Evaluation of Endocrine Disrupting Effects in Potomac River Fish

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Prepared by

Beth L. McGee
Alfred E. Pinkney
U.S. Fish and Wildlife Service
Chesapeake Bay Field Office
Annapolis, Maryland

and

Daniel J. Fisher
Lance T. Yonkos
University of Maryland
Wye Research and Education Center
Queenstown, Maryland

Prepared for

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ABSTRACT

The Potomac River estuary, the second largest tributary to Chesapeake Bay, is an important nursery and spawning area for both migratory and resident fish species of commercial and recreational importance. The objective of the study was to evaluate the potential for endocrine disrupting effects on fish collected from this watershed. The study sites were located downstream of a major wastewater treatment plant for the District of Columbia and in the Anacostia River, a tidal tributary. These sites were selected because they were located in an urbanized area, near multiple sources of endocrine disrupting compounds. The reference site was located on the tidal Patuxent River adjacent to the Jug Bay Wetlands Sanctuary. We measured vitellogenin (Vtg) production and gonadal histopathology in field collected male common carp (*Cyprinus carpio*) from these sites as indicators of exposure and response to estrogenic compounds. Vtg was found at detectable levels in 100 % (16 of 16) of the male carp from all sites. Vtg concentrations were all less than those measured in females, and ranged from 0.001 mg/ml (the detection limit) in the Potomac River to 0.058 mg/ml measured in an Anacostia carp. There were no statistical differences in the mean Vtg concentrations in male carp collected from the two potentially impacted sites and the reference site; however, Vtg levels in fish from all three sites appear to be above normal concentrations based on a comparison to data from several hundred carp collected in the Mississippi River Basin. The measurement of detectable levels of Vtg in all male carp collected suggests there may be widespread low level exposure of fish populations to endocrine disrupting compounds in the Potomac River. Histopathological analyses did not indicate corresponding effects on reproduction; however the sample size is limited. The finding of elevated Vtg in male carp from the reference site is somewhat surprising and may be related to exposure to agricultural chemicals, several of which are presumed endocrine disruptors. We recommend additional study including sampling a larger number of fish and the histopathological evaluation of both females and males at all sites.

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INTRODUCTION

The Potomac River estuary, the second largest tributary to Chesapeake Bay, is an important nursery and spawning area for both migratory and resident fish species of commercial and recreational importance, including: striped bass (*Morone saxatilis*), white perch (*Morone americana*), American shad (*Alosa sapidissima*) and American eel (*Anguilla rostrata*). Previous studies by the U.S. Fish and Wildlife Service's Chesapeake Bay Field Office (CBFO) in the Potomac estuary have reported elevated tissue concentrations of polychlorinated biphenyls (PCBs) and DDT (both presumed endocrine disruptors, Tyler *et al.* 1998) in carp and channel catfish (Pinkney *et al.* 1995a, b, 1997). These data prompted Maryland and Virginia to issue a fish consumption advisory, in 1999, for a 38 mile stretch of the river, from Aquia Creek to the District of Columbia. The advisories warn the public to limit consumption of channel catfish greater than 18 inches due to PCB concentrations and also warn against eating carp and eels. In the District of Columbia, both the Potomac and the Anacostia River, a Potomac River tributary, also have a fish consumption advisory for bottom feeding fish based on elevated levels of PCBs and chlordane (Velinsky and Cummins 1994). These advisories suggest that fish, and wildlife that consume fish, are being exposed to endocrine disrupting contaminants in the Potomac estuary. The ultimate effects of this exposure are unknown.

The objective of the study was to evaluate the potential for endocrine disrupting effects on fish collected from the tidal Potomac River watershed. We measured vitellogenin (Vtg) production and gonadal histopathology in male common carp (*Cyprinus carpio*) as indicators of exposure and response to estrogenic compounds. Vtg is normally synthesized in the liver of females during oogenesis and is a precursor of egg yolk (Goodbred *et al.* 1997). The presence of Vtg in the plasma of male fish indicates exposure to an estrogenic stimulus and high Vtg concentrations have been associated with a decrease in testes weight and changes in the development of sperm (Jobling *et al.* 1996). Carp were chosen for study because it is one of the species for which simple methods to measure Vtg production are available, it appears to be relatively sensitive to endocrine disrupting effects (Folmar *et al.* 1996, Goodbred *et al.* 1997), and the species is abundant throughout the Potomac River. Furthermore, previous studies by CBFO in the Potomac estuary have indicated elevated tissue concentrations of PCBs and DDT in carp.

