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SHORT COMMUNICATIONS

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Detection by Microsatellite Analysis of Early Embryonic Mortality in an Alligator Population in Florida

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ABSTRACT: In the 1980s, alligators (*Alligator mississippiensis*) of Lake Apopka (Florida, USA) underwent a population decline associated with decreased egg viability, effects that have been associated with endocrine-disrupting, persistent organochlorine pesticides. It is currently unknown whether the decreased egg viability is due to fertilization failure or early embryonic death. Therefore, we conducted a preliminary study to evaluate the use of microsatellite DNA loci to determine the fertilization status of nonviable eggs. Using microsatellite analysis, we compared genotypes from blastodisks and embryos with the genotypes from females trapped at the nest. Four of five nonviable egg samples tested yielded evidence of fertilization. No evidence of unfertilized eggs was obtained, but amplifiable DNA could not be obtained from one entirely nonviable clutch. Thus, we demonstrate that early embryonic mortality in alligators can be detected by microsatellite analysis, but also suggest substantial effort is needed to improve the recovery of DNA and amplification of alligator microsatellite loci.

Key words: American alligator, *Alligator mississippiensis*, blastodisk, DNA, fertilization, genetics.

The population decline among American alligators of Lake Apopka (Florida, USA) has figured prominently in concerns about the potential health effects of endocrine disrupting contaminants (Guillette et al., 1994; Semenza et al., 1997), which have been associated with reproductive tract and endocrine changes (Gross et al., 1994; Crain et al., 1997) and with environmental contamination by persistent organochlorine pesticides (Schelske and Brezonik, 1992; Masson, 1995). Wildlife biologists observed that proportions of viable eggs among clutches collected during the 1980s was decreased to as low as 5%, whereas the number of nests and number of eggs per nest remained unchanged (Woodward et al., 1993). Also observed were increased hatchling mortality rates and a severe decline in the number of juveniles.

The Lake Apopka alligator population remains below that expected based on historical data and in comparison with other central Florida lakes (Woodward, pers. comm.). Reproductive success is still poor and continues to result from losses at different early life stages (Giroux, 1998). However, the nature of these losses and their causes are still unknown, and their relationship to pesticide exposure is unclear. Reproductive success is significantly better for clutches from regions of the lake where significantly higher concentrations of DDE and DDT occur in eggs and maternal fat (Giroux, 1998), and experimental *in ovo* exposures have not caused decreased embryonic or hatchling viability (Gross, 1998). Furthermore, decreased egg viability recently has been observed among alligators of other, less contaminated lakes, such as Lake Griffin (Florida, USA).

Determining the roles of fertilization failure and early embryonic mortality in decreased egg viability is an important step

TABLE 1. PCR primer characteristics used to amplify microsatellite loci from American alligators.

Locus	Primer sequences[a]	Label[b]	PCR conc.[c]
Amiμ-6a	TTCTTTCCCAGATACACACTT	TET	0.4 μM
Amiμ-6b	AGTAGAAGGGGACAGGTTATT		0.4 μM
Amiμ-8a	CCTGGCCTAGATGTAACCTTC	FAM	0.2 μM
Amiμ-8b	AGGAGGAGTGTGTTATTTCTG		0.2 μM
Amiμ-15a	CACGTACAAATCCATGCTTTC	HEX	0.4 μM
Amiμ-15b	GGGAGGGTTCAGTAAGAGACA		0.4 μM
Amiμ-17a	GCTGACCTTGGTTGGAAACTCTA	FAM	1.0 μM
Amiμ-17b	CCTGTTCTTGCATAAANCTGATA		1.0 μM
Amiμ-18a	ATCTCCGAGGGGAAAAATACA	FAM	0.4 μM
Amiμ-18b	AATAGATGGAGTGATGTTATAGTCAG		0.4 μM

[a] Primer sequences are 5′ to 3′.
[b] ABI fluorescent label used for each primer pair.
[c] Concentration of primer used in multiplex amplifications.

in investigating effects of contaminant exposure on reproductive success. For example, fertilization failure could result from defective viability, structure, or function of sperm or oocytes; anatomical or physiologic alterations of male or female reproductive systems; or behavioral changes that decrease mating success, whereas early embryonic mortality could be the result of direct embryotoxic effects or of alterations in egg structure or composition due to oviduct dysfunction.

