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POTENTIAL NATURAL EXPOSURE OF MISSISSIPPI SANDHILL CRANES TO AFLATOXIN B_1

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ABSTRACT: A survey was conducted to determine if carcinogenic mycotoxins were present in foods consumed by Mississippi sandhill cranes (*Grus canadensis pulla*). Samples of field corn (*Zea mays*) (n = 111) and chufa (*Cyperus esculentus*) (n = 20), obtained in 1987, 1988 and 1989 on the Mississippi Sandhill Crane National Wildlife Refuge (MSCNWR) and nearby private lands were analyzed for aflatoxin B_1(AB_1), ochratoxin A and sterigmatocystin using thin layer chromatography. Chufa samples were negative for all three mycotoxins. Aflatoxin B_1 was found in corn at concentrations from 5 to 5,000 ppb; the other mycotoxins were not found in corn. Contaminated corn was found in 72% of all corn fields, but the proportion of contaminated fields was 57 to 100% for the 3-yr period. Contamination with AB_1 was greatest in corn obtained from the ground post-harvest. Overall, 32% of corn samples from the ground had levels ≥200 ppb with a mean of 427 ppb (range = 5 to 5,000 ppb) in contaminated fields. In 1989, mean AB_1 concentration in corn on the ground was 5 to 1138 ppb for individual fields. The concentration of AB_1 was ≤200 ppb in all corn samples from upright stalks. The study demonstrated that AB_1 is available to sandhill cranes and at levels that may pose a serious health threat.

Key words: Aflatoxin B_1, avian neoplasia, carcinogens, chufa, corn, *Grus canadensis pulla*, Mississippi sandhill crane, mycotoxins, neoplasia, tumors.

INTRODUCTION

The Mississippi sandhill crane (*Grus canadensis pulla*) is an endangered subspecies that occurs only on the Mississippi Sandhill Crane National Wildlife Refuge (MSCNWR) and contiguous areas in Jackson County, Mississippi (USA). Currently, the crane population is comprised of approximately 75 birds. Increase in crane numbers since 1975 has been accomplished though intensive management and restoration (Dewhurst, 1985) because natural recruitment was insufficient to ensure survival of the population.

Numerous factors jeopardize natural growth of the Mississippi sandhill crane population; recent attention was focused on the potential impact of disease. Necropsies of 16 free-ranging cranes that died from 1974 to 1990 revealed malignant neoplasms of unknown etiology in five (31%) birds. The neoplasms were considered to have caused death in four cranes. Biliary hyperplasia was noted in five birds with neoplasms and one crane without neoplasms (N. Thomas, pers. comm.).

Because the high prevalence of neoplasia was unparalleled in other populations of wild animals, an intensive investigation was begun to determine the etiology. Environmental toxins were considered to be the most important potential etiologic agents and thus, emphasis was placed on ruling out their involvement. Although the relationship between malignant neoplasms and biliary hyperplasia in Mississippi sandhill cranes is unknown, both lesions are produced by mycotoxins in other birds and in mammals (Wogan and Newberne, 1967; Norred, 1986). Among mycotoxins, aflatoxin B_1 (AB_1), ochratoxin A (OCA), and sterigmatocystin (SMC) are carcinogenic (Wogan, 1977; Richard et al., 1989). Mycotoxins, especially AB_1, occur in corn (*Zea mays*) and other field crops in the southeastern United States where climatic conditions contribute to crop stress and lead to growth of toxin-producing molds (Lillehoj and Hesseltine, 1977). Corn is planted as a winter source for cranes on the MSCNWR; and cranes utilize waste corn from small privately owned farms adja-

cent to the MSCNWR (Dewhurst, 1985). Chufa (*Cyperus esculentus*) also is planted for food and readily utilized by cranes (Dewhurst, 1985) and could be a source of mycotoxins.

The objectives of this study were to (1) determine if AB_1, OCA, and SMC were present in field corn and chufa on the MSCNWR and surrounding private lands, (2) determine within season and among year variation in occurrence of the above mycotoxins, and, (3) assess the impact of carcinogenic mycotoxins, if present, on the health of sandhill cranes on the MSCNWR.

