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Flow Cytometry for Monitoring Contaminant Exposure in Black-Crowned Night-Herons

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Abstract. The flow cytometry method (FCM) was employed to determine cellular DNA content of black-crowned night-heron (*Nycticorax nycticorax*) embryos and 10-day-old chicks collected at sites differing in types of chemical contamination. The coefficient of variation of DNA content (CV) in blood collected from embryos suggested cytogenetic damage at a site in Louisiana known to be contaminated with petroleum. Blood CV from chicks suggested genetic damage at a site in Texas also known to be contaminated with petroleum. Spleen CVs in chicks were significantly lower than respective means from the reference site. The CVs of chick blood and liver and spleen negatively correlated, suggesting recovery of spleen and liver cells after exposure to a clastogenic compound. Thus, the lower CVs may also have been indicative of genetic damage. Based on the findings of this study, FCM is a potential indicator of certain environmental contaminants in black-crowned night-herons.

The U.S. Fish and Wildlife Service is evaluating herons and egrets as indicators of estuarine contamination. These birds are good candidate indicators, because they are high in the food chain, bioaccumulate contaminants, have a wide geographic distribution, and nest in large colonies (Custer *et al.* 1991). Earlier monitoring focused on chemical evaluation of eggs and tissues and biochemical monitoring of cytochrome(s) P450 induction and related monooxygenase (MFO) activities in livers of three heron species (Custer *et al.* 1991).

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The flow cytometry method (FCM) is a rapid and simple quantification of cellular characteristics from suspended cells (Bickham 1990, Dallas and Evans 1990). Mice exposed to a mutagen in the laboratory had a significantly higher coefficient of variation (CV) in DNA content than controls, and the magnitude and duration of the increase were dose dependent (Otto *et al.* 1981).

Studies with FCM as an indicator of the effects of environmental mutagens on natural wildlife populations are limited. Spleens of white-footed mice (*Peromyscus leucopus*) from a site contaminated with a complex mixture of oil, grease, polychlorinated biphenyls, hexachlorobenzene, and several trace elements had a significantly higher CV in DNA content than mice from a control site (McBee and Bickham 1988). An earlier study of white-footed mice and cotton rats (*Sigmodon hispidus*) with standard karyology demonstrated significantly more chromosomal aberrations at the contaminated site (McBee *et al.* 1987). The CV in DNA content was higher in slider turtles (*Trachemys scripta*) inhabiting waters contaminated with low concentrations of radioactive and non-radioactive contaminants (Bickham *et al.* 1988) and radioactive contaminants alone (Lamb *et al.* 1991) than in conspecifics from reference sites. When game-farm mallard ducks (*Anas platyrhynchos*) were released near an abandoned nuclear-reactor cooling reservoir, increased variation in cellular DNA and DNA anuploidy was detected in red blood cells with flow cytometry as the ducks accumulated radioactive ^{137}Cs (George *et al.* 1991).

The objective was to evaluate the potential of FCM as a tool to monitor contaminants in black-crowned night-herons (*Nycticorax nycticorax*). Specifically, we wanted to determine whether genetic damage was greater in wild populations of this species inhabiting areas known to be contaminated with petroleum (Houston Ship Channel, TX; Sabine National Wildlife Refuge [NWR], LA), polychlorinated biphenyls (PCBs) (Green Bay, WI), or heavy metals (Baltimore Harbor, MD) than in wild populations in a reference location (Chincoteague, VA). If genetic damage was found, we also wanted to determine which developmental stages (eggs or chicks) and tissues (blood, liver, or spleen) were the most sensitive to contamination.

Table 1. Geometric mean coefficient of variation of tissues of black-crowned night-heron embryos and chicks collected at five locations in the United States in 1991

	Geometric mean coefficient of variation (n)				
	Blood		Liver		Spleen
Location	Embryo	Chick	Embryo	Chick	Chick
Reference (VA)	3.57 BC[a] (15)	3.44 B (10)	3.78 A (20)	4.01 AB (10)	4.89 A (10)
Baltimore, MD	3.45 C (8)	3.45 B (10)	3.73 A (13)	4.12 A (10)	5.20 A (10)
Green Bay, WI	3.87 B (12)	3.59 AB (10)	3.58 A (12)	3.76 AB (10)	4.11 B (10)
Houston, TX	3.83 B (14)	3.86 A (12)	3.72 A (14)	3.64 B (12)	3.98 B (11)
Sabine NWR, LA	4.37 A (7)	3.54 B (4)	3.70 A (7)	3.39 B (4)	4.23 B (4)

[a]Among site means not sharing the same letter are significantly different (1 way ANOVA, $P \leq 0.05$, Bonferroni multiple separation test)

Materials and Methods

Black-crowned night-heron pipped eggs and 10-day old chicks were collected live in 1991 from five colonies in the United States: Chincoteague Bay, Northhampton Co., VA (37°56'N, 75°25'W); Baltimore Harbor, Baltimore Co., MD (39°15'N, 76°35'W); Rabbit Island, Cameron Co., LA (29°50'N, 93°25'W); Alexander Island, Harris Co., TX (29°45'N, 95°05'W); and Lonetree Island, Brown Co., WI (44°30'N, 88°00'W).