METHODS

Study Sites

Adult carp were collected from two sites in the tidal Potomac River estuary and a reference site on the tidal Patuxent River (Figure 1). The Potomac study sites were located downstream of a major wastewater treatment plant for the District of Columbia (Blue Plains) and in the Anacostia River, a tidal tributary (Figure 1). These sites were selected because they were located in urbanized area, near multiple sources of endocrine disrupting compounds (EDCs). Several studies have indicated that effluents from waste water treatment plants are estrogenic and effects can be manifest many kilometers downstream (Jobling *et al.* 1998; Folmar *et al.* 1996). Compounds such as ethinylestradiol (a component of birth control pills) and nonylphenols (a degradation product of surfactants used in common household cleaners and paints) are known

EDCs and, along with natural estrogens, are frequently found in sewage effluents. In addition, studies have shown that mixtures of different estrogenic chemicals are more potent than individual chemicals (Sumpter and Jobling 1995). The Anacostia River is highly urbanized and contaminated with PCBs, also known endocrine disruptors. In addition, during high rainfall, untreated sewage enters the Anacostia through numerous combined sewer outfalls. The reference site was located on the tidal Patuxent River adjacent to the Jug Bay Wetlands Sanctuary. The land use in this watershed is primarily forested and agricultural, with very little urbanization (U.S. EPA Chesapeake Bay Program, Middle Patuxent River Watershed Profile, http://www.chesapeakebay.net/wshed.htm).

Fish collection and sample processing

Adult carp were collected by electroshocking between May 30, 2001 and August 7, 2001. We originally planned to sample four sites on the Potomac River; however, several collection attempts failed due to equipment malfunctions on the electrofishing boats. At least five adult male fish were collected from each site. Males were initially identified by external examination as those that "milked" or produced sperm when the ventral portion of the fish was squeezed. Sex was confirmed upon necropsy and internal examination of the gonads. Several females were also collected in the Potomac (n=2) and Anacostia (n=4) Rivers to serve as "positive" controls for the Vtg analyses.

Collected fish were held in live wells until processing, which usually occurred within 3 hours of collection. Methods followed those in Goodbred *et al.* (1997) and are briefly described here. Fish were assigned a sample number that included collection site, species identifier and sample number. In this study, the three sites were identified as follows: CA = Anacostia River, CP= Potomac River downstream of the wastewater treatment plant and JB= reference site located at Jug Bay on the Patuxent River. Fish were anaesthetized in MS 222, weighed and measured. A 3ml or 5 ml syringe was used to draw approximately 1.5 ml of blood from the posterior caudal artery. Blood was ejected into heparinized centrifuge tubes and spun for 10 minutes at 4000 rpm to obtain plasma. The plasma was aspirated with a transfer pipette into a new container and placed on dry ice. Within 24 hours, samples were placed into liquid nitrogen. Vtg samples were shipped on dry ice to Nancy Denslow, University of Florida, who analyzed them for Vtg using Enzyme-Linked Immunosorbent Assay (ELISA) (Folmar *et al.* 1996). The detection limit for plasma Vtg was 0.001 mg/ml.

Gonads were dissected from the body and weighed to calculate the gonadosomatic index (GSI), expressed as the gonad weight as a percentage of total body weight. After weighing, five to six 1-cm pieces were cut from the bottom end of the testes, fixed with 10% neutral buffered formalin then paraffin embedded using standard histological procedures for thick section light microscopy. Five μm thick sections were mounted on glass slides and stained with hematoxylin and eosin, according to Luna (1968). Liver samples were collected from the Potomac and Anacostia River fish and processed in a similar way. These samples were collected and analyzed opportunistically under the auspices of a companion study focused on the health of resident fish in the Potomac River estuary. Although histopathological effects on the liver have not yet been linked to endocrine disruption, we have included the results because they are a good indicator of overall fish health (Adams *et al.* 1993).

In addition to specimens being examined for pathology, a semi-quantitative method was used to assess the level of maturity of testicular tissues. Briefly, a scale from 0 to 5 was devised to rank specimens based on the proportion of spermatogenic cells at various stages of differentiation (e.g., spermatogonia, spermatocytes, spermatids and mature spermatozoa). A greater proportion of mature spermatozoa relative to less mature cells was assumed to indicate a more advanced level of maturity. Multiple sections from different regions of gonad were assessed to determine ranks for individual fish with higher ranks indicating more advanced maturity. A maturity index for each location was calculated as the mean of all individual ranks from that location.