MATERIALS AND METHODS

Field sampling

The study was conducted at the Mississippi Sandhill Crane National Wildlife Refuge (88°40'N, 30°27'W) which consists of 6,940 ha located in southern Jackson County, Mississippi near the West Pascagoula River between Biloxi and Pascagoula, Mississippi (USA) (Dewhurst, 1985). Field corn and chufa were randomly selected from fields on the MSCNWR and adjacent privately owned farms over a 3 yr period from 1987 through 1989. Selection of private farms for sampling was based on previous sightings of feeding cranes (Dewhurst, 1985), telemetry data and availability of corn.

Samples of corn were collected from available fields at bimonthly intervals during early fall 1987 and winter and fall 1988. In 1989, attempts were made to collect corn at weekly intervals from mid-August through mid-December. Corn was obtained as whole ears form upright stalks or was sampled from the ground post harvest. For sampling corn from stalks, fields were divided into approximately equal quadrants and an ear of corn was obtained from 10 locations equally spaced along the rows in each quadrant. The location of the first sampling station in each quadrant was determined by using a random numbers table. At each sampling station the ear of corn closest to the sampler was taken from the stalk without regard to condition. Kernels were removed from ears and combined to form a single sample (250 g) from each quadrant resulting in four separate samples from a field on each sampling date.

Methods of sampling corn from the ground varied with size of field and availability of corn. For large areas, sampling was according to Taylor (1939); beginning at a randomly selected corner of the field, samples were taken at 10 equidistant locations by walking a "W" pattern. Two "W" sampling formations were completed in each field. A random numbers table was used to select the first sampling station and the nearest recognizable ear or fragment of an ear comprised the sample. In fields where the amount of corn was small, the first available 50 g of corn was obtained at random by recovering individual kernels from the ground.

Chufa was obtained from sites on the MSCNWR at the time of corn sampling. Random sampling was conducted using the method described above (Taylor, 1939). At each sampling station several plants were pulled up and edible tubers were removed. A sample of 250 to 500 g of tubers was taken from each field at each sampling date. Samples were sealed in plastic whirlpak bags, frozen in the field on dry ice and then transferred to a freezer (−80 C) pending analyses.

Analysis for mycotoxins

For each corn field one sample was analyzed for each sampling period; if found negative, a second sample was analyzed if sufficient material was available. One chufa sample was analyzed from each field and sample period. Using 50 g samples, mycotoxins were extracted and quantified by thin layer chromatography (TLC) according to modifications of the method of Rottinghaus et al. (1982): Chufa was washed free of dirt and dried overnight at 40 C. Corn was processed without further drying. A 50 g sample was ground to a fine powder and extracted with 100 ml acetonitrile : 4% aqueous postassium chloride (9:1) on a wrist action shaker for 30 min. The extract was filtered through number 4 Whatman filter paper. To 10.0 ml of extract, 1.0 ml of petroleum ether was added and the mixture was vortexed. Ether was removed and discarded. Step 3 was repeated. Distilled water (8.5 ml) was added to 10.0 ml of extract. The extract/water solution was applied to an activated C_{18} column in a vacuum extraction box. The column was washed with 3.0 ml of acetonitrile : 4% aqueous potassium chloride : distilled water (18:2:17). The wash solution was collected along with the extract/water solution. Chloroform (5.0 ml) was added to the solution in step 5, and the mixture was vortexed, then centrifuged for five minutes at 750 rpm. The chloroform (bottom) later was removed and filtered through Whatman No. 1PS filter paper. Steps 6 and 7 were repeated. The filtrate was evaporated to dryness at 40 C using a nitrogen blower. Approximately 0.25 ml of chloroform : acetonitrile (98:2) was added to the dried residue, vortexed, and transferred to a 2.0 ml vial. Step 9 was repeated and the content of the vial was evaporated to dryness as in step 8. Chloroform : acetonitrile (98:2) (100 μl) was added to the

