PIGMENTED SUBCUTANEOUS SPINDLE CELL TUMORS IN GIZZARD SHAD (*Dorosoma cepedianum*): OCCURRENCE IN TWO ADDITIONAL LAKES AND FURTHER INVESTIGATIONS INTO THE ETIOLOGY OF THE LESIONS

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PREFACE

Recently, an epizootic of pigmented subcutaneous spindle cell neoplasms was reported in a population of gizzard shad (*Dorosoma cepedianum*) from Lake of the Arbuckles, a man-made lake in central Oklahoma, USA (Ostrander et al. 1995; Jacobs and Ostrander, 1995). The lesions affected about 22% of adult shad, but not juveniles and the occurrence did not appear to be seasonal. The cell of origin of the poorly differentiated neoplasms was not determined precisely. Histologically it appeared to be neural, being either a pigment cell, probably the melanophore, or a nerve sheath cell. Studies aimed at the identification of an etiological agent such as a chemical carcinogen or a retrovirus were unsuccessful. Cases of poorly differentiated neoplasms have occurred in other species from that lake. A lesion diagnosed as a hemangiopericytoma was reported from a white bass (*Morone chrysops*) (Hawkins et al. 1996) and two threadfin shad (*Dorosoma petenense*) were recently captured and diagnosed with lesions similar to those of the gizzard shad (Geter et al. in prep.).

The major objectives of the present study were to determine: (1) if lesions similar to those in Lake of the Arbuckles gizzard shad occurred in gizzard shad from nearby lakes; (2) if carcinogenic elements, detectable by inductively coupled plasma-mass spectroscopy (ICP-MS), could be identified in sediment, water, or fish tissues; (3)

whether naturally occurring uranium deposits could be contributing significant radioactivity to the study sites; (4) examine genetic markers produced by RAPD and DS-PCR for possible banding differences between tumor-bearing and nontumor-bearing gizzard shad; and (5) whether tumor location on the fish could be used to suggested a cause for the lesions. This thesis has been prepared as one chapter that has been submitted as a manuscript. This manuscript was submitted with collaborators to *Environmental Toxicology and Chemistry.*

The author would like to thank Dr. Gary K. Ostrander for the opportunity to work on such a fantastic scientific study. With his guidance, patience, and advice the research became an evolving and developing learning experience. Thanks are also extended to my committee members, Drs. Roman Lanno and Ron Van Den Bussche, for their advice and review of this manuscript.

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ABBREVIATIONS

BS	band sharing
DNA	deoxyribonucleic acid
DS	double stringency
EPA	Environmental Protection Agency
ICP-MS	inductively coupled plasma mass spectrometry
MCA	multi-channel analyzer
NTB	nontumor-bearing
PCR	polymerase chain reaction
PDD	pulse decay discrimination
RAPD	randomly amplified polymorphic DNA
SAS	statistical analysis software
SRM	standard reference materials
SURRC	Scottish University Research and Reactor Center
TB	tumor-bearing

CHAPTER I

PIGMENTED SUBCUTANEOUS SPINDLE CELL TUMORS IN GIZZARD SHAD (*Dorosoma cepedianum*): OCCURRENCE IN TWO ADDITIONAL LAKES AND FURTHER INVESTIGATIONS INTO THE ETIOLOGY OF THE LESIONS

ABSTRACT

Pigmented subcutaneous spindle cell neoplasms affected approximately 20% of the gizzard shad (*Dorosoma cepedianum*) in Lakes Murray and Texoma in southcentral Oklahoma. No neoplasms were found in shad from a reference site, Lake Carl Blackwell, Oklahoma. Similar neoplasms were previously reported in approximately 22% of adult gizzard shad from Lake of the Arbuckles in Oklahoma. Inductively coupled plasma mass spectrometry (ICP-MS) did not identify significant levels of potentially carcinogenic trace elements (beryllium, chromium, nickel, arsenic, selenium, cadmium, mercury, and lead) in the water, sediment, or tissue. Analysis of radon and gross alpha/beta radiation by liquid scintillation counting failed to reveal levels of radioactivity above background. Genetic marker analysis could not separate tumor-bearing gizzard shad from nontumor-bearing gizzard shad using random amplified polymorphic DNA (RAPD) and double-stringency polymerase chain reaction (DS PCR) analysis. Band sharing analysis of both techniques showed no statistical difference between the tumor-bearing and nontumor-bearing shad. Of 2128 fish examined from all lakes, 387 exhibited lesions distributed over the head,

trunk, and fins with a significantly higher number of tumors occurring dorsally (79.5%) versus ventrally (20.5%). Although the precise etiology of these tumors remains unknown, available data appears to preclude the involvement of know carcinogens or radioactivity.

INTRODUCTION

An epizootic of pigmented subcutaneous spindle cell neoplasms was reported in a population of gizzard shad (*Dorosoma cepedianum*) from Lake of the Arbuckles, a man-made lake in central Oklahoma, USA [1,2]. The lesions affected about 22% of adult gizzard shad, but not juveniles, and the occurrence did not appear to be seasonal [2]. The cell of origin of the poorly differentiated neoplasms was not determined but appeared to be neural, probably a pigment cell or a nerve sheath cell. Studies aimed at identification of an etiological agent such as a chemical carcinogen or a retrovirus were unsuccessful. Since that study, additional cases of poorly differentiated dermal neoplasms have been found in other fish species from Lake of the Arbuckles. These included a lesion diagnosed as a hemangiopericytoma reported from a white bass (*Morone chrysops*) [3], and poorly differentiated spindle neoplasms from two threadfin shad (*Dorosoma petenense*) [Geter et al., in prep.].

The major objectives of the present study were to determine: (1) if lesions similar to those in Lake of the Arbuckles gizzard shad occurred in gizzard shad from other lakes in the same drainage; (2) if trace elements that might affect carcinogenesis such as

beryllium, chromium, nickel, arsenic, selenium, cadmium, mercury, and lead, detectable by inductively coupled plasma mass spectrometry (ICP-MS), could be identified in sediment, water, or tissues; (3) whether naturally occurring uranium deposits were contributing significant radioactivity to the study sites; (4) if genetic markers produced by RAPD and DS PCR could distinguish tumor-bearing from nontumor-bearing gizzard shad; and (5) whether the anatomic distribution of the tumors might provide a clue to their etiology.