Data Analysis

Data on total length, weight, GSI and Vtg concentrations in carp from each site were first tested to determine if they met the assumptions for parametric statistics, equal variance and a normal distribution. Mean concentrations were compared with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Data not meeting assumptions for parametric statistics were log-transformed. If assumptions were still not satisfied, the data were analyzed via the Kruskal-Walllis (K-W) test, followed by Dunn's method for multiple comparisons. A p value of 0.05 was used for all tests of significance.

RESULTS AND DISCUSSION

There were significant differences in the size (both weight and length) of male carp collected from each site (Table 1). Jug Bay male carp were significantly heavier than Potomac River carp (K-W test, $p=0.006$; Dunn's Method, $p < 0.05$) and carp from both Jug Bay and the Anacostia River were significantly longer than fish from the Potomac River (ANOVA, $p < 0.001$; Tukey's, $p < 0.05$). Due to the small sample size and the number of variables affecting size structure of fish populations, we cannot draw any conclusions concerning the reasons for size differences among sites.

Vtg was found at detectable levels in 100 % (16 of 16) of the male carp from all sites (Table 1). Vtg concentrations were all less than those measured in females (Table 1), and ranged from 0.001 mg/ml (the detection limit) in the Potomac River to 0.058 mg/ml measured in an Anacostia carp. McDonald *et al.* (2002) reported detecting Vtg in only 28 of 384 male carp collected from the Mississippi River Basin. They detected trace amounts of Vtg (0.001 - 0.01 mg/ml) in at least one male carp from 15 stations and greater concentrations (0.01 - 0.03 mg/ml) in only one carp from each of two stations. Vtg concentrations equivalent to that commonly found in females were detected at only two stations (0.958 and 2.645 mg/ml). In the present study, there were no statistical differences in the mean Vtg concentrations in male carp collected from the two potentially impacted sites and the reference site (K-W test, $p = 0.451$). Vtg concentrations in all fish, including the reference, appear to be above normal concentrations based on the results of McDonald *et al.* (2002), but less than concentrations in male carp collected in a sewage effluent canal near St. Paul, Minnesota (Folmar *et al.* 1996). It is possible that seasonal effects on Vtg levels may explain some of the observed differences between our results and those of McDonald et al (2002), as they collected fish in the fall and ours were collected in early summer. Nonetheless, our results suggest low level exposure to endocrine modulating compounds may be

occurring at all three sites; however, the potential seasonal effects on Vtg levels needs to be explored.

The finding that concentrations of Vtg in male carp from the reference site, Jug Bay on the Patuxent River, also suggest exposure to EDCs, is somewhat surprising. The surrounding land use is a combination of agriculture (~ 40%), forest and wetlands (~ 50%), with very little urbanization (~ 6%). However, there are some agricultural chemicals, most notably the herbicide atrazine, that are thought to have endocrine modulating effects (Goodbred *et al.* 1997). In another study conducted by our office, we found fairly high levels of atrazine in the Patuxent River near Jug Bay (McGee 2002). Therefore, it is possible that this herbicide, possibly in conjunction with other EDCs, is inducing Vtg production in indigenous carp.

Vtg in females ranged from 0.116 to 89.309 mg/ml in the Anacostia River (Table 1). McDonald *et al.* (2002) measured Vtg in 384 female carp of comparable size from 45 stations in the Mississippi River Basin. They reported concentrations ranging from not detected (i.e., below 0.001 mg/ml) to a high value of 16.201 mg/ml. Interestingly, Vtg concentrations in the Anacostia female carp were several times higher than the highest value measured in the McDonald *et al.* (2002) study; however, their samples were collected in the fall whereas our samples were collected in early summer. Vtg concentrations are affected by many factors including reproductive stage, season, temperature and chemical composition of the environment. Any one or a combination of these factors may explain the high concentrations of Vtg in female carp from the Anacostia River.

The gonadosomatic index (the ratio of gonad weight to total body weight) was estimated for the collected male carp. There were no significant differences among the mean GSI values from the three sites (ANOVA, $p=0.71$) and no significant correlation between Vtg concentrations and the GSI (Spearman, $p > 0.05$). The GSI, like concentrations of Vtg, is affected by factors such as the reproductive cycle, season, age, sex, as well as environmental influences. A reduction in GSI and impaired gonadal development has been reported in response to chemicals such as mercury and several insecticides. The range in values in the present study (1 - 6%) is comparable to the 1-10% GSI values typically observed by McDonald *et al.* (2002).