dried residue. From this vial 4 μl was spotted onto an activated silica gel HL TLC plate and developed in toluene : ethyl acetate : acetic acid (4:1:1). On the same plate, external standards containing aflatoxin B_1, ochratoxin A, and sterigmatocystin were spotted at five levels including: (1) aflatoxin B_1 (5, 10, 20, 40, and 60 ppb) and (2) ochratoxin A and sterigmatocystin (50, 100, 200, 400, and 600 ppb). Also, an internal standard with one of the concentrations above was spotted with each sample. Under long wavelength UV light the locations of sample spots were compared to that of the internal and external standards to verify the presence of aflatoxin B_1 and ochratoxin A in the samples. For positive samples the brightness of sample spots was compared to external standards to determine the quantity of mycotoxin present. To detect sterigmatocystin the plate was sprayed with aluminum chloride : ethanol (20:80) and heated for 10 minutes at 150 C. The location and brightness of sample spots were compared with standards as in step 14. In samples where the identity of mycotoxins was unclear, two-dimensional TLC was done. TLC plates were developed using methanol : distilled water : acetic acid (3:2:1). Sample spots were compared to internal and external standards as indicated above. A single sample of mycotoxin-free corn or chufa was included as a negative control on each sample run. Also, a single mycotoxin-free sample spiked with 20 ppb AB_1, 200 ppb OCA, and 200 ppb SMC was analyzed along with unknown samples on each run as a positive control.

Efficiency of recovery of mycotoxins and limits of detection were determined for corn and chufa prior to analysis of unknowns by a modification of the method of Gimeno (1979). Briefly, AB_1 (20 ppb), OCA (200 ppb), and SMC (200 ppb) were mixed with each of 10 samples of mycotoxin-free corn or chufa. Samples were extracted and mycotoxins quantified as described above. For each mycotoxin, percentage recovery was calculated by dividing the amount recovered by the total amount added to each sample. Limits of detection were estimated by spotting various concentrations of standards of each mycotoxin onto TLC plates. For AB_1, concentrations were 1, 5, 10, and 20 ppb; for OCA and SMA, concentrations were 10, 50, 100, and 200 ppb. Ten replicates of each concentration were spotted. The limit of detection for each mycotoxin was taken to be the lowest concentration that was clearly visible for each replicate.

Extracts of four different corn samples were sent to the Department of Veterinary Public Health, Texas A&M University (College Station, Texas 77843, USA) and the Animal Health Diagnostic Laboratory (Lansing, Michigan 48824, USA) for confirmation by gas chromatography-mass spectroscopy (GC-MS).

RESULTS

Field sampling

Two hundred fifty five samples including field corn (n = 203) and chufa (n = 52) were obtained from 14 locations on and adjacent to the MSCNWR during 1987 through 1989. In the first year of study, corn samples were obtained during November 1987 and January and March 1988 from two fields on the MSCNWR and three privately-owned fields north of the MSCNWR. Poor corn production on the MSCNWR fields limited sampling; thus, most samples were collected from privately-owned fields. Corn obtained from fields on the MSCNWR was found only on upright stalks whereas on some private fields corn was sampled from the ground during some sampling periods.

During fall 1988, corn was sampled from seven fields, four on the MSCNWR and three privately-owned fields, during August, October and December. Thus, sampling was initiated earlier than in 1987 and additional fields were cultivated on MSCNWR to alleviate corn shortages. In spite of this effort, corn was not available after October due to use by wildlife. In August 1988, corn from private fields was in good condition and was available only from upright stalks. However, by October corn was harvested or knocked down for livestock use on two private farms (Bosarge and H. P. Davis Farms) and samples were collected from the ground. Corn was unavailable on these farms by December. On a third private farm (Tanner Farm), standing corn was available in all three sample periods, and corn was visibly moldy in December.

In 1989, corn samples were obtained during 11 periods at 5 to 30 day intervals from August through December. Samples were taken from six fields on the MSCNWR and three privately-owned fields. After the initial August sampling, a portion of the corn on four MSCNWR fields (Table 1)

was harvested weekly by mowing to simulate management practices that made corn available to feeding cranes. Sampling of available waste corn was conducted at approximately weekly intervals. Corn was quickly utilized by wildlife and samples were not available after October. For the remaining MSCNWR fields corn was obtained either from stalks or from the ground depending upon availability, but samples were not available after September. Corn from two private fields was harvested using standard farming practices and waste corn was collected from the ground weekly (Table 1).

Chufa was available at most sampling periods in all 3 yr but sampling was sometimes limited by wildlife use, burning, or plowing. Increase in the number and size of fields enabled more uniform sampling during 1988 and 1989. Chufa always was in apparently good condition; tubers were occasionally damaged by insect grubs but were rarely visibly moldy.