MATERIALS AND METHODS

Materials

Unless noted otherwise, analytical grade reagents purchased from Sigma Chemical Company, St. Louis, MO, USA, were used.

Fish collection

Gizzard shad were collected from Lake of the Arbuckles, Lake Texoma, Lake Murray (Figure 1), and Lake Carl Blackwell either by seining with a 18-m, 1 cm mesh beach seine for gizzard shad (<1 year old) or by gill netting for mature gizzard shad (2-3 years old) with 100-m gill nets with 6 cm mesh. Nets were set perpendicular to the water flow and examined every 6 hours for 24-72 hours. Gizzard shad were weighed, measured, and examined grossly for tumor occurrence. Liver and muscle tissue were excised for

inductively coupled plasma mass spectrometry (ICP-MS) analysis and liver tissue excised for both random amplified polymorphic DNA (RAPD) and double-stringency polymerase chain reaction (DS PCR) analysis. Gizzard shad were collected from the reference site, Lake Carl Blackwell, which is a man-made lake located in a separate drainage in north central Oklahoma, and sampled as described above.

Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS analysis was conducted on sediment, water, and tissues (gizzard shad liver and muscle) from Lake of the Arbuckles and Lake Texoma to determine the presence of trace elements such as beryllium, chromium, nickel, arsenic, selenium, cadmium, mercury, and lead that might affect the occurrence or development of the neoplasms [4]. Sample sites from Lake of the Arbuckles and Lake Texoma are shown in Figure 1. Sediment samples were taken with an Ekman dredge, hypolimnetic water samples with a Van Dorn sampler, and epilimnetic water samples by hand 5 cm below the water surface. Sediment and water samples were placed in 50-ml acid-washed polyethylene tubes (Fisher Scientific Co, Pittsburgh, PA). Samples and blanks were placed on ice, transported to the laboratory in darkness, and stored at 4°C. Water samples and trip blanks were filtered with Whatman glass microfibre filters. Filtered water and filter were placed in separate pre-cleaned, acid-washed, aluminum-wrapped 100 ml glass sample vials. About 1.0-1.5 g (wet wt.) liver and muscle were excised from six tumor-bearing and six nontumor-bearing gizzard shad from both Lake of the Arbuckles and Lake Texoma. Dissected tissues were

placed in sterile 2-ml centrifuge tubes, placed on dry ice for transport to the laboratory, and then stored at -20°C. Sediment and water samples were shipped on ice, and tissue samples were shipped on dry ice for ICP-MS analysis.

Clean techniques were followed in all phases of sample preparation and analysis. All reagents and chemicals utilized in these procedures were of the ultra-pure grade to minimize the introduction of metals. Standard reference materials included National Institute of Standards and Technology's Standard Reference Materials (SRM) 1646 Estuarine Sediment and SRM 1635 Oyster Tissue were processed and analyzed in parallel with each set of samples to control for contamination and define recovery. Techniques for sample processing, acid digestion, and trace element solubilization were conducted to maximize recovery of *in situ* trace elements, and to minimize or exclude extraneous metal contamination using a modification of Environmental Protection Agency Method 6020. All steps in which contamination could be extraneously introduced were carried out under a level 100 laminar flow hood. Water samples were analyzed following a 1 to 10 dilution in 3% ultra-pure nitric acid solution.

Sediment and tissue samples were thawed and the entire sample transferred to a metal-free vessel for thorough homogenization. An aliquot of the homogenate was transferred to a metal-free polypropylene digestion vessel for digestion using a CEM 2000 (CEM Corp., Matthews, NC) microwave digestion system. Digestions were performed using approximately 1 g (wet wt.) sediment or tissue in 10 ml 50% ultra-pure nitric acid for approximately two hours. Samples were diluted to 50-ml final volume and a 1-ml aliquot was analyzed for trace element concentrations. Samples of MilliQ water and

reagent acids were retained for trace element determinations as blanks or reagent blanks with each set of digest. Yttrium 89 was used as an internal standard in all samples.

Analyses of elements were performed on each sample using a Fisons PlasmaQuad II+ Inductively Coupled Plasma Mass Spectrometer. All samples were analyzed using triplicate, one-minute data acquisition/integration times. Final trace element concentrations were blank subtracted and corrected for internal standard recovery, analysis dilution, digestion volume, and the original mass of the sample.

Environmental radiation

Water samples for gross alpha/beta and radon-222 radiation analysis were taken from the same locations as water samples for ICP-MS. Alpha/beta radiation samples were treated by the following procedure. Filtered (Whatman #1) and nonfiltered samples were placed into two sterile, acid-washed 50-ml polyethylene tubes (Fisher Scientific Co) to a total volume for 98 ml. To each tube, 1 ml of 5N HCl was added to preserve the sample until processing. Samples were placed on ice and kept in the dark for transport to the laboratory where they were stored at 4°C. For analysis, samples were placed into 200-ml acid-washed glass beakers and incubated at 80°C to reduce the volume to 8-10 ml. The samples were then quantitatively transferred into 20-ml polyethylene vials and further incubated at 80°C to a final volume of less than 1 ml. Samples were shipped to the Scottish University Research and Reactor Center (SURRC) in Glasgow, Scotland, for analysis.

