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Initial Survey of Plasma Vitellogenin and Gonadal Development in Male Carp (*Cyprinus carpio*) From Three Locations in New Jersey, USA

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The presence of vitellogenin (Vtg), an egg yolk protein precursor, in the blood of male fish can be used as a biomarker for exposure to endocrine-disrupting chemicals (EDCs). Normally, vitellogenesis is induced in mature females by endogenous estrogens. However, males also possess the Vtg gene, which can be activated by exposure to exogenous estrogenic compounds (Jobling et al. 1998). High plasma Vtg levels have been measured in male carp downstream from sewage treatment plants (STPs), and some of the carp have displayed intersex testes, in which oocytes are present in the testicular tissue (Solé et al. 2003).

The Gonadal Somatic Index (GSI) and gonad histopathology have been used as indicators of the reproductive health of fish populations (McDonald et al. 2000). Findings have shown that male carp collected from waters receiving municipal and industrial effluents have a lower GSI and a higher incidence of gonadal abnormalities than carp from areas receiving lower levels of contaminants (Lee et al. 1999; Patino et al. 2003). The proliferation of macrophage aggregates in carp testes has been used as a biomarker for exposure to contaminants (Patino et al. 2003) and may be an indicator of oxidative stress (Blazer 2002).

Because New Jersey is the most densely populated state in the United States, there is considerable potential for the release of EDCs into its aquatic environment. However, very little information exists on exposure of fish to EDCs in the state. Zhou et al. (1999) found evidence of thyroid dysfunction in mummichog (*Fundulus heteroclitus*) from a contaminated salt marsh creek. McArdle et al. (2004) found no evidence of hepatic Vtg induction in mummichog from a contaminated estuarine site in the New York–New Jersey harbor complex. Data on exposure of freshwater fish are lacking.

The purpose of the current study was to search for evidence of EDC exposure and assess gonad histology in male carp (*Cyprinus carpio*) from three sites in New Jersey, and to develop strategies and methods for future research.

Materials and Methods

Location, flow, and other related information on surface water discharges was obtained using ArcView 3.2 Geographic Information System (GIS) software (ESRI, Redlands, CA, USA), with data layers downloaded from the New Jersey Department of Environmental Protection (NJDEP) GIS web page (http://www.state.nj.us/dep/gis/lists.html). Some additional information was obtained by personal communications with NJDEP staff (NJDEP Division of Compliance and Enforcement, Northern Office, Cedar Knolls, New Jersey).

Three sites were selected for study: the lower Passaic River, Crosswicks Creek, and Spruce Run Reservoir. The Passaic River is considered one of the most impacted rivers in the northeastern United States. Approximately 2 million people, one-fourth of the state's population, live within the watershed, and more than 80% of the lower Passaic watershed is urbanized. Water quality is impaired as a result of industrial and municipal treatment plant discharges as well as non–point source runoff. A total of 19 major wastewater treatment plants discharge into the nontidal

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portion of the river (United States Environmental Protection Agency 2005).

Carp were collected in the nontidal section above the Dundee Dam at Elmwood Park. Crosswicks Creek is a tidal freshwater stream. Carp were collected below a large municipal wastewater treatment plant that discharges 40 million L/day of treated effluent. The carp were collected in a zone extending from the discharge to a point approximately 3 km downstream.

We were unable to locate a reference site totally free of sewage discharges at which carp were abundant. Spruce Run Reservoir, a water supply impoundment, was selected as the reference site although four small discharges to the reservoir or its tributaries are permitted. Two package plants serving a school and a psychiatric hospital discharge a total of approximately 0.23 million L/day of treated domestic wastewater into tributary streams. A filtration/carbon absorption facility to remediate fuel-oil contaminated groundwater discharges approximately 0.19 million L/day of treated groundwater into another tributary. Also, a rock quarry discharges stormwater runoff into the lake via a storm sewer.

Carp were collected by pulsed-DC electrofishing during late-summer low-flow conditions. All the fish were collected in a 3-week period from September 8 to 30, 2005 to minimize any potential confounding effects from seasonal variations in hormonal response. Because carp spawn in the late spring/early summer period (Scott and Crossman 1973), we expect that the collections took place during the period of gonadal recrudescence. We attempted to select for males based on the appearance of the vent. Fish that could be positively identified as females were released.

We established a target sample size of 10 males per site, which was sufficient to detect EDC exposure in other studies of feral carp (Folmar et al. 1996; Petrovic et al. 2002). Carp were returned to the laboratory alive, well, and anesthetized with MS-222. Kahl et al. (2001) reported that anesthesia with MS-222 did not effect plasma Vtg in male fathead minnows (*Pimephales promelas*).

