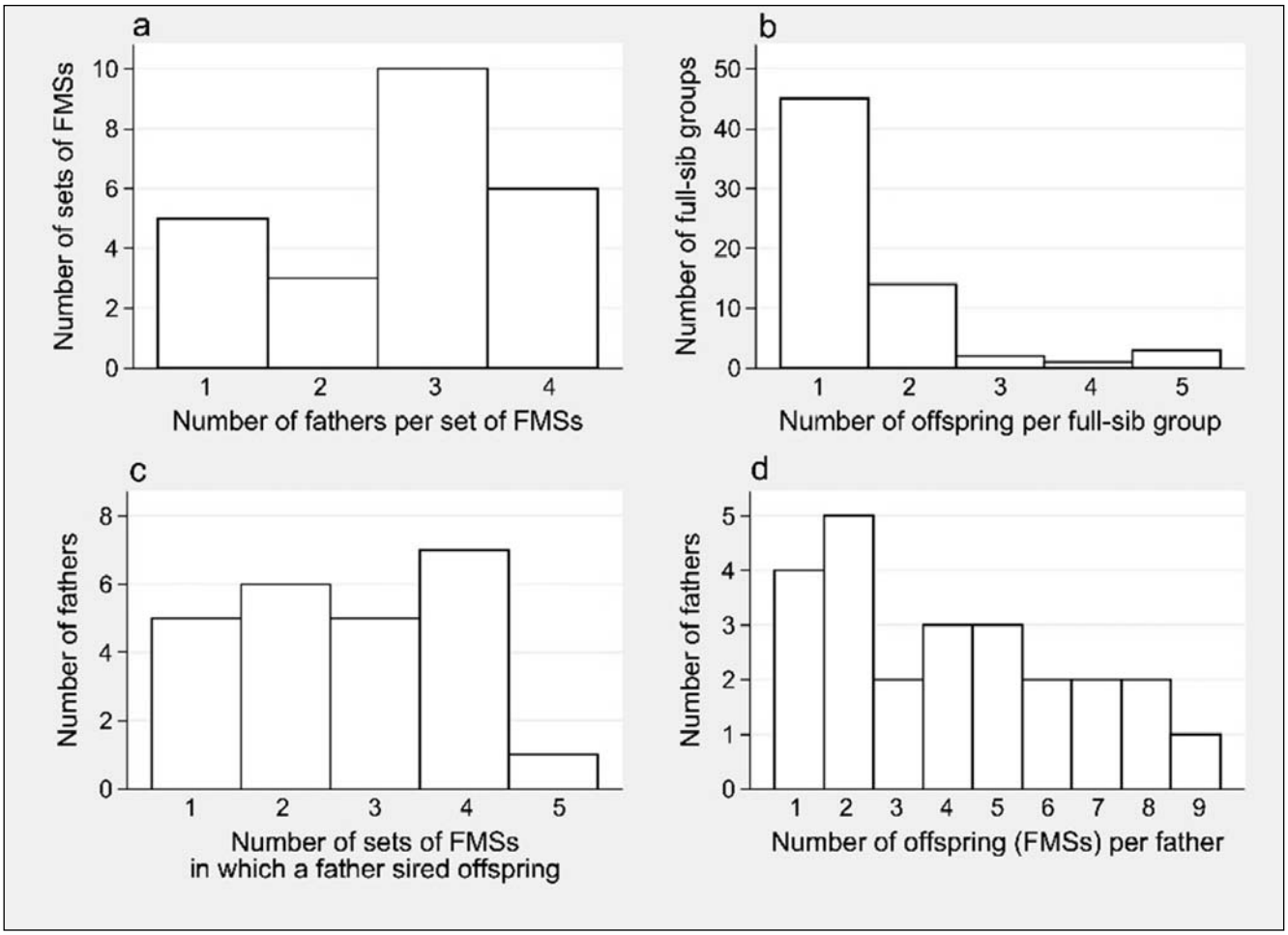

FIGURE 1: Extent of polyandry (a), distribution of full-sib group sizes (b), extent of polygyny (c) and mating success of fathers (d), where fathers and full-sib groups were inferred from the genotypes of sets 3 to 6 foetal membrane specimens (FMSs) from each of 24 Nile crocodile (*Crocodylus niloticus*) broods

identifying polyandry used in this study, revealed that some Nile crocodile broods had more than one father.