Upon receipt at SURRC, the samples were quantitatively transferred to 20-ml glass vials, placed on a hot plate, and slowly taken to dryness. Residual organic matter was oxidized using a small volume (<10 ml) of concentrated nitric acid and hydrogen peroxide. In preparation for liquid scintillation analysis, the samples were again taken to dryness, cooled, and 2 ml of 1N HCl and 10 ml of Ultima Gold AB scintillation cocktail were added to each vial. The samples were counted in a Packard Instruments 2770 TR/SL (time-resolved/ super low level) scintillation counter equipped with pulse decay discrimination (PDD) circuitry to separate alpha from beta events. The instrument was optimized by counting an alpha (Am^{241}) and a beta (Cl^{36}) standard in the same volumes of HCl and Ultima Gold AB as the samples, at a range of PDD settings. The spill of alpha events into the beta multi-channel analyzer (MCA) and the spill of beta events into the alpha MCA were then plotted against PDD setting. The cross-over of these two plots (<1% spill) was taken as the optimum setting. This was determined to be a setting together with 2 blanks containing no measurable activity. Net sample count rates were then determined.

The counting efficiency for alpha events is nearly 100%. Therefore, the only correction between net count rate (counts per second) and Bq (disintegrations per second) is for the approximate 1% spill which has been determined. The counting efficiency for beta emitters depends on the E_{max} of the nuclide of interest and the degree of quenching of the sample. Typically efficiencies for beta emitters will range between approximately 25 and 100%. Applying an efficiency of 25% provides an upper limit for the activity: however, an efficiency range of 50-100% yields a more reasonable measurement. To

correct beta counts per second to Bq with a 50% counting efficiency, a multiplication factor of 2X was applied to the original beta results. Limits of detection were determined according to the method of Currie [5]. Samples for radon analysis were taken with "Radon in Water Liquid Scintillation Sampling Kits" (Niton Corporation, Bedford, MA), according to the manufacturer's recommendations and analyzed by Niton Corporation.

Random amplified polymorphic DNA (RAPD) and double-stringency polymerase chain reaction (DS PCR) analysis

Tumor-bearing and nontumor-bearing gizzard shad from Lake of the Arbuckles were weighed, measured, and livers excised. Dissecting scissors were soaked in 100% ethanol and thoroughly cleaned prior to each dissection. Tissues were individually wrapped in 30 x 30 cm sheets of autoclaved aluminum foil, placed in plastic freezer bags, and kept on dry ice until they were brought to the laboratory, and stored at -20°C. DNA extraction was accomplished by standard phenol/chloroform separation followed by ethanol precipitation and stored in a Tris EDTA (TE) buffer at 4°C [6]. For this study, we used both RAPD and DS PCR techniques to produce genetic markers. Double-stringency polymerase chain reaction mixtures used two primers with different annealing temperatures [7]. The first primer, a M13 (CTCCCACCRCCRAGT) core microsatellite primer (Oklahoma State University Recombinant DNA/Protein Resource Facility, Stillwater, OK) amplifies a region between microsatellites, whereas the second primer, a standard 10-mer RAPD primer (Operon Technologies Inc., Alameda, CA) amplifies the

products of the first primer. For analysis we used the 'Kit B' set of RAPD primers, which contained 20 individual sequences. The DS PCR reactions were carried out in two phases. The first 15 cycles used high annealing temperatures (47°C) that amplified template DNA by only the M13 primer. After 15 rounds of replication, the annealing temperature was reduced to 35°C for an additional 25 cycles allowing the RAPD primer to anneal and elongate the higher concentrated DNA fragments produced by the M13 primer. DS PCR reactions were performed in 25 μl volumes, containing 10 mM tris-HCL, pH 8.4, 200 μM each dNTP (Perkin-Elmer, Branchburg, NJ), 50 mM KCl, 6 pmol of M13 primer, 20 pmol of 10-mer primer, and 1 U AmpliTaq DNA polymerase (Perkin-Elmer). Individual RAPD reactions were identical except for 50 pmol of the RAPD primer and deletion of the M13 primer. All reactions were performed with a GeneAmp PCR System 9600 (Perkin-Elmer) thermal cycler. The RAPD temperature profile was denaturation at 94° C for 30 s, annealing at 35° C for 30s, and extension at 72° C for 30s, for 35 rounds of replication. The DS PCR temperature profile was as follows: denaturation at 94°C for 30 s, annealing at 47°C for 30 s, and extension for 30 s at 72°C. The final 25 cycles were completed with the following conditions: denaturation at 94°C for 30 s, annealing at 35°C for 30 s, and extension at 72°C for 30 s. The products were electrophoresed in 1 X TBE (9 mM Tris-borate, 0.2 mM EDTA, pH 8.0) at 25 V for 5 h in 5.0% polyacrylamide in 1 X TBE, [6], stained with ethidium bromide, and examined under UV light, and photographed. Eight individuals were used for genetic marker analysis, four tumor-bearing (lanes 1-4) and four nontumor-bearing (lanes 5-8). Lane 9 contained one μg of 100 bp size standard (Cat. # 15628-050, Gibco-BRL, Gaithersburg, MD) used as a size

reference during visualization and scoring.

Bands were hand scored according to migration distance and incorporated into a presence-absence matrix. From this matrix, a band-sharing index (BS) was calculated as $BS = 2 N_{ab}/(N_a + N_b)$, where N_{ab} is the number of shared bands, N_a is the number of bands in one lane, and N_b is the number of bands in the other lane [8]. Band sharing indices were calculated for tumor bearing (lanes 1-4), nontumor-bearing (lanes 5-8), tumor bearing vs. non-tumor-bearing (lanes 1-4 vs. 5-8), and a total comparison of all individuals (lanes 1-8). All individuals scored were present on the same gel for a total of 40 gels (20 RAPD and 20 DS PCR).

Tumor location

The anatomical location of grossly visible tumors was noted and analyzed statistically to determine whether a pattern emerged. To systematically record tumor location, a schematic diagram of a gizzard shad was produced which was divided into dorsal and ventral sections by a horizontal line from the opening of the mouth to the middle of the caudal fin. The dorsal and ventral sections were then divided into three zones by vertical lines running down from the anterior base of the dorsal fin and the base of the caudal fin (Figure 2). The dorsal fin was included in zone 3 and all ventral fins were included in zone 6 to assess fin tumor occurrence.