Embryos and chicks were killed by decapitation within 2 h of collection. Immediately after death, blood, spleen, and liver samples were removed from the embryos and chicks. Blood was collected with heparinized capillary tubes and aspirated into a cryotube with freezing media (Ham's F10 media with 18% fetal calf serum and 10% glycerine). The cryotube was maintained at ambient temperature, rotated every 10–15 min for about 2 h to ensure penetration of the cells with glycerin, and subsequently frozen in liquid nitrogen. A section of liver (about 0.25 cm^3) and the entire spleen were removed, and each was placed in a cryotube and frozen in liquid nitrogen, usually within 5 min of death. Both samples were later transferred to an ultracold freezer and stored at −80°C until analysis.

Blocks of tissue (2–3 mm^2) or blood were excised from the frozen samples. Nuclear suspensions, prepared by the method of Vindelov *et al.* (1983), were treated with RNase for at least 30 min and stained with propidium iodide for 15 min. Propidium iodide intercalates between the bases (Deitch *et al.* 1982; Waring 1971) and stains RNA and DNA. Nuclear DNA content was analyzed on a Coulter Profile II flow cytometer by quantification of nuclear fluorescence. This instrument uses a 488 nm Argon laser and is equipped with a computer program that determines half-peak CV and mean, standard deviation, and number of cells for any particular region designated by the operator. Cells were simultaneously analyzed for nuclear fluorescence and side scatter. The latter is an indication of the amount of cytoplasm still attached to the nucleus. Therefore, cells with high levels of side scatter were excluded (gated) from the analysis to minimize variation from sample preparation. We counted 10,000 cells in the G1 peak and recorded mean and standard deviation of each individual. Full-peak percent CV of the gated G1 cell population, which is the mean divided by the standard deviation × 100, was calculated for each individual. Spleen samples from all embryos were not suitable for analysis. Cell proliferation was so great for this tissue that it was difficult to accurately differentiate G1 and S populations.

Because the variances of the gated CVs of blood of embryos (Bartlett's test, $B = 10.8$, $df = 4$, $0.025 < P < 0.05$) and spleens of chicks ($B = 12.3$, $df = 4$, $0.001 < P < 0.025$) were significantly different among location, all data were log-transformed prior to statistical analysis. Geometric means of the gated CV of blood, liver, and spleen (10-day old chicks only) were compared among locations by age with analysis of variance (ANOVA). The Bonferroni multiple comparison test was used to determine differences among means. Pearson correlation was used to compare gated CVs among tissues within age groups. Geometric means are presented in text and tables.

Results

The gated mean CV in DNA content of blood of embryo black-crowned night-herons was significantly greater at LA than at all other locations (Table 1, 1-way ANOVA; $F = 10.3$; $df = 4, 51$; $P \leq 0.0001$; Bonferroni multiple comparison method); gated mean CV in DNA content from WI and TX locations were greater than MD but did not differ significantly from the reference site. The gated mean CV in blood of 10-day old chicks was greater in TX than in MD, LA, and the reference site ($F = 5.38$; $df = 4, 41$; $P = 0.0014$).

The gated mean CV in livers of embryo black-crowned night-herons did not differ among locations (Table 1; $F = 1.04$; $df = 4, 61$; $P = 0.3946$). In contrast, the gated mean CV in livers of 10-day old chicks was greater at the MD than TX and LA sites ($F = 4.54$; $df = 4, 41$; $P = 0.004$). The gated mean CV in spleens of 10-day old chicks was greater at the VA and MD than WI, TX, and LA sites ($F = 27.06$; $df = 4, 40$; $P = 0.0001$).

Gated CV in liver and gated CV in blood in embryos did not correlate (Table 2). However, gated CVs in chick blood negatively correlated with gated CVs in liver, and spleen, and gated CVs in liver positively correlated with gated CVs in spleen.

Discussion

Based on significantly greater CVs in DNA content compared to the reference site, blood cells in embryos at the Sabine NWR, LA, and in chicks at Houston, TX, exhibited genetic

Table 2. Correlation matrix between the gated coefficient of variation of blood and liver from black-crowned night-heron embryos and blood, liver, and spleen from 10-day-old black-crowned night-heron chicks

	Pearson correlation coefficient | P | (n)		
	Embryos	Chicks	
	Liver CV	Liver CV	Spleen CV
Blood CV	−0.12	−0.378	−0.49
	0.24	0.0088	0.0007
	(59)	(47)	(44)
Liver CV	—	—	0.56
	—	—	0.0001
	—	—	(44)

damage. Based on significantly lower CVs, genetic damage occurred in spleen cells of black-crowned night-heron chicks at the LA and TX sites and in Green Bay, WI. No genetic damage was suggested based on the CV of liver samples. A simultaneous study of flow cytometry and standard karyology (*e.g.*, McBee *et al.* 1987, McBee and Bickham 1988) would confirm the relation between chromosome breaks and CV in DNA content.