Apart from inferring which FMS sets have more than one father, Colony also inferred the most likely full-sib structure and the most likely number of sires in each set of FMSs. The accuracy of these inferences depends on the probability of Colony excluding any male in the population that is unrelated to the real father from being the father. Considering the genotypes of all offspring in each of the 65 inferred full-sib groups (Wang, 2007), the probability of exclusion was 0.9724. Thus, there is a 2.8% probability that Colony wrongly assigned a father to an FMS. The assignment of fathers is therefore expected to be correct for the large majority of FMSs.

Although the high probability of exclusion suggests that Colony mostly assigned fathers correctly to the 3–6 FMSs in the 24 FMS sets, the broods from which the FMSs were sampled were generally large, with an average of 23 hatchlings. Having genotyped an average of 29.2, 19.6 and 18.8 hatchlings per brood in the American alligator (Davis et al., 2001; Lance et al., 2009) and in Morelet's crocodile (McVay et al., 2008) the authors showed that the proportions of hatchlings sired by the primary sire compared to other sires in broods with multiple paternity was highly skewed. In the light of this it is likely that the number of fathers per brood used in the current study was higher than the numbers inferred per set of 4.1 (average) FMSs. The low number of FMSs from a brood that were genotyped does not permit any inference about the number of fathers in the whole brood.

Sefc et al. (2008) showed that MinCol (minimum Colony as they referred to it) correctly inferred the number of fathers per brood if there were two or four fathers per brood and underestimated the number by one in 35% of broods with five fathers, whereas Colony commonly overestimated the true number of fathers per brood. Using broods of 25 consisting of four small full-sib groups of three and one of 13 Sefc and Koblmüller (2009) showed that MinCol (MIN as they referred to it) underestimated the number of fathers per brood but that Colony on average estimated the number of fathers per brood to within one of the actual number. The findings of the current study that the MinCol model inferred 56–58 full-sib groups over all 24 sets of FMSs and Colony (first replicate of model 5) slightly more at 65, are in accordance with the trends identified by Sefc et al. (2008)

and Sefc and Koblmüller (2009). The 38 full-sib groups identified over all 25 sets of FMSs using Al3p1c are substantially fewer than those inferred with MinCol and Colony. Likewise, Sefc et al. (2008) showed that the single locus minimum method inferred lower numbers of sires per brood than the MinCol method, which inferred lower numbers of fathers per brood than Colony.

Davis et al. (2001) showed that American alligator males may sire offspring in more than one brood during the same season. Similarly, the current study shows that Nile crocodile males on a commercial farm sire offspring in more than one brood, with one third of the 24 inferred fathers having sired offspring in at least three broods.

The alleles of the parents inferred with model 5 are unlikely ($P < 0.05$) to have been in Hardy-Weinberg equilibrium in four of the 11 loci. Accounting for the effect of inbreeding and deviation from Hardy-Weinberg equilibrium with model 5ib did not result in a material difference to the inferred paternity obtained with model 5.

Further research is required in which more offspring per brood are genotyped to more precisely determine the extent of polyandry and polygyny in breeding ponds on commercial farms. This study suggests a need for research to determine whether phenotypic characteristics of interest to commercial farming such as growth, failure to thrive and mortality of hatchlings (Brien et al., 2014) are related to differences in paternity.

Population density, migration and territorial behaviour of Nile crocodiles in the wild may differ from what it is on commercial farms. The extent of polyandry, polygyny, full-sib sizes and the mating success of males in wild populations may therefore differ from those reported in the current study for a commercial farm. These phenomena need to be studied in wild Nile crocodile populations as well.

In the wild, a Nile crocodile mother opens the nest once hatching occurs, collects the hatchlings in her mouth and carries them to the water (Combrink et al., 2016). Modern technology should make it possible to monitor wild nests and note when the mother removes the hatchlings from a nest. This would allow one to harvest the foetal membranes from the egg remains soon after and determine whether they yield uncontaminated genotypes of the hatchlings. If this proves successful, it would provide a non-invasive model by which to study mating patterns in Nile crocodiles in the wild.

Conclusion

Foetal membranes from hatched Nile crocodile eggs are a suitable source of DNA for genetic profiling of hatchlings. Determining the genotype of four to five offspring per clutch (six in one clutch) demonstrated that at least half and likely more than 19 of 24 Nile crocodile broods from a communal breeding pond on a commercial farm had more than one father and that males commonly sire offspring in more than one brood.

Author contributions

CH selected the panel of STR markers, oversaw the procedures in the Veterinary Genetics Laboratory and confirmed the identification of alleles. JGM and JON collected the foetal membrane specimens. JAN wrote the computer algorithms for Al3p1c. JON did the data analysis and wrote the manuscript.

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