Statistics

A one-way ANOVA was used to determine differences between Lake Texoma and Lake Murray to Lake of the Arbuckles for neoplasm occurrence in the gizzard shad population. A paired T-test was used to determine if differences existed between the prior studies and the present studies neoplasm occurrence in Lake of the Arbuckles.

Concentrations for ICP-MS were reported as μg/L for water samples and μg/g wet weight for sediment, liver, and muscle tissues. Concentrations below the detection limits of the ICP-MS were represented as zero for statistical analysis. For analysis of the concentrations of trace elements (beryllium, chromium, nickel, arsenic, selenium, cadmium, mercury, and lead), all data were initially tested for normality and homogeneity of variance. Trace element concentrations for the liver and muscle tissues were not normally distributed, and appropriate nonparametric statistics were performed. A one-way Wilcoxon signed ranks two sample test was performed on the data from the liver and muscle tissues.

Paired T-tests were conducted on the RAPD and DS PCR band sharing data to determine whether differences existed between tumor-bearing and nontumor-bearing gizzard shad and also to test differences between the two methods.

A one way ANOVA was performed to distinguish differences from the tumor location data area matrix and a paired T-test applied to test differences between dorsal and ventral locations. All statistical analysis were performed at the $p = 0.05$ level of significance with the Statistical Analysis Software from SAS Institute, Cary, NC.

RESULTS

Neoplasm prevalence

Gizzard shad collected from the Lake of the Arbuckles for the present study had a total neoplasm prevalence of 15.4% (73/474) which was lower than that reported in previous studies, 21.01% (208/990) [1,2] (Table 1). However, statistical analysis testing for differences between previous studies and the current study failed to reveal a significant difference in neoplasm prevalence ($p = 0.469$). Collections from Lakes Texoma and Murray showed neoplasm prevalences of 16.8% (111/660) and 20% (4/20), respectively. No significant differences were noted in neoplasm prevalence from Lakes Texoma and Murray shad populations when compared to Lake of the Arbuckles ($p = 0.515$ and 0.801 respectively). Also, neoplasm occurrence was rather evenly distributed among collection sites in Lake Texoma including the Glasses (14.8%), Caney (12.5%), and Lebannon (17.1%) sites. No neoplasms (0/32) were found in shad from the reference site, Lake Carl Blackwell.

Inductively coupled plasma mass spectroscopy (ICP-MS)

ICP-MS analyses for beryllium, chromium, nickel, arsenic, selenium, cadmium, mercury, and lead [4] were conducted on sediment, water, and tissues (liver and muscle) from Lake of the Arbuckles and Lake Texoma. Figure 1 (Table 2) shows trace metal

concentrations in sediments as the means of three samples taken from each lake. Water concentrations are shown as the means of three samples from the epilimnion and three samples from the hypolimnion taken from each lake as illustrated in figure 1. Water values are given as total recoverable trace elements by adding both the dissolved and suspended fraction concentrations (Table 3). Analysis of tumor-bearing and nontumor-bearing tissues showed statistical differences between beryllium (<0.05 vs. 0.79 μg/g) and nickel (<0.05 vs. 21.25 μg/g) in the liver and nickel (10.35 vs. 4.48 μg/g) in the muscle at $p \leq 0.05$ (Table 4).

Environmental radiation

Radioactivity levels in forty-five water samples from Lake of the Arbuckles, Lake Texoma, and Lake Carl Blackwell ranged from <0.07-0.51 Bq liter $^{-1}$ for alpha, <0.40-1.60 Bq liter $^{-1}$ for beta, and <100 pCi liter for radon 222 radiation (Table 5).

Random amplified polymorphic DNA (RAPD) and double-stringency polymerase chain reaction (DS PCR) analysis

Tumor-bearing gizzard shad were indistinguishable from nontumor-bearing gizzard shad by genetic marker analysis performed in this study. Band sharing analysis was accomplished by calculating the band sharing value for the tumor-bearing markers (lanes 1-4), nontumor-bearing markers (lanes 5-8), tumor-bearing vs. nontumor-bearing markers

(1-4 vs. 5-8), and a total value for all individuals (lanes 1-8). Band sharing analysis showed no difference between the tumor-bearing and nontumor-bearing gizzard shad with RAPD ($p = 0.294$) or DS PCR ($p = 0.236$) (Tables 6 and 7, respectively). However, band sharing comparisons between the two techniques revealed significant differences between RAPD and DS PCR for three of the four levels of comparison. Differences were noticed in the tumor-bearing ($p = 0.006$), tumor-bearing vs. nontumor-bearing ($p = 0.027$), and total (0.019), while no significant difference was detected in the nontumor-bearing comparisons ($p = 0.056$). RAPD analysis generated a greater number of bands than DS PCR ($p = 0.001$) with a total of 303 and 201 scorable bands, respectively.

Tumor location

The location of 577 tumors from 346 tumor-bearing gizzard shad (Table 6) was scored. The occurrence of tumors in the dorsal section (459/577=79.5%) was significantly higher than that in the ventral section (118/577=20.5%), ($p = 0.001$). Lesions were particularly abundant in the dorso-anterior portion of the fish (zone 1) with 42.3% (244/577) occurring there ($p = 0.001$). Of the 229 tumor-bearing gizzard shad that exhibited a single tumor, 44.5% (102/229) had the tumor in zone 1 ($p = 0.001$), and 82.1% (188/229) had the tumor in one of the three dorsal zones ($p = 0.001$). About one-third of the tumor-bearing gizzard shad had multiple tumors (117 of 346). Of those 117 specimens with multiple tumors, 86 (73.5%) had at least one tumor in zone 1, and 98.3% (115/117) had at least one tumor in one of the three dorsal zones.