Analysis for mycotoxins

The mean recovery of AB_1, OCA, and SMC from spiked corn was ≥75%. In all 10 spiked samples recovery ranged from 75 to 100% for AB_1 and OCA; however, there was more variation in the efficiency of recovery for SMC. Similar results were obtained for chufa. In all analyses where spiked samples were included (n = 18), ≥75% of all three mycotoxins were recovered. Samples of mycotoxin-free corn (n = 14) and mycotoxin-free chufa (n = 5) analyzed were negative for mycotoxins. Limits of detection for AB_1, OCA, and SMC were determined to be 5, 50 and 100 ppb, respectively.

Analyses for mycotoxins were conducted on a total of 111 field corn samples obtained during the three-year study; three sampling periods during 1987 (n = 14 corn samples), three during 1988 (n = 27 corn samples), and 11 during 1989 (n = 70 corn samples). Overall AB_1 was found in corn at levels from 5 to 5,000 ppb. During 1987, AB_1 was found in a single stalk sample

TABLE 1. Levels of aflatoxin B_1 (ppb) in corn obtained from the ground post-harvest at various sites on the Mississippi Sandhill Crane National Wildlife Refuge (MSCNWR) and adjoining private lands in 1989.

Site	Aug 14	Aug 23	Aug 29	Sep 6	Sep 14	Sep 19	Oct 2	Oct 12	Oct 26	Nov 28	Dec 12	x
Private land												
Bosarge Farm	0	0	0	0	100	500	500	200	NA[a]	NA	NA	163
Tanner Farm	500[b]	2,000	500	0	0	0	0	0	100	100	0	291
MSCNWR												
Ben Williams Crop Unit	NA	0	20	100	500	4,000	NA	0	NA	NA	NA	770
Green Pond Crop Unit	NA	0	0	200	200	1,000	NA	0	0	NA	NA	200
South Spray Field	0	0	100	5,000	4,000[b]	0	0	0	NA	NA	NA	1,138
Utah Crop Unit	5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	5

[a] NA, indicated corn not available for sampling.
[b] Two samples analyzed; one analysis conducted on all other samples.

from one MSCNWR field and four samples collected from the ground from two private fields. In 1988, AB_1 was found in a single stalk sample from one MSCNWR field and three stalk samples and one sample collected from the ground from three privately owned fields. In 1989, all six fields on the MSCNWR and the three privately-owned fields were contaminated with AB_1 (Table 1). Seventy-two percent of fields were contaminated with AB_1; 60%, 57%, and 100% were contaminated in 1987, 1988, and 1989, respectively.

Prevalence and intensity of AB_1 contamination varied with site, sampling date, year, and whether corn was on upright stalks or on the ground. Half of the corn samples from the ground contained AB_1 whereas only 30% from upright stalks were contaminated. In corn from upright stalks, levels of AB_1 were <200 ppb for all three years, and the mean level in contaminated fields was 51 ppb (range = 5–200 ppb). In contrast, 32% of corn samples obtained from the ground post-harvest had levels that exceeded 200 ppb with a mean level of 427 ppb (range = 5–5,000 ppb). Mean AB_1 concentration in corn on the ground from individual fields sampled in 1989 ranged from 5 to 1,138 ppb. Means from fields sampled in 1987 and 1988 were not computed because few samples were obtained from the ground. The GC-MS analyses of extracts of four corn samples confirmed the presence of AB_1 at levels equal to or exceeding TLC analyses. Neither OCA nor SMC was found in corn. Twenty chufa samples were analyzed for AB_1, OCA, and SMC; all were negative.

DISCUSSION

During 1987 and 1988, the prevalence of AB_1 contamination was greater for private fields with at least two-third of the fields affected in most sampling periods; and levels of AB_1 were much higher than in the few MSCNWR fields that were contaminated. Higher contamination in private fields probably resulted from scattering of corn on the ground at harvest. Although the prevalence of AB_1 contamination of MSCNWR fields appeared to be lower than for private fields, fewer MSCNWR fields were sampled and samples were small, if not inadequate, especially during 1987. Also, standard harvest practices were not used on the MSCNWR; available corn was taken from upright stalks. In 1989, corn on the MSCNWR was scattered on the ground by mowing to provide greater foraging opportunity for cranes. Thus, AB_1 contamination was more prevalent and of greater intensity. Greater contamination of corn on the ground was due probably to environmental conditions, especially increased moisture, that promoted mold growth and toxin production.