The total length and weight of the carp were recorded, and blood was collected in a plastic centrifuge tube by severing the caudal peduncle. Blood samples also were taken from a few females collected inadvertently. The blood was immediately centrifuged. Aprotinin was added (2 TIU/mL), and the samples were frozen at –80° C. The GSI was calculated as gonad weight × 100/body weight, and condition factor was calculated as weight × 10^5/length^3 (Anderson and Gutreuter 1983).

A sample from the middle of each testis was removed and fixed in 10% formalin for histologic processing. After dehydration, the tissues were embedded in paraffin, cut into 7-µm sections using a rotary microtome, and stained in hematoxylin and eosin (H&E). A subsample of histologic sections from each location also was stained with periodic acid-Schiff reagent to demonstrate the presence of polysaccharides. Histologic sections of gonads were examined by light microscopy. The area of each testis section occupied by macrophage aggregates was quantified using an ocular grid at a magnification of 100.

Plasma Vtg was measured using a commercially available carp enzyme-linked immunoassay (ELISA) kit (Amersham Biosciences, Piscataway, NJ, USA). The sensitivity of the assay, calculated as the concentration on the standard curve equal to three standard deviations above the mean blank absorbance (n = 10), was 0.028 µg/mL. The minimum dilution factor used for plasma samples was 2:1, resulting in a method detection limit of 0.056 µg/mL. The percentage of recovery for a plasma sample spiked with 0.200 µg/mL of Vtg was 86.5%.

Statistical analyses were performed using SAS software (Release 8.02; SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was used to compare total length, weight, and percentage of macrophage aggregates in the testes among sites. Tukey's test was used for post hoc multiple comparisons. Macrophage aggregate data were arcsine transformed. A Kruskal-Wallis test was used to compare GSI among sites. A nonparametric post hoc multiple comparison test was performed using a SAS macro written by Juneau (2004) based on a procedure suggested by Dunn (1964). The incidence of Vtg detection in male carp from different sites was compared using Fisher's exact test. An alpha level of 0.05 was used to determine statistical significance.

Results and Discussion

All the males collected were sexually mature according to histologic examination of the testes. Carp collected at Crosswicks Creek were significantly larger than those from the Passaic River or Spruce Run Reservoir ($p < 0.01$) (Table 1).

The highest incidence of plasma Vtg detection was found among the males from Crosswicks Creek (Table 1), although among-site differences were not statistically significant ($p = 0.08$). However, the incidence of plasma Vtg detection in Crosswicks Creek males was significantly higher ($p = 0.04$) than the combined incidence at the other two locations. A carp from Crosswicks Creek also had the highest Vtg concentration measured in any male in the study. The exact Vtg concentration was not determined in two Crosswicks Creek samples due to the lack of sufficient plasma to run additional dilutions. Therefore, these concentrations were recorded as greater than 0.5 and greater than 5.0 µg/mL. The concentrations for the remaining four males in which Vtg was detected ranged from 0.248 to

Table 1 Mean biologic parameters of male carp from three sites in New Jersey[a]

Site	Total length (cm)	Weight (g)	Condition factor	GSI	Macrophage aggregates (%)	Incidence of Vtg detection	Vtg concentration range (μg/mL)
Crosswicks Creek	63.5 ± 5.8 A (11)	3545 ± 808 A (11)	1.37 ± 0.11 A (11)	6.11 ± 2.88 AB (11)	0.42 ± 0.34 A (11)	6/11 A	0.248 to > 5
Passaic River	50.3 ± 2.1 B (10)	1888 ± 274 B (10)	1.47 ± 0.10 A (10)	8.28 ± 1.44 B (7)	0.41 ± 0.26 A (10)	1/10 A	3.038
Spruce Run Reservoir	54.3 ± 4.8 B (9)	1949 ± 549 B (9)	1.20 ± 0.15 B (9)	5.14 ± 1.71 A (9)	0.75 ± 0.66 A (9)	2/9 A	0.149–0.995

GSI, Gonadal Somatic Index; Vtg, vitellogenin

[a] Sample sizes are in parentheses. Means within a column followed by the same letter are not significantly different ($p > 0.05$)

0.522 μg/mL. Elevated levels of plasma Vtg in male carp from Crosswicks Creek were consistent with exposure to EDCs. Numerous studies have documented elevated plasma Vtg in carp downstream from STPs (Lavado et al. 2004; Petrovic et al. 2002; Solé et al. 2002; Solé et al. 2003).

Currently, no systematic testing is performed in New Jersey for EDCs in surface waters or effluents. Therefore, we cannot assess actual exposure of fish at the three sites. However, alkylphenol polyethoxylates have been reported in Passaic River tributaries (Barnes et al. 2002), and bis(2-ethylhexyl)phthalate has been reported in effluents discharged into Crosswicks Creek and the Passaic River Basin (http://www.nj.gov/dep/opra/online.html). In future studies, Vtg assays may be useful in targeting sites for detailed chemical analyses.

