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Environmental Contamination and Toxicology

Use of Flow Cytometry to Screen for the Effects of Environmental Mutagens: Baseline DNA Values in Cottonmouth Snakes

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Since the late 1970's flow cytometry has been accepted as a fast and accurate technique for the quantification of nuclear DNA content (Deaven 1982). However, use of the technology has typically been confined to clinical settings due to the high cost of the instrumentation and the requirement for trained personnel. Flow cytometry recently has been applied to the study of genetic damage in wild populations. McBee and Bickham (1988) detected DNA damage in wild rodents inhabiting a dumpsite contaminated with petrochemicals, and Bickham et al. (1988) detected DNA aneuploidy in turtles found in seepage basins contaminated with radiation. Given these few studies, there is a need to establish a data base for the application of flow cytometry to environmental screening.

We chose to study the western cottonmouth snake, *Agkistrodon piscivorus leucostoma*, which is a common inhabitant of aquatic environments of the Southeastern United States. Accumulation of a variety of contaminants could potentially reach high levels in this predatory species given its generalized diet which includes fish, rodents, birds, turtles, and snakes (Burkett 1966). Our objectives in this study were: a) to examine the variation in nuclear DNA content within and among three populations of cottonmouth snakes, and b) to survey for evidence of possible genetic damage in individual snakes.

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MATERIALS AND METHODS

Animals used in the study were collected on the Ames Plantation which lies in the headwater basin of the North Fork Wolf River watershed. The watershed is located in the Mississippi Embayment section of the Gulf Coastal Plain physiographic province, approximately 80 km east of Memphis, Tennessee. Topography is characterized by rolling hills with broad flat floodplains. A layer of Pleistocene loess was deposited on top of fluvial materials. Erosion has removed much of the unconsolidated loess and fluvial deposits exposing more highly erodible sands in many upland areas. Recent alluvial deposits of sand, silt, clay, and fine gravel from upland sources are found in the floodplain areas of the watershed.

The study sites were three separate impoundments. Two sites, Hancock Pond and Pine Pond, are 40-year-old manmade basins located in highly-agricultural upland areas, with water supplies dependent almost entirely on runoff from adjacent agricultural areas. The third site is a beaver impoundment, May Farm Pond, with water supplies provided mainly from free-flowing springs, but also influenced by occasional flooding.

The snakes were killed and measured; within 5 min of death blood was collected from the tail into sodium citrate (from Becton-Dickinson vacutainer # 4606) with a syringe. The samples were kept refrigerated, and analyzed in randomized order within 24 hr as described elsewhere (Tiersch et al. 1989).

In brief, the DNA content of the cells was estimated by use of the EPICS V flow cytometer (Coulter Electronics, Hialeah FL) with argon-ion laser operated at a wavelength of 488 nm at 300 mW in light-stabilized mode. Filters included two 515 nm long pass, 560 nm dichroic, and a 570 nm long pass in front of the photomultiplier tube. The instrument was aligned using fluorescent beads and human leukocytes to obtain best resolution. The blood cells of snake and thawed blood cells of a male domestic chicken *Gallus gallus* were analyzed as a mixture in 0.5 mL of lysis-staining buffer containing 25 µL buffered RNase (1.0 mg/mL), 0.1% sodium citrate, 0.1% Triton X 100, and 25 µg propidium iodide. The fluorescence values of at least 40,000 stained nuclei from snake and chicken were digitized individually to yield distribution histograms (Fig 1). DNA content was calculated by dividing the fluorescence value of the snake cells (peak channel number) by the fluorescence value of the simultaneously-analyzed chicken cells (internal reference). Percent coefficient of variation (CV), which provides an estimate of heterogeneity of DNA values, was calculated from the fluorescence distributions of each sample.

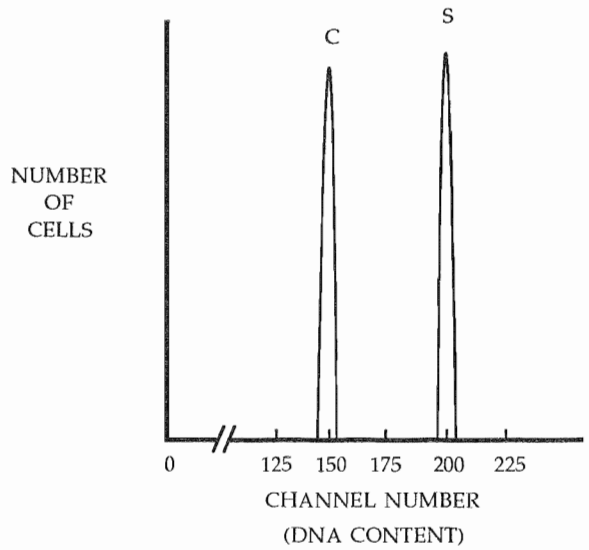

Figure 1. Graphic representation of a typical DNA fluorescence histogram showing chicken blood cells used as an internal reference (C) and blood cells of cottonmouth snake (S). Nuclear DNA content is directly proportional to fluorescence intensity, expressed here as channel numbers.