Testes from all five male carp from the Anacostia River appeared healthy and without pathology. The mean maturity index for the Anacostia samples was 3.5 with one individual ranking only 2.0. Hepatic tissue from two of five Anacostia samples had evidence of mild pathology. In one case (CA05), a mild acute inflammatory response was indicated by a focal accumulation of leukocytes. In the other (CA04), several regions of marked hepatocytic hypertrophy occurred in association with areas of mild hemorrhage and necrosis. Pathology was consistent with handling stress during capture.

Tissues from two of five male carp from the Potomac River site contained multiple granulomata. Granuloma formation is a chronic inflammatory response which may be initiated by a variety of infectious and/or noninfectious agents. Lesions are typically characterized by large aggregates of immune cells (macrophages, lymphocytes, fibroblasts, giant cells, etc.) in varying states of degradation often surrounded by one or several layers of lymphoid cells. In one case (CP05), 4

small granulomata were found within a hepatic portal region containing numerous individual macrophages and evidence of fibrosis. These granulomata, including one heavily encased in epithelioid cells, along with the macrophage aggregates and fibrosis are all consistent with a chronic and ongoing mycobacterial infection. Testicular tissue from this specimen also contained at least 5 small granulomata dispersed within the organ (i.e., not in proximity to one-another). The second case (CP07) contained numerous small to medium sized granulomata associated with mesenteric pancreatic tissue and many granulomata in testicular tissue (Figure 2). Those lesions found within testicular tissue were predominantly associated with the dense connective tissue at the posterior end of the organ. Again, the granulomata were consistent with a mycobacterial infection. While the occurrence of granulomata in feral fish is not surprising, observation of such a lesion within testicular tissue is uncommon and therefore, worth noting. The other three fish captured in the Potomac River were free from apparent pathology. The mean maturity index for this site was 3.2 with a range of 2.5 to 4.0.

Testes from six male carp captured at Jug Bay appeared healthy and without evidence of pathology. The mean maturity index for the site was 3.0 with a range of 2.5 to 3.5. Because hepatic tissue was not collected from fish from this site, an assessment of this organ was not possible.

Overall, the histopathological results do not show any evidence of intersex, or other, more subtle, effects on the reproductive system of collected male fish. Intersex, defined by the simultaneous presence of both male and female gonadal characteristics, has been found in a high proportions of roach (*Rutilus rutilus*), a cyprinid related to the common carp, collected downstream of sewage treatment plants in Europe (Jobling *et al.* 1998).

Conclusions

The measurement of detectable levels of Vtg in all male carp collected suggests there may be widespread low level exposure of fish populations to endocrine disrupting compounds in the Potomac River. Histopathological analyses do not indicate corresponding effects on reproduction; however the sample size is limited. We recommend additional study including a larger number of fish and the histopathological evaluation of both females and males.

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Table 1. Characteristics and biomarkers for adult carp.

Location	Species	Sample ID #	Sex	Length (mm)	wt (kg)	VTG (mg/ml)	GSI	Maturity Index
Anacostia River	common carp	CA01	F	402	1.59	0.553	ND	
Anacostia River	common carp	CA02	F	435	2.95	76.759	ND	
Anacostia River	common carp	CA03	M	423	1.47	0.016	0.05	4.0
Anacostia River	common carp	CA04	M	505	2.72	0.058	0.06	3.0-3.5
Anacostia River	common carp	CA05	M	354	1.02	0.007	0.03	2.0
Anacostia River	common carp	CA06	M	531	2.95	0.007	0.06	4.0-4.5
Anacostia River	common carp	CA07	F	390	ND	89.309	ND	
Anacostia River	common carp	CA08	M	504	ND	0.011	ND	4.0
Anacostia River	common carp	CA09	F	306	0.68	0.116	ND	
		Avg. males only		**463**	**2.04**	**0.020**	**0.05**	**3.5**
Potomac River	common carp	CP01	F	325	0.79	0.946	ND	
Potomac River	common carp	CP02	F	305	0.45	ND	ND	
Potomac River	common carp	CP03	M	310	0.45	0.021	0.03	3.5-4.0
Potomac River	common carp	CP04	M	297	0.34	0.038	0.04	3.0-3.5
Potomac River	common carp	CP05	M	185	0.34	0.002	0.04	2.5-3.0
Potomac River	common carp	CP06	M	225	0.11	0.001	0.08	3.0-3.5
Potomac River	common carp	CP07	M	265	0.34	0.006	0.03	3.0
		Avg. males only		**256**	**0.32**	**0.014**	**0.04**	**3.2**
Jug Bay	common carp	JB01	M	620	3.52	0.004	0.03	3.0
Jug Bay	common carp	JB02	M	630	3.29	0.026	0.06	3.0-3.5
Jug Bay	common carp	JB03	M	577	2.72	0.048	0.04	3.0-3.5
Jug Bay	common carp	JB04	M	586	2.84	0.014	0.06	2.5-3.0
Jug Bay	common carp	JB05	M	771	7.84	0.025	0.04	3.0-3.5
Jug Bay	common carp	JB06	M	333	0.79	0.012	0.01	2.5
		Avg. males only		**586**	**3.50**	**0.020**	**0.04**	**3.0**