DISCUSSION

An epizootic of pigmented subcutaneous spindle cell neoplasms in gizzard shad (*Dorosoma cepedianum*) which was first observed in 1991 was thought to be limited to Lake of the Arbuckles, Oklahoma. The epizootic now been documented in two additional lakes, Texoma and Murray, which are about 55 kilometers south of Lake of the Arbuckles but share drainages. The reference site, Lake Carl Blackwell, is 180 kilometers north and lies in a different drainage. Neither the geographical extent nor when the date the epizootic began are known. Both Lake of the Arbuckles and Lake Murray were stocked by the Oklahoma Department of Water Quality with gizzard shad and threadfin shad (*D. pentenense*) from Lake Texoma in 1980 [J. Pigg, personal communication] to provide forage for game fish. This suggests that antecedents of tumor-bearing shad from Lake of the Arbuckles and Lake Murray were introduced from the same source at the same time. Department of Water Quality records for Lake Texoma show shad species occurring there naturally [J. Pigg, personal communication].

The cause of the gizzard shad neoplasm epizootic has not been determined. Although the etiology of neoplasia in wild fish generally varies with species and site, chemical contamination and oncogenic viruses are known causes of fish neoplasia [9]. Neoplastic lesions are known to affect nearly every cell and tissue type and in fishes from freshwater [10], estuarine, and marine habitats [11]. Hepatic neoplasia is usually considered to be caused by exposure to carcinogenic chemicals, particularly polynuclear

aromatic hydrocarbons in sediments. Hepatic neoplasia considered to be carcinogen induced has been reported from winter flounder (*Pleuronectes americanus*) from Boston Harbor [12], white croaker (*Genyonemus lineatus*) from near Los Angeles [13], mummichog (*Fundulus heteroclitus*) from the Elizabeth River, VA [14], brown bullhead (*Ameiurus nebulosus*) [15,16], white sucker (*Catostomus commersoni*) [17] from several locations in the Great Lakes region and several other species and locations. Non-hepatic neoplasia is rarer than hepatic neoplasia in teleosts and is not as clearly related to environmental contamination [9,11]. Epizootics of non-hepatic neoplasia that have been associated with environmental contamination in the Great Lakes region include epidermal neoplasms in brown bullhead [14,18] and white suckers [17], dermal pigment cell neoplasms (chromatophoromas), and neurilemmomas in freshwater drum (*Aplodinotus grunniens*) [15], gonadal neoplasms in carp/goldfish hybrids [19,20,21], and several types of non-hepatic as well as hepatic neoplasms in sauger (*Stizostedion canadense*) and walleye (*S. vitreum*) [22]. Several epizootics of neoplasia appear to have a viral origin. These include lymphoma in the northern pike (*Esox lucius*) [23], plasmacytoid leukemia in chinook salmon (*Oncorhynchus tschawytscha*) [24], dermal sarcoma in walleye [25], and neurofibromatosis in bicolor damselfish (*Pomacentrus partitus*) [26].

At the sites of the gizzard shad neoplasm epizootic, the presence of trace elements (beryllium, chromium, nickel, arsenic, selenium, cadmium, mercury, and lead) that might play some role in carcinogenesis was determined by ICP-MS, which allows detection of low levels of most elements in the periodic table [27]. The technique has also been used for simultaneous analysis of multiple elements in biological materials [28,29]. ICP-MS

analysis of the sediment and water samples revealed trace element concentrations below suggested levels of both the EPA and the State of Oklahoma [30,31]. Comparison of analyses between tumor-bearing and nontumor-bearing tissues showed statistical differences among several elements including beryllium (<0.05 vs. 0.79 μg/g) and nickel (<0.05 vs. 21.25 μg/g) in the liver and nickel (10.35 vs. 4.48 μg/g) in the muscle at $p \leq 0.05$. However, a comparison of tested trace elements to concentrations in other teleosts species showed gizzard shad tissue to be within average concentrations [32]. Although significant analytical differences were noted between tumor-bearing and nontumor-bearing gizzard shad, it is likely that these levels do not have biological significance. We must therefore conclude from the data at hand that trace elements are not involved in shad neoplasm formation.

Naturally occurring radiation can harm aquatic systems by producing a range of syndromes from reduced vigor to lethality, shortened life span, diminished reproductive rate, and genetic transmission of radiation-altered genes [33]. In this study we investigated whether background radioactivity might be a cause or contributor to the gizzard shad neoplasia. An Oklahoma Geological Survey minerals map published in 1969 showed deposits of uranium within the watershed around Lake of the Arbuckles. The uranium was mostly disseminated in gray sandstone and gray to black shales, and occurred in small low-grade deposits ranging from 0.2-70 ppm uranium. Also, local deposits of crude oil and asphalt contained higher than normal amounts of uranium [34,35,36]. Evaluation of environmental alpha/beta radiation and radon-222 levels in the watersheds of both Lake of the Arbuckles and Lake Texoma revealed values below U.S. EPA

drinking water guidelines [37,38], suggesting that radiation probably is not be a factor in gizzard shad neoplasms.

Random amplified polymorphic DNA (RAPD) and double-stringency polymerase chain reaction (DS PCR) were used in an effort to generate a genetic marker to identify and separate tumor-bearing and nontumor-bearing gizzard shad. RAPD analysis has been used for genetic mapping, plant and animal breeding applications, and population genetics [39]. The major advantages of this technique are its ability to detect polymorphisms, small amount of DNA needed, ease of use, and no requirement for sequence information on the organism. A major drawback of RAPD markers is that they are usually dominant since polymorphisms are detected as a presence or absence of bands after PCR amplification. These markers result from size changes in the amplified region or base changes that alter primer binding. Another drawback is population genetic parameters used to calculate genetic variability cannot be used, nor can RAPD data be directly compared with other techniques such as allozymes or restriction fragment length polymorphism (RFLP) analysis. For these reasons, we analyzed variation between the tumor-bearing and nontumor-bearing gizzard shad as a binary (presence/absence) value using tests which do not require explicit genetic assumptions. With RAPD and DS PCR analysis, a genetic marker which could separate the two groups was not identified. Also, no statistical difference was noticed in the band sharing values between tumor-bearing and nontumor-bearing gizzard shad using both techniques. However, comparisons revealed differences between the two techniques with RAPD analysis producing a third more markers than DS PCR. This difference in marker production is accountable to a first phase of high-

stringency amplification in the DS PCR technique which reduces the concentration of different DNA that can be amplified by the RAPD primers. Studies have shown genetic markers produced by the RAPD technique do not follow Mendelian segregation [40,41]. By including an early first phase of high-stringency amplification, DS PCR genetic markers alleviate that problem [7]. Although the use of RAPD analysis has its drawbacks, the technique may potentially yield clearer and more comparative information especially for the analytical methods using RAPD markers for population genetic estimates [42,43].