Aflatoxicosis is manifested by a variety of clinical signs and disease states depending on animal species, dosage and duration of exposure (Newberne, 1974; Norred, 1986). Among avian species, ducklings were most susceptible to acute aflatoxicosis with an oral LD_{50} of 0.335 mg AB_1/kg body weight (Lijinsky and Butler, 1966). Turkeys were of intermediate sensitivity with rapid death loss at 500 ppb (Norred, 1986) whereas chickens were relatively resistant with gradual death losses at 2,500 to 4,000 ppb (Norred, 1986). In domestic turkey poults, 100 ppb AB_1 in the diet daily for five weeks was immunotoxic and lowered resistance to infectious agents; at 300 ppb turkeys exhibited reduced weight gain and feed efficiency (Giambrone et al., 1984). The carcinogenicity of AB_1 is well documented in some species such as ducklings, and male rats being especially sensitive (Barns and Butler, 1964; Carnaghan, 1965). In general, the total dosage of AB_1 required for carcinogenesis is low, and long term exposure is required. For instance, hepatic carcinomas were produced in ducklings after feeding AB_1 at levels of 30 ppb for 14 mo. A dosage of 15 ppb in feed produced liver tumors in male rats after 17 mo (Wogan and Newberne, 1967).

The risk of aflatoxicosis in sandhill cranes is influenced in part by availability of corn,

its relative importance in the diet, and AB_1 concentration. Observations of Mississippi sandhill cranes in and near corn fields indicate that corn is an important part of the fall/winter diet (Dewhurst, 1985); however, there is no quantitative information on the amount of corn available or the daily intake by cranes on the MSCNWR. In the western United States, waste corn was a major food source for other sandhill crane subspecies and comprised approximately 90% of esophageal contents of birds that had fed in corn fields (Lewis, 1978; Reinecke and Krapu, 1978). Data on the weight of corn ingested by sandhill cranes feeding in corn fields is limited. Lewis (1978) recorded a mean of 350 ml (approximately 250 g) in cranes that were killed during trapping but did not specify the moisture content of the corn. In another area, however, the average dry weight of corn ingested by sandhill cranes collected after foraging in corn fields for one hour was only 23 to 30 g (Reinecke and Krapu, 1978). Wide variation in the amount of corn ingested would be expected depending on availability of corn at feeding sites.

In 1989, mean AB_1 concentration in corn on and near the MSCNWR was 427 ppb but varied from 5–5,000 ppb (Table 1). At these levels a variety of acute and chronic toxic effects might occur in sandhill cranes. Acute toxicosis would require ingestion of relatively large quantities of contaminated corn over a short period; however, if the LD_{50} for sandhill cranes is similar to ducklings, an adult crane weighing 3.5 kg (Lewis, 1978) would have to consume 2.75 kg of corn containing 427 ppb AB_1 to produce acute toxicity. Average daily food consumption of captive cranes is 0.250 kg (G. Gee, pers. comm.). At that level, acute toxicity is unlikely at AB_1 concentrations <6,700 ppb, but food consumption by wild birds may be higher (Lewis, 1978). Indeed, confirmed cases of aflatoxicosis have not been diagnosed in the 16 cranes presented for necropsy to date. Because of lack of information on relative sensitivity of Mississippi sandhill cranes, and high levels of AB_1 in corn in certain fields, the possibility of acute toxicity should not be ruled out, however.

Despite low probability of acute toxicity, intake of moderate amounts of contaminated corn could contain daily dosages of AB_1 comparable to that received by domestic turkey poults (Giambrone et al., 1984). Depending on the level of AB_1 intake, cranes could be subject to subacute effects of AB_1 such as retardation of growth and immunotoxicity which increase susceptibility to predation and disease. Long term exposure to low concentrations of AB_1 could lead to development of tumors months after exposure.

This study demonstrates that AB_1 is widespread and occurs at high levels in corn fields potentially utilized as food sources by Mississippi sandhill cranes. Although AB_1 may be a serious health threat to cranes, it is difficult to assess the risk of acute and chronic effects because there is no information on susceptibility of cranes to aflatoxicosis. Further research is needed to quantify intake of waste corn by Mississippi sandhill cranes and determine availability of corn in and around the MSCNWR. Also, toxicity studies using an abundant subspecies should be done to assess acute and chronic effects of exposure to AB_1. Planting of alternative crops such as chufa or small grains that are less susceptible to growth of toxin-producing molds could minimize exposure to AB_1. This option probably is not feasible on private lands which are an important source of corn during fall; however, monitoring of private fields for AB_1 along with the use of devices is to discourage crane use might prevent exposure.

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