Previous studies have shown that nonviable eggs (those that do not develop an opaque circumferential band indicating chorioallantoic membrane development) commonly contain a 1–4 mm blastodisk composed of 80 to 200 cells (Masson, 1995). This non-viable blastodisk could be due to abortive parthenogenesis, which commonly occurs in poultry (Mittwoch, 1978; Harada and Buss, 1981), or it could indicate early embryonic mortality. Because parthenogenetically derived cells usually have 2N chromosome numbers due to automixis or apomixes, simply counting the chromosomes will not elucidate fertilization status. The fertilization status of such blastodisks clearly could be established by demonstration of nonma-

TABLE 2. Results of the microsatellite fragment analysis of females (F) and their respective nonviable (NV) and viable (V) eggs. Alleles of the same length as the maternal loci are shared indicating the maternal contribution; the second differing allele (in italics) indicates the paternal contribution. Similar alleles shared between nonviable and viable eggs from clutch 10 demonstrate fertilization by the same male. Dashed lines (–) indicate no fluorescent peaks above background (alleles) were obtained.

		Microstatellite primer loci/Fragment length			
Clutch number	F/NV/V	8	15	17	18
	F	—	159/159	245/245	192/196
	NV	—	159/*163*	237/245	—
	NV	—	159/*163*	—	—
	V	132/134	159/*163*	237/245	192/*194*
	F	134/134	153/161	237/253	188/188
	NV	—	—	—	—
134/134	V	134/*136*	153/161	*245*/253	188/*192*
—	F	134/138	149/163	249/265	188/190
—	NV	—	149/*161*	—	—
—	NV	—	149/*149*	*261*/265	188/*192*
—	F	130/132	163/159	237/257	188/192
—	NV (all)	—-	—	—	—

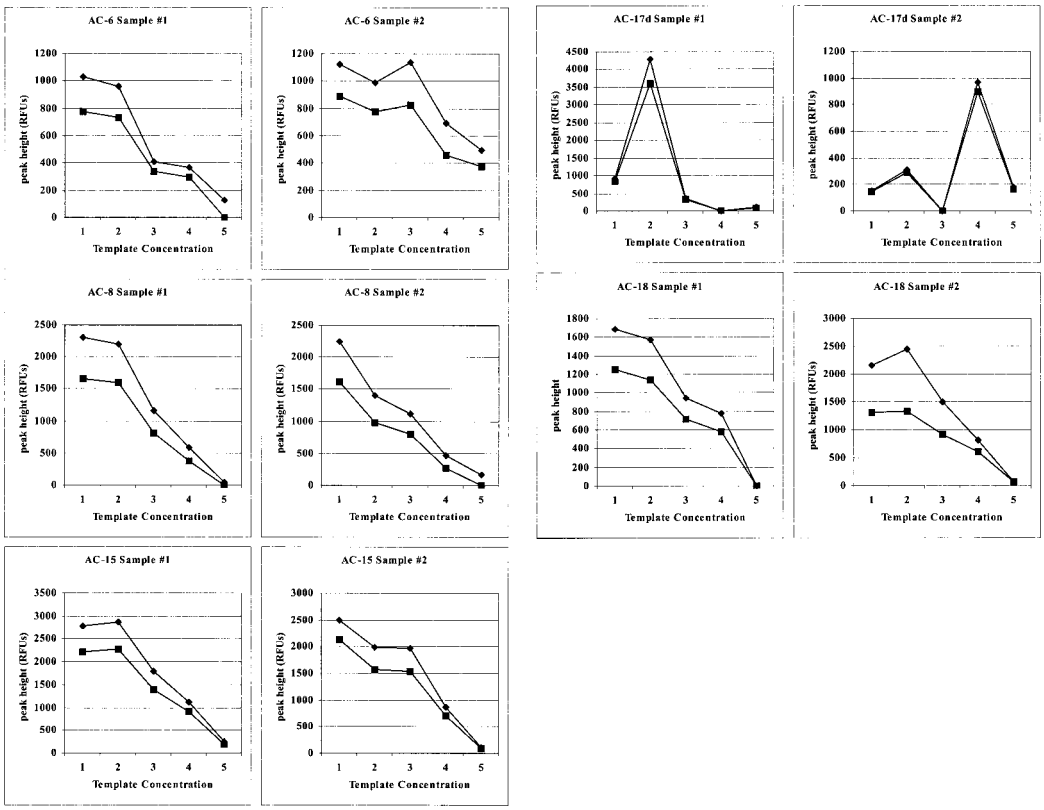

Figure 1. Fluorescent signal of alligator microsatellite alleles using 10-fold serially diluted alligator DNA as template. Sample 1 concentration 1 was 135 ng/μl, sample 2 concentration 1 was 170 ng/μl. All loci are heterozygous. The shorter allele is plotted with diamonds.

ternal genetic material (i.e., heterozygosity for one allele of nonmaternal origin). Thus it would be ideal to identify genotypes with high levels of allelic diversity, so that most matings would produce heterozygous offspring with unique alleles for each parent. One method of obtaining such genotypes is through analysis of microsatellite DNA loci. Microsatellites are small tandem arrays of simple DNA sequences with core repeat units of 1 to 5 base pairs (bp) and total array sizes of less than a few hundred bp (Charlesworth et al., 1994). Polymorphisms result from differences in the number of repeats; thus, alleles are distinguished according to size (Tautz, 1989). Eleven microsatellite loci have been characterized in alligators (Davis et al., 2000; Glenn et al., 1998).