RESULTS AND DISCUSSION

Collection site and snake identification numbers, DNA content values, CV values, and snake lengths are listed in Table 1. On average, the snakes possessed 1.35 times the DNA content of chicken. DNA content values, expressed as fluorescence relative to chicken, did not differ significantly among locations (ANOVA, $P > 0.05$). The CV values for DNA content of individual snakes ranged from 1.98 to 2.63%, with a mean of 2.23 ± 0.17% (SD). These CV values were comparable to the values obtained for human leukocytes and repeated tests of the chicken internal reference. The CV of the snake values was not different among locations (ANOVA, $P > 0.05$). Snakes ranged from 38.0 to 114.5 cm in length; the mean length was 77.0 ± 17.5 cm. The length of individual snakes was not correlated with CV values ($r^2 = 0.00$).

Aneuploidy, as evidenced by subpopulations in the fluorescence distributions, and mosaicisms due to mixtures of ploidy levels within an individual were not detected. One specimen, not included in the statistical analyses above, had a fluorescence value 1.48 times the mean of the values for the other snakes. This apparently triploid value was confirmed by repeated analyses of the specimen.

Table 1. Identification number, nuclear DNA content, Percent coefficient of variation, and total body length of cottonmouth snakes collected at three sites.

Study site and snake number*	DNA content**	Percent CV***	Length (cm)
A1	1.34	2.37	67.5
A2	1.35	2.13	71.0
A3	1.28	2.12	68.5
A4	1.37	2.63	99.0
B1	1.36	2.08	86.0
B2	1.34	2.38	68.5
B3	1.35	2.18	61.0
B4	1.34	2.15	87.5
B5	1.36	2.60	66.0
B6	1.36	2.07	108.0
B7	1.34	2.13	71.0
B8	1.34	1.98	73.5
B9	1.36	2.32	114.5
B10	1.36	2.23	86.4
B11	1.36	2.14	96.5
B12	1.30	2.10	70.0
B13	1.36	2.15	82.5
B14	1.36	2.30	38.0
B15	2.01	2.31	53.5
B16	1.36	2.43	94.0
B17	1.34	2.31	80.0
C1	1.37	2.33	82.5
C2	1.36	2.01	58.5
C3	1.36	2.20	87.5
C4	1.36	2.18	59.5

*Study site A = Hancock Pond; B = May Farm Pond; C = Pine Pond. Numbers indicate snakes in order of capture.
**Nuclear DNA content of erythrocytes expressed as a ratio of the snake fluorescence value (channel number) divided by the fluorescence value of the chicken internal reference.
***Percent coefficient of variation.

Flow cytometry offers many advantages over conventional cytogenetic procedures. The technique requires minutes for an analysis of thousands of cells, compared to hours or days for the analysis of tens or rarely, hundreds of cells by cytogenetic analysis. Flow cytometry, however, should not be considered as a substitute for cytogenetic analysis because certain chromosomal anomalies, such as balanced translocations, are not detectable by measurement of DNA content.

A combination of the approaches would be useful, such as in employing flow cytometry for initial screening of large numbers of organisms in order to identify specimens for further cytogenetic study.

Flow cytometry does provide a sensitive and rapid technique for detection of anomalies in cellular DNA content (Otto et al. 1981; Fantes et al. 1983) and has been used to show aneuploidy in wild populations of turtles and rodents (Bickham et al. 1988; McBee and Bickham 1988). We found no evidence of aneuploidy or mosaicism in cottonmouth snakes, and there was no difference in DNA content among snakes from the three sites. We found a single normal-appearing snake (B15) that had a triploid (3C) level of DNA content. Although viable triploids are not uncommon in the lower vertebrates (Thorgaard and Gall 1979) triploidy can only arise through anomalies of fertilization (Ohno et al. 1963). Polyploidy has been induced experimentally in other animals by the application of thermal shock, or chemicals to fertilized eggs (e.g. Purdom 1983), but the etiology of the triploid in this study is uncertain.

Chromosomal abnormalities within subpopulations of cells can occur due to exposure to mutagens (Al-Sabti 1985; McBee and Bickham 1988). Small changes can be detected by flow cytometry as increases in the CV of cell populations (Otto et al. 1981). Bickham et al. (1988) reported a significant difference between mean CV values for turtles from radiation-contaminated pools (3.84%) and those from a control population (2.98%). Significantly higher CV values were also found in rodents from a petrochemical-contaminated site (3.94 ± 1.58%) compared to those (3.23 ± 0.30%) for the control population (McBee and Bickham 1988). The mean CV in our study (2.23 ± 0.17%) was lower than that reported in these other studies. The variability in CV values observed among studies is problematic as it may be due to factors such as species differences, differential exposure to mutagens or to differences in technique.

Bickham et al. (1988) found a significant positive correlation between plastron length and CV in turtles. They suggested that the increased CV in older, larger turtles could be the result of gradual accumulation of mutations in cell populations during the lifetime of the animals. We found no relation between CV and snake length, and no evidence of genetic damage in the snakes from the three sites studied. Although measurements of actual pesticide levels were not made, each pond in this study received agricultural runoff from actively-managed fields. It should be noted that cottonmouths may or may not be as susceptible to environmental mutagens as other snakes (Hall 1980). Stafford et al. (1976) reported higher levels of catabolic activity in the liver of *A. piscivorus* than in other snake species which may

reflect an enhanced ability to detoxify and excrete pesticides. It would be informative to compare the values we observed in these populations with values for snakes from other locations.

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