It is suspected that the higher CV in blood and lower CV in spleen at the Sabine NWR and the Houston Ship Channel were in response to the petrochemical contamination at these sites. Genetic damage has been associated with exposure to petrochemical wastes (McBee *et al.* 1987, McBee and Bickham 1988). The Sabine NWR site is in Calcasieu Lake, which is heavily affected by oil and gas exploration and production, as well as by discharges associated with an extensive petrochemical industry located upstream on the Calcasieu River in the vicinity of Lake Charles. Sediments and biota on and adjacent to the refuge are moderately to heavily contaminated with petroleum hydrocarbons and trace elements (Schultz 1991). Wildlife in the Houston Ship Channel, Texas, are exposed to industrial and petroleum contaminants. Black-crowned night-heron chicks from the Houston Ship Channel had significantly higher concentration of aromatic hydrocarbons than chicks collected from Narragansett Bay, Rhode Island (Custer, in preparation). Double-crested cormorants (*Phalacrocorax auritus*) wintering in the Houston Ship channel accumulated polychlorinated styrenes and selected aromatic hydrocarbons in their tissues (King *et al.* 1987). Chlorostyrenes were also detected in heron and egret eggs and chicks from the Houston Ship Channel (Rice and Custer 1991).

The lower CV in night-heron spleen cells from Green Bay, Wisconsin, was unexpected. Earlier studies did not reveal a relation between genetic damage and PCB contamination in birds and Green Bay is a PCB-contaminated site. Organochlorine contaminants and chromosome aberrations were not associated in herring gull (*Larus argentatus*) eggs collected from five locations in the Great Lakes and one Atlantic Coast location (Ellenton *et al.* 1983); samples were not collected from Green Bay in that study. Perhaps a compound other than PCBs not present in locations investigated by Ellenton *et al.* (1983, 1985) was present in Green Bay and elicited a clastogenic response in the spleen cells of the night-heron chicks. Further research is needed to address this question.

The lowered CV of spleen cells may be indicative of recovery after exposure to a clastogenic compound. In some cases, tissues recover to a significantly lower CV than control values (Bickham *et al.* in press). The lower CV may result from a high turnover of damaged cells and replacement by a population of cells with less variability. The inverse correlation between blood CVs and liver and spleen CVs may be the result of a clastogenic effect from increased CV (blood) followed by recovery that lowers CV (liver and spleen).

The recovery hypothesis implies a single or brief exposure followed by a period without further exposure but recovery. This could occur if the adults switched to feeding less contaminated prey to their chicks. The suggested recovery of spleen CV could be tested by feeding chicks oil-dosed food and measuring CV in DNA content of blood and spleen at different intervals post-dosing.

Blood of night-heron embryos and chicks and spleen of chicks were more suitable than liver samples for the detection of a clastogenic response. Differences among tissues were also found in other studies. Spleen was the most sensitive tissue to assay in slider turtles, blood was the next most sensitive; and kidney and heart were relatively insensitive (Lamb *et al.* in press). In rats (*Rattus norvegicus*), spleen, bone marrow, and white blood cells were most sensitive to the chemical triethylenemelamine, a clastogenic drug; kidney, large intestine, and testis were less sensitive (Bickham *et al.* in press).

Embryos in this study may have been exposed to petroleum transferred to the egg externally by the adults (Albers 1991) or during egg development inside the female. To test whether external egg-oiling was responsible for the lower CV in DNA content, CVs from embryos of artificially and naturally incubated eggs from reference and petroleum-contaminated sites could be measured.

Based on the findings in this study, FCM is a potential indicator of environmental contaminants in black-crowned night-herons. Differences in CV in DNA content were found among locations and seem to be related to petrochemical wastes. The FCM assay is sensitive to genetic aberrations from petroleum (McBee and Bickham 1988; McBee *et al.* 1987) and radiation (Bickham *et al.* 1988, George *et al.* 1991) exposure and would complement the MFO assay, which is sensitive to TCDD and related compounds (Rattner *et al.* 1989). Finally, a major advantage of FCM is that if blood is used, it is nondestructive to the organism (Bickham 1993, Shugart 1993).

We envision that FCM could be used with a suite of other indicators to evaluate contaminant response in individuals or populations (Depledge 1989). If differences were encountered, more detailed research could then be directed in a cost effective manner, especially in allocation of costly chemical analyses. To further pursue FCM, controlled comparisons between clastogenic properties of chemicals in the environment have to be made.

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