ND = No data

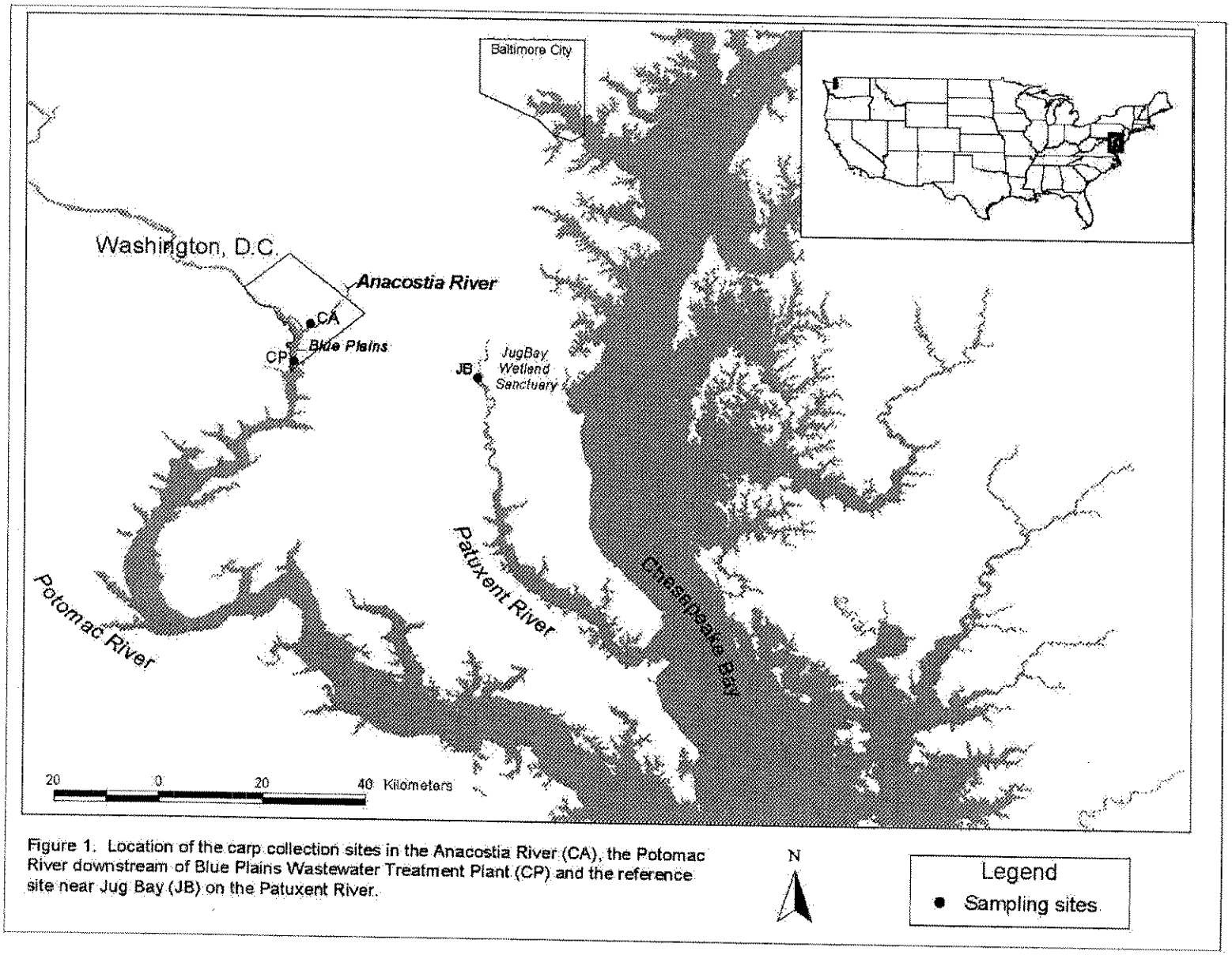

Figure 1. Location of the carp collection sites in the Anacostia River (CA), the Potomac River downstream of Blue Plains Wastewater Treatment Plant (CP) and the reference site near Jug Bay (JB) on the Patuxent River.