We conducted a preliminary study to evaluate use of microsatellite analysis to determine fertilization status of alligator eggs, testing for presence of maternal and non-maternal alleles at five microsatellite loci (Table 1) in blastodisks and embryos from nonviable and viable eggs. Egg clutches and blood samples were collected in mid to late June, 1999. Nest sites were designated with an identifier number, and mound site characteristics and clutch sizes were recorded. Eggs were collected into plastic pans lined with natural substrate. A snare trap was set at each site and checked daily for the presence of a female. Twenty ml of blood was collected from the occipital sinus of captured females, transferred to sodium heparin tubes, and kept on ice pending delivery to the laboratory. Eggs were candled with a commercial candler (Lyon Electric Company, Chula Vista, California, USA) and numbers of viable and nonviable eggs were recorded. Nonviable

eggs were cleaned with a detergent solution, opened with scissors, and the contents decanted into a plastic weighing dish for blastodisk identification. Blastodisks were removed, transferred to 1.5 ml microcentrifuge tubes and frozen at −80 C. For clutches containing viable eggs, one such egg was sacrificed for developmental staging and as a positive control for microsatellite analysis (Ferguson, 1985). Heparinized blood was frozen in 2 ml portions at −80 C.

Blood samples and egg clutches were obtained from nine alligators. One clutch contained only viable eggs, seven clutches contained one to five nonviable eggs, and one clutch contained no viable eggs. In four clutches, nonviable eggs were putrefied and contained no identifiable blastodisks; these clutches were excluded from the study. The four clutches studied were: clutches 10 (50 eggs with 2 nonviable), 27 (40 eggs with 2 nonviable), 30 (53 eggs with 4 nonviable), and 32 (44 eggs with none viable). Suspected blastodisks from five eggs from clutch 32 were processed routinely for histological examination.

DNA was extracted with a commercial kit (DNAeasy, Qiagen Inc., Valencia, California) according to the manufacturer's instructions. Briefly, 50 to 100 μl of thawed blood, or entire blastodisks, were treated with 80 to 100 μl of digestion buffer. The samples were centrifuged through a silica matrix, followed by two washes with a second buffer and elution into 1.5 ml microcentrifuge tubes. In the case of blastodisk samples, extracted DNA was concentrated by standard cold ethanol precipitation after addition of sodium acetate, then air dried overnight and dissolved in 50 μl of ultra pure water. DNA used to investigate the minimum amount of DNA necessary for amplication was purified according to the methods of Davis et al. (2000) and 10-fold serially diluted in TLE (10 mM Tris pH 8, 0.2 mM EDTA pH 8).

Extracted DNA was analyzed by PCR using primers for alligator microsatellite loci Amiμ6, Amiμ8, Amiμ15, Amiμ17, and Amiμ18 (Table 2) as previously described (Davis et al., 2000). For each locus, one primer was tagged with a fluorescent label (FAM, HEX, or TET). Multiplex amplifications were performed in a Techne Genius thermocycler (Techne, Inc., Princeton, New Jersey, USA), using 25 μl reaction volumes with a final concentrations of 250 mg/mL bovine serum albumin (BSA), 150 mM of each dNTP, 2 mM $MgCl_2$, either 1.25 units of Promega Taq polymerase or 1 unit AmpliTaq Gold (with appropriate buffer from the supplier), 0.2 to 1.0 mM of each primer, and 2–5 μL DNA. Thermal cycling with Promega Taq polymerase consisted of an initial denaturation step of 95 C for 3 min, followed by 30 cycles of 95 C for 30 sec, 55 C annealing for 15 sec, and 72 C extension for 30 sec, with a final extension step of 72 C for 5 min. The same cycling parameters were used with AmpliTaq Gold except that the initial denaturation step was continued for 10 min. Reaction mixtures were then tested for presence of amplicons by electrophoresis through a 1.2% agarose gel, staining with ethidium bromide, and UV transillumination. Amplicon sizes were determined on an ABI Prism 377 DNA Sequencer (PE Biosystems, Foster City, California). A cocktail of 3.0 μL dextran/formamide loading buffer, 0.65 μL Promega CXR fluorescent ladder, and 25 ng of PCR product was prepared, denatured by incubation at 95 C for 5 min, and placed on ice. Of this, 1.2 μl 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 over a 1.5 hr period. GeneScan and Genotyper software (PE Biosystems) were used to identify microsatellite alleles from the fluorescent chromatograms.