The cell of origin in the gizzard shad neoplasm has not been definitively determined [1,2]. Two major possibilities considered are pigment cells and peripheral nerve sheath cells. Pigment cells, particularly melanocytes, were likely because the tumors were usually darkly pigmented, whereas the swirling patterns of the tumors suggested an origin from peripheral nerve sheath cells although poorly differentiated pigment cell neoplasms can express similar patterns. Tumor location analysis revealed a significantly higher number of tumors occurring in the dorsal section (459 of 577=79.5%) versus the ventral section (118 of 577=20.5%). The area of highest occurrence was from the occiput to the dorsal fin origin. This dorso-anterior area was the location for 244 of the 577 (42.3%) tumors scored and also correlates with a high concentration of nerve sheath cells that arise from the neural crest. However, pigment cells also arise from the neural crest and the dorsal area of the gizzard shad is more heavily pigmented than the ventral area. Further histopathological and immunocytochemical studies are underway to determine the cell of origin (Geter et al., in prep.). Tumor location analysis show the lesions are not randomly distributed but that most (79.5%) occur on the dorsal surface. This argues

strongly that the shad neoplasms are not carcinogen induced, at least from direct exposure from sediments. Most epizootics of carcinogen induced hepatic neoplasia and nearly all examples of skin neoplasia in wild fishes, as listed above, occurred in bottom feeding species with the skin neoplasms thought to have been initiated by direct exposure to carcinogenic compounds in the sediments.

In summary, the cause of an epizootic of dermal neoplasia in gizzard shad (*Dorosoma cepedianum*) from lakes in Oklahoma and Texas remains unsolved. The present study suggests that an etiology from trace elements or radiation cannot be supported. Furthermore, tumor location analysis suggests that direct exposure to sediment-related carcinogens is not a likely cause of the neoplasia. Future studies will investigate the geographic range of the disease, determine tumor prevalence in other species including the threadfin shad and examine additional tumor specimens to determine the cell of origin.

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Table 1. Dates, total gizzard shad caught and percent of tumor-bearing shad per catch for Lake of the Arbuckles, Lake Texoma and Lake Murray, Oklahoma, USA.

Lake of the Arbuckles

Date	Shad caught	Tumor-bearing	% with tumors
6-23/25-95*	142	27	19.0
10-2/3-95	10	2	20.0
11-11/12-95	15	2	13.0
11-24/26-95	129	17	13.2
5-5-96	77	9	11.7
8-11/12-96	85	7	8.2
Totals	**1448**	**272**	**18.8**

Lake Texoma

Date	Shad caught	Tumor-bearing	% with tumors
5-6/10-96	81	12	14.8
5-13/17-96	16	2	12.5
5-21&23-96	498	85	17.1
8-13-96	65	12	18.4
Totals	**660**	**111**	**16.8**

Lake Murray

Date	Shad caught	Tumor-bearing	% with tumors
5-22/23-96	20	4	20

* Fish sampled before 1995 were previously reported [1,2].

Table 2. Sediment quality guideline values and average ICP-MS values in μg/g from sediment samples taken from Lake of the Arbuckles (N=3) and Lake Texoma (N=3). Detection limits for shown elements are 0.05 μg/g.

Element	Guideline	Arbuckle	Texoma
Beryllium	40*	3.80	2.40
Chromium	25†	10.90	4.95
Nickel	20†	9.55	2.83
Arsenic	3†	1.76	1.89
Selenium	10*	3.30	2.73
Cadmium	1*	0.70	0.40
Mercury	1†	0.00	0.00
Lead	40†	14.13	6.83

*Fishbein et al. 1987[4], †U.S. Environmental Protection Agency, 1977[30].

Table 3. Water quality guideline values and average ICP-MS values in μg/L from water samples reported as total recoverable metals (dissolved + suspended) from Lake of the Arbuckles (N=6) and Lake Texoma (N=6). Detection limits for shown elements are 0.001 μg/L.

Element	Guideline	Arbuckle	Texoma
Beryllium	1*	0.14	0.34
Chromium	50†	11.67	7.54
Nickel	600†	2.23	7.38
Arsenic	100†	1.60	2.18
Selenium	10†	2.02	2.04
Cadmium	20†	<0.001	0.45
Mercury	2†	0.15	0.09
Lead	100†	1.95	1.66

*U.S. Environmental Protection Agency, 1995, †Oklahoma Department of Water Quality.

Table 4. Average ICP-MS values and range in $\mu g/g$ wet weight from liver and muscle tissue taken from tumor-bearing (TB) *N=12* and nontumor-bearing (NTB) *N=12* gizzard shad from Lake of the Arbuckles and Lake Texoma with detection limits for listed elements at $0.05\mu g/g$. Average biota reference values are also given.

	Liver				Muscle				Average
Tissue	**TB**	**Range**	**NTB**	**Range**	**TB**	**Range**	**NTB**	**Range**	**Biota‡**
Beryllium*	<0.05	*0.00-0.08*	0.79	*0.00-1.01*	<0.05	*0.00-0.05*	<0.05	*0.00-0.04*	<1
Chromium	2.02	*0.33-6.18*	6.74	*0.77-10.82*	4.68	*1.30-11.32*	4.62	*0.54-7.89*	<1-11
Nickel*†	<0.05	*0.00*	21.25	*2.23-34.44*	10.35	*0.00-21.13*	4.48	*0.00-8.78*	<2-36
Arsenic	3.55	*1.51-6.77*	6.08	*4.01-6.23*	<0.05	*0.00*	0.49	*0.00-2.24*	<0.2-6.7
Selenium	0.42	*0.00-1.04*	0.56	*0.00-2.06*	1.68	*0.53-4.76*	1.03	*0.00-3.66*	<0.3-7.9
Cadmium	0.33	*0.00-0.89*	0.51	*0.06-2.44*	0.364	*0.00-0.64*	0.189	*0.00-0.87*	<0.3-9.5
Mercury	0.09	*0.00-0.12*	<0.05	*0.00-0.07*	<0.05	*0.00-0.37*	<0.05	*0.00-0.22*	<0.5
Lead	4.83	*1.33-8.02*	5.97	*1.87-12.04*	0.56	*0.00-2.53*	0.32	*0.00-2.2*	<5-22

Stastistically significant difference of element between tumor-bearing and nontumor-bearing gizzard shad liver (*) and muscle (†). ‡U.S. Geological Survey, Water-Resources Investigations Report 95-4045.