Although the Passaic River is an urban, effluent-dominated stream, Vtg was detected only in 1 of the 10 males tested. One possible explanation is that carp in the Passaic River were sampled approximately 20 km downstream from the nearest domestic sewage discharge, whereas those in Crosswicks Creek were sampled within 3 km of a discharge. Estrogenicity could be reduced by dilution or biodegradation with increasing distance from the point of discharge. Jürgens et al. (2002) reported that 17β-estradiol had a half-life as short as 0.2 days in river water during the summer. Solé et al. (2000) reported higher plasma Vtg concentrations in male carp collected near STPs than in carp collected further downstream. Future surveys in the Passaic basin should include sites closer to wastewater discharges.

The timing of the sample collection also could have affected the results. Fish were collected over a short period (3 weeks). Both the concentration of EDCs in sewage effluent and Vtg induction may vary seasonally (Petrovic et al. 2002; Solé et al. 2002).

The finding of two male carp with detectable Vtg at the reference site may reflect, at least in part, the difficulty of finding uncontaminated reference sites in a densely populated state such as New Jersey. Although point source discharges to the reservoir are minimal, EDCs also may be present in non–point source runoff from agricultural operations or leachate from home septic systems (Koplin et al. 2002), which also could contribute to the observed Vtg induction. Better reference data may be obtained by using laboratory control subjects (Jobling et al. 1998), multiple reference sites (Snyder et al. 2004), or both.

There is some discrepancy in the literature regarding the interpretation of low plasma Vtg levels such as those we report. Plasma Vtg levels in the low μg/mL range have been reported in carp captured at reference sites or maintained as laboratory control subjects (Snyder et al. 2004; Solé et al. 2002; Villeneuve et al. 2002). Lee et al. (1999) considered plasma Vtg up to 10 μg/mL in male carp to represent background levels. Others have reported much lower plasma Vtg levels (in the ng/mL range) in feral male carp from both polluted and unpolluted habitats (Carballo et al. 2005; Matsumoto et al. 2002). The reasons for the wide range of reported values is not clear, but they could be attributable to factors such as differences in analytical methods, genetic variation among fish populations, and different levels of background EDC exposure. Because it is not currently possible to establish a generally applicable normal range of plasma Vtg for diagnostic purposes, evidence of exposure to EDCs is best established statistically by comparison with suitable controls or reference fish. We believe that the plasma Vtg levels in carp from Crosswicks Creek indicate some degree of EDC exposure. However, larger sample sizes than those used in this study may be needed for reliable detection of low-level Vtg induction.

Carp from Spruce Run Reservoir had a GSI lower than those from the Passaic River, although the latter site is known to have impaired water quality. Although we cannot rule out a contaminant-related cause for the lower GSI, it appears more likely that the lower GSI was caused by other factors. Condition factor, a general measure of nutritional status (Bagenal and Tesch 1978), also was lowest in carp from Spruce Run Reservoir ($p < 0.01$). Nutritional status can have a large influence on reproductive development, thus affecting GSI as well (Adams et al. 1999). At the time the fish were collected, the reservoir had been drawn down

extensively for an extended period because of the dry conditions, which could result in increased stress and decreased food availability for a bottom-feeding species such as carp.

No evidence of the intersex condition was observed in the fish from any site either at necropsy or during microscopic examination of histologic sections of testes. All testes sections examined contained varying numbers of cells identified as macrophage aggregates. The cells were granular with a yellow-tan color in H&E stained sections and red in sections stained with periodic acid-Schiff reagent, indicating the presence of polysaccharides. The tissue area occupied by macrophage aggregates did not differ significantly among sites ($p = 0.35$).

Plasma Vtg levels in all the male carp were well below those measured in females incidentally captured at the three study sites (mean, 6,840 µg/mL; range, 3,090–13,400 µg/mL; n = 10). Some studies have found male carp below STPs with plasma Vtg concentrations similar to those in females. In some males, the highly elevated Vtg was associated with gonadal abnormalities such as the presence of oocytes in the testes, testicular atrophy, macrophage aggregates, and decreased GSI (Lavado 2004; Solé et al. 2003). Our findings regarding carp in Crosswicks Creek are similar to those of Carballo et al. (2005) and Matsumoto et al. (2002), who reported low levels of plasma Vtg but normal GSI and gonad histology in male carp from waters that receive treated sewage effluent. Differences in the estrogenicity of effluents (Harries et al. 1999) and the degree of dilution in the receiving water (Jobling et al. 1998) could explain some of the reported differences. Fish movement also could affect the degree of exposure. The lower end of the sampling reach on Crosswicks Creek is only 3 km from its confluence with a much larger river. Fish could move in and out of Crosswicks Creek, resulting in lower exposure to EDCs in the effluent.

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