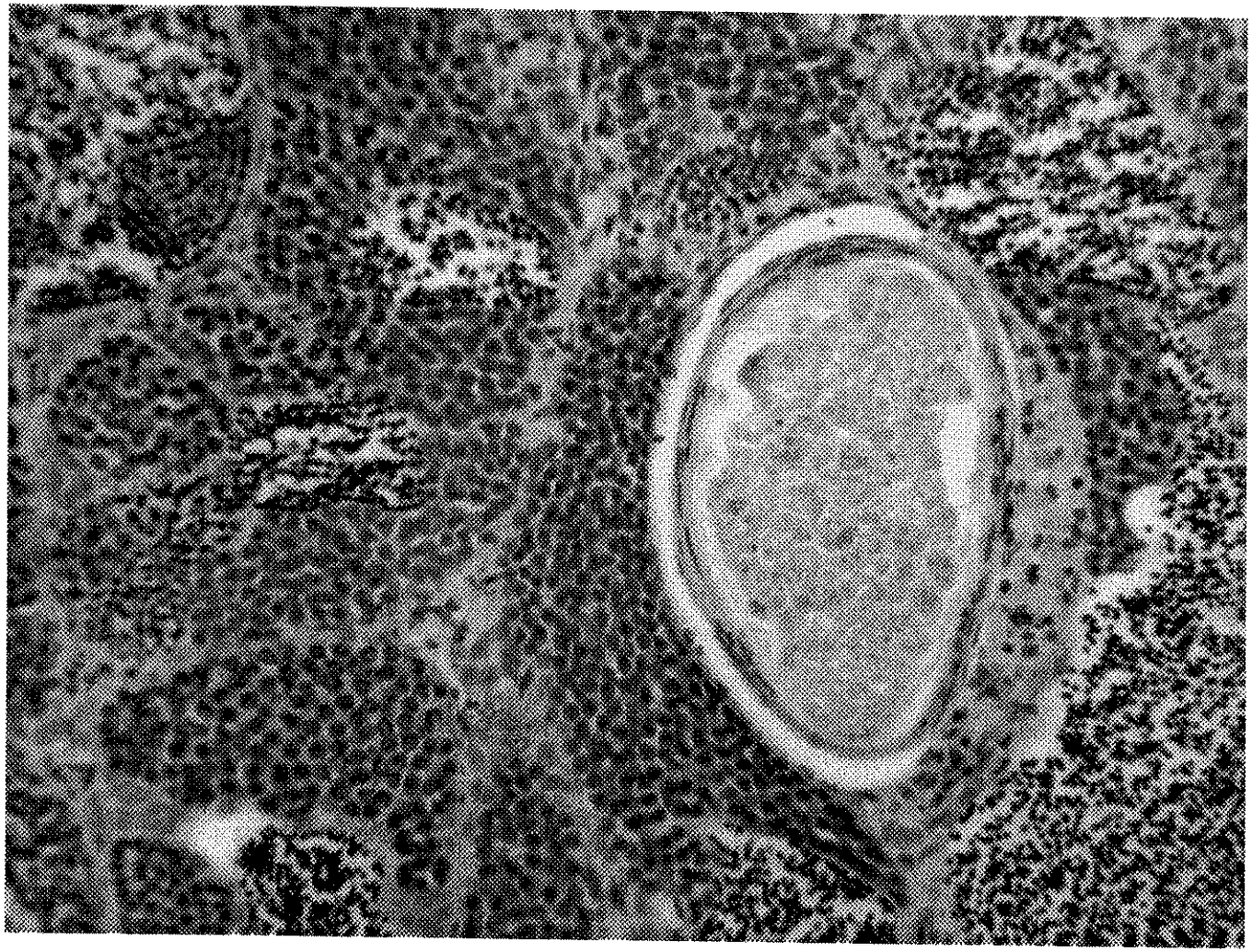

Figure 2. Granuloma in the testicular tissue of carp (specimen CP07) collected from the Potomac River site.