Table 5. Environmental radiation levels (alpha/beta and radon 222) in Lake of the Arbuckles, Lake Texoma and Lake Carl Blackwell. Alpha and beta values from filtered (F) and unfiltered (UF) samples reported in Bq liter $^{-1}$ and radon 222 values in p Ci/L. U.S. EPA drinking water guidelines for alpha, beta, and radon radiation are 0.56 Bq liter $^{-1}$, 1.85 Bq liter $^{-1}$, and 400 pCi liter, respectively.

Lake of the Arbuckles	**Alpha (F/UF)**	**Beta (F/UF)**	**Radon 222***
Guy Sandy (E)	<0.07/<0.07	<0.40/<0.40	<100
Guy Sandy (H)	<0.07/<0.07	<0.40/<0.40	<100
Rock (E)	<0.07/<0.07	<0.40/<0.40	<100
Rock (H)	0.15/<0.07	1.60/<0.40	<100
Buckhorn (E)	<0.07/<0.07	<0.40/<0.40	<100
Buckhorn (H)	<0.07/<0.07	<0.40/<0.40	<100
Lake Texoma			
Glasses (E)	<0.08/0.11	0.80/1.00	<100
Glasses (H)	<0.08/<0.51	1.12/0.64	<100
Caney (E)	<0.07/0.12	0.52/1.24	<100
Caney (H)	<0.08/0.09	0.68/<1.00	<100
Lebannon (E)	0.09/<0.07	<0.40/<0.40	<100
Lebannon (H)	<0.07/<0.07	<0.40/0.48	<100
Lake Carl Blackwell			

(E)	<0.07/<0.07	<0.84/1.08	<100
(H)	<0.07/<0.07	<0.84/1.00	<100

**Radon water samples were not filtered.*

(E)=epilimnion (H)=hypolimnion

Table 6. Average band-sharing indices derived by comparison of RAPD markers from four tumor-bearing (TB) and four nontumor-bearing (NTB) gizzard shad from Lake of the Arbuckles.

Probe	Bands	TB	NTB	TBvsNTB	Total
B1	13	0.952	0.883	0.925	0.922
B2	12	0.903	0.835	0.875	0.872
B3	13	0.890	0.929	0.915	0.912
B4	19	0.790	0.819	0.844	0.827
B5	19	0.643	0.715	0.725	0.705
B6	12	0.894	0.943	0.926	0.923
B7	15	0.924	0.915	0.925	0.923
B8	17	0.785	0.816	0.831	0.818
B9	13	0.850	0.769	0.806	0.808
B10	17	0.893	0.852	0.878	0.876
B11	18	0.879	0.905	0.909	0.902
B12	14	0.810	0.823	0.838	0.829
B13	18	0.665	0.728	0.729	0.715
B14	14	0.810	0.843	0.825	0.826
B15	15	0.830	0.785	0.803	0.805
B16	13	0.868	0.894	0.911	0.898

B17	13	0.872	0.904	0.912	0.902
B18	13	0.800	0.847	0.835	0.830
B19	19	0.695	0.745	0.733	0.727
B20	16	0.795	0.823	0.815	0.812
Average	**15.15**	**0.827**	**0.839**	**0.848**	**0.842**

Table 7. Average band-sharing indices derived by comparison of DS PCR markers from four tumor-bearing (TB) and four nontumor-bearing (NTB) gizzard shad from Lake of the Arbuckles.

Probe	Bands	TB	NTB	TBvsNTB	Total
M13/B1	11	0.901	0.845	0.832	0.850
M13/B2	10	0.965	1.000	0.974	0.978
M13/B3	12	0.894	0.943	0.926	0.913
M13/B4	13	0.928	0.908	0.924	0.921
M13/B5	6	0.906	0.867	0.880	0.882
M13/B6	13	0.908	0.870	0.901	0.896
M13/B7	11	0.923	0.921	0.909	0.915
M13/B8	7	0.762	0.756	0.805	0.785
M13/B9	8	0.835	0.636	0.755	0.747
M13/B10	13	0.830	0.845	0.862	0.852
M13/B11	14	0.903	0.949	0.941	0.934
M13/B12	12	0.908	0.895	0.923	0.914
M13/B13	9	0.929	0.900	0.898	0.905
M13/B14	7	0.916	0.906	0.907	0.909
M13/B15	8	0.870	0.919	0.880	0.886
M13/B16	10	0.943	0.922	0.924	0.928