PCR products were consistently obtained with blood samples from female alligators and from viable embryos (see results in Table 2), except for Amiμ6. Amiμ6 was, therefore, excluded from subsequent analyses due to concerns about null alleles (cf. Davis et al., 2000). Generation of am-

plicons with samples from nonviable eggs was less consistent; however, results were recorded for at least one locus for two nonviable eggs from each of two clutches. For the three clutches containing mostly viable eggs (clutches 10, 27, and 30), amplicons were obtained most consistently for Amiμ15 (4 of 5 samples tested) and 17 (2 of 5 samples tested). No results for any locus were obtained from the nonviable eggs from clutch 32. However, all embryos and blastodisks from which results were obtained were heterozygous for at least one locus and shared at least one allele per locus with the female caught at the nest. In clutch 10, the blastodisks from the viable and nonviable eggs had the same alleles, and in clutch 30 the nonviable eggs had different paternal alleles.

Although the genotypes obtained form the nonviable eggs clearly showed a paternal contribution, the overall success of amplification was quite low. Therefore, we conducted amplification attempts on two sets of 10-fold serially diluted alligator DNA (Fig. 1). Extracted DNA was initially quantified using OD_{260} absorbance from which serial dilutions were obtained. Two μl of each dilution were then used in the PCR amplification. The two DNA samples used for this assay were heterozygous at all loci. These results clearly show that the shorter of the two alleles consistently yields a stronger signal than the larger allele. Scorable amplification (>100 RFU) of the lowest concentration of DNA (13.5 pg/μl and 17 pg/μl) was achieved only for locus Amiμ15, and sample 2 of locus Amiμ17 is clearly seen from these results. However, the inconsistency of amplification with Amiμ17 is also seen from these results. In general, a template concentration of 135 (270 total DNA) or 170 (340 total DNA) pg/μl was required for successful amplification. Because the blastodisks are expected to yield no more that about 20 pg/μl of DNA, it is easy to see why Amiμ15 was amplified most often from the nonviable eggs.

In some cases it will not be possible to obtain results with any method because of insufficient DNA. For example, a proportion of nonviable eggs might not contain sufficient material for analysis due to a lack of a viable oocyte, failure to undergo cell division, or very early death of the embryo followed by autolysis. This appeared to be the case with eggs from clutch 32. Although these eggs contained tiny masses interpreted as blastodisks, no cells were evident histologically. Thus, it may be appropriated to screen such samples for amplification of mitochondrial DNA, which can be obtained from significantly less DNA than nuclear loci (Glenn et al., 1999) and is highly conserved among alligators (T. C. Glenn, unpubl. data). If no amplification of mitochondrial DNA can be achieved, then it can be objectively concluded that no amplifiable DNA was recovered from that sample. After samples have been shown to contain amplifiable DNA, they can then be screened for microsatellites or other variable nuclear loci.

The methods used need further refinement, because DNA recovery and PCR conditions were not specifically re-optimized to achieve amplification from the limited quantities of potentially degraded DNA obtained from the nonviable eggs. Also, amplicons were not obtained with samples from nonviable eggs for Amiμ6 and Amiμ8. Amiμ6 failed to amplify in most blood samples, indicating potential problems with the conservation of the primer annealing sites (Glenn et al., 1998). Modifications to sample preparation, DNA purification, and PCR conditions would improve amplification efficiency as well as focusing on primer pairs with the highest sequence conservation (Glenn et al., 1998) and PCR amplification efficiency (Glenn et al., 1999). The development of additional microsatellite loci and detailed investigations to optimize PCR from limited amounts of alligator DNA will be necessary to improve the proportion of blastodisks which can be genotyped successfully.

In summary, we conclude that microsatellite analysis can be used to determine

fertilization status of nonviable alligator eggs and that early embryonic mortality occurs in nonviable eggs from viable clutches. Further studies to optimize the molecular genetic tools used and to assess the occurrence of early embryonic mortality are needed to develop a better understanding of causes of reproductive failure in alligators and its relationship to previously reported effects of endocrine-disrupting contaminants.

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