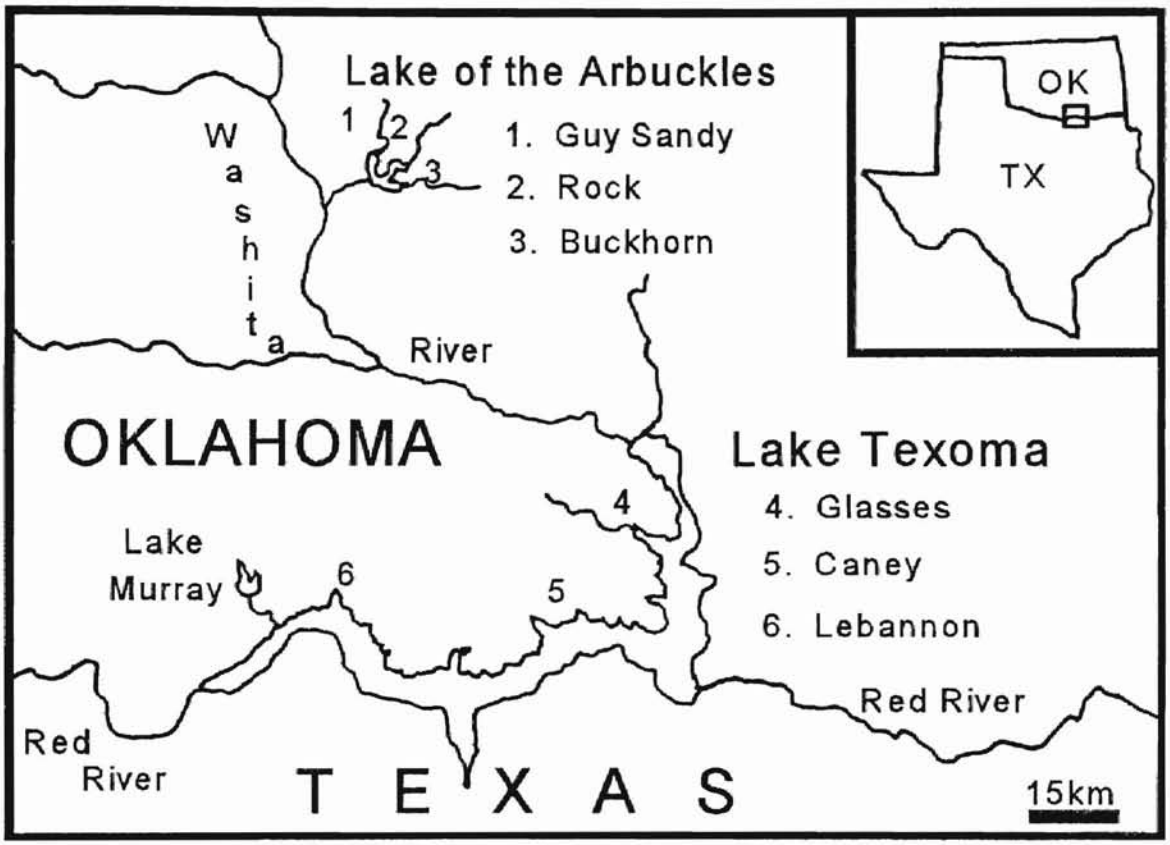
M13/B17	14	0.868	0.860	0.859	0.861
M13/B18	8	0.956	0.919	0.940	0.939
M13/B19	7	0.789	0.788	0.807	0.799
M13/B20	8	0.879	0.874	0.878	0.877
Average	**10.05**	**0.891**	**0.876**	**0.886**	**0.885**

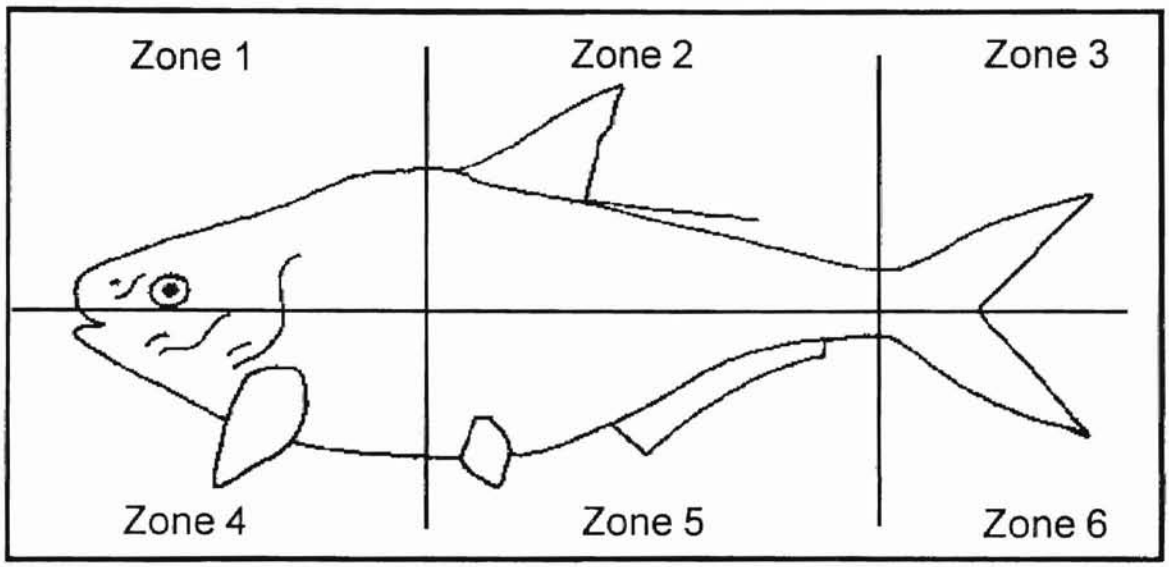
Table 8. Location analysis of tumors as illustrated in Figure 2 reported as frequency of tumors per zone from shad from Lake of the Arbuckles (N=243; 1.72 tumors/shad), Lake Texoma (N=103; 1.5 tumors/shad) and total from both lakes.

Zone	Arbuckle	Texoma	Total (%)
1	39.05	50.10	42.29
2	17.62	12.10	16.12
3	19.52	25.50	21.14
4	7.14	3.20	6.07
5	9.38	0.60	6.93
6	7.38	7.60	7.45

Figure 1. Map showing sampling sites within Lake of the Arbuckles, Lake Texoma and Lake Murray.

Figure 2. Schematic diagram of a gizzard shad used in statistical analysis of tumor location.


Lake of the Arbuckles
1. Guy Sandy
2. Rock
3. Buckhorn
Washita River
OK
TX
OKLAHOMA
Lake Texoma
4. Glasses
5. Caney
6. Lebannon
Lake Murray
Red River
Red River
TEXAS
15km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VITA

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