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A survey of indicators for reproductive endocrine disruption in *Fundulus heteroclitus* (killifish)
at selected sites in the Chesapeake Bay

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Abstract—Plasma vitellogenin and related parameters in the killifish *Fundulus heteroclitus* were measured at selected sites in the Chesapeake Bay. In males, vitellogenin was above the detection limit 14 % of the time, and detections did not differ between sites or seasons. Few differences in plasma vitellogenin levels were found between sites during fall in either male or female *F. heteroclitus*, the time of natural gonadal regression for this species. There was some variation in the ratio of male to female *F. heteroclitus*, but was not consistent at most sites. Significant negative correlations were found between reported sediment polycyclic aromatic hydrocarbons (PAHs) and GSI, and PAHs and plasma vitellogenin in females in both Spring 1999 and Spring 2000. Gonadal anomalies in *F. heteroclitus* included slight reductions in certain tissue types. Overall, reproductive endocrine disruption in the killifish *F. heteroclitus* at the sites sampled in the Chesapeake Bay appeared somewhat minimal.

Keywords: *Fundulus heteroclitus*, killifish, Chesapeake Bay, endocrine disruption, vitellogenin, estuaries

1. Introduction

Endocrine disrupting compounds (EDCs) are exogenous agents that cause adverse health effects in an intact organism or its progeny, subsequent to changes in endocrine function (European Commission, 1996). The occurrence of wildlife populations, particularly in the aquatic environment, exhibiting effects thought to be associated with endocrine disruption has prompted a significant amount of research to determine the extent and severity of impacts. A number of studies have found effects in fish from various locations (Jobling et al., 1998; Hinck et al., 2007; Blazer et al., 2007; Kleinkauf et al., 2004; Purdom et al., 1994; Harries et al., 1997; Lye et al., 1998; Allen et al., 1999; Collier et al., 1998; Scott et al., 2007; Kleinkauf et al., 2004).

EDCs include such diverse chemical groups as the alkylphenol polyethoxylate surfactants (Jobling and Sumpter, 1993), polycyclic aromatic hydrocarbons or PAHs (Anderson et al., 1996; Santodonato, 1997), the industrial intermediate bisphenol-A (Sumpter and Jobling, 1995), polychlorinated biphenyls (PCBs) (Thomas, 1989), and certain trace elements (Ruby et al., 2000; Thomas, 1989). There is also acknowledgement that when present in the aquatic environment as is sometimes seen adjacent to sewage treatment plants (STPs), natural and synthetic estrogens can contribute significantly to observed effects (Jobling et al., 2007; Purdom et al., 1994; Harries et al., 1997).

Much of the research to date on endocrine disruption has focused on reproductive effects. Part of the impetus for investigations with fish can be traced back to casual observations by anglers in the mid-1980s of hermaphrodite roach (*Rutilus rutilus*) inhabiting STP lagoons in the U.K.

(Routledge et al., 1998). Since then, reproductive effects observed in wild fish have ranged from the presence of biomarkers of exposure, to changes in gonadal structure that would likely impair reproduction.

Vitellogenin has become one of the most widely used biomarkers of exposure to EDCs. In egg laying fish, this phosphoglycolipoprotein produced by the liver is the precursor of egg yolk (Anderson et al., 1996). Although vitellogenin is typically associated with females, male fish also possess hepatic estrogen receptors, and the presence of elevated levels of vitellogenin in males has been used as an indicator of exposure to estrogen or estrogenic compounds (Sumpter and Jobling, 1995). Purdom et al. (1994) found clear evidence of increased levels of plasma vitellogenin in both sexes of rainbow trout (*Oncorhynchus mykiss*) exposed to STP effluents in the U.K. In addition, the presence of intersex (presence of ovotestes) roach in a number of rivers in the U.K. has been linked to exposure to STP effluents (Jobling et al., 1998). Endocrine disruption has also been found in fish in the estuarine and marine environments, and in some cases, effects have been linked to fish migration patterns (Kirby et al., 2004; Kleinkauf et al., 2004) or possible accumulation of estrogenic compounds through the food chain (Scott et al., 2006; Scott et al., 2007). Finally, there is recent evidence that upgrades to STP treatment processes can result in reduced levels of estrogenic contamination and associated endocrine disruption (Kirby et al., 2004; Kleinkauf et al., 2004).

Studies have also linked organic chemical contamination with effects on the endocrine system in fish. Female English sole (*Parophrys vetulus*) from a contaminated area in Puget Sound had significantly lower levels of 17 β -estradiol compared with those from less contaminated sites

(Johnson et al., 1988). In addition, inhibited gonadal development was found in this same species in an area of Puget Sound heavily contaminated with PAHs (Collier et al., 1998).

This paper presents the results of a survey of plasma vitellogenin and related parameters in the killifish *Fundulus heteroclitus*, a common inhabitant of the Chesapeake Bay. Pait and Nelson (2003) demonstrated that male *F. heteroclitus* respond to a number of estrogenic EDCs in the laboratory, producing plasma vitellogenin in a dose dependant manner. *F. heteroclitus* is a ubiquitous, nonmigratory species inhabiting salt, brackish, and occasionally freshwater marshes from Nova Scotia to northeast Florida, and is found characteristically close to shore (Abraham, 1985). Bigelow and Schroeder (1953) called *F. heteroclitus* “one of the most stationary of fishes”. Because *F. heteroclitus* is a nearshore, nonmigratory, and relatively nonmobile species, effects seen in the field would likely be related to conditions, activities or events occurring in that location. For this study a second species of fish, the sheepshead minnow (*Cyprinodon variegatus*), was collected when available at the same sites as *F. heteroclitus*, in order to compare vitellogenin concentrations in males of another species. Hemmer et al. (2001) showed that male *C. variegatus* readily produce vitellogenin when exposed to estrogenic EDCs.

2. Materials and Methods

2.1. Sampling sites

Fish were sampled from a total of eight sites in the middle and upper portions of the Chesapeake Bay (Figure 1). Sites ranged from pristine (reference) to industrialized, urbanized and agricultural areas, to sites influenced by discharges from STPs. Industrial activities near the

sampling sites in Baltimore (Fort Armistead, Patapsco River and Stonehouse Cove) included petroleum bulk storage, organic chemical and plastics manufacture, sewage treatment and a landfill. The Back River site in Baltimore County was located 1.7 km southeast of the Back River Wastewater Treatment Plant, which discharges approximately 150 million gallons of secondary treated water per day to the river. The primary reference site chosen for this study was Beaverdam Creek (Figure 1), located on the eastern shore of the Chesapeake Bay. Together with the Taylor Wildlife Management Area and the Blackwater National Wildlife Refuge, the area of marsh is approximately 100 km². There were no dwellings or commercial developments within 5 km of the site. A second reference site was located at the Point Lookout State Park at the confluence of the Potomac River and the Chesapeake Bay.

2.2. Sample collection

Fish were collected twice a year, between Fall 1998 and Spring 2000, once in spring/summer, the reproductive season for both *F. heteroclitus* and *C. variegatus*, and again in fall after reproduction had ceased. Sites were sampled up to four times over the course of the study. Stonehouse Cove in Baltimore was only sampled once as fish were not found during other visits; Beaverdam Creek was not sampled in Fall 1998. *F. heteroclitus* were collected using standard minnow traps (Frabill, Inc., Allenton, WI) baited with bread. Traps were set from shore or placed in the salt marsh at a depth of 1 meter or less from a canoe. The traps were checked after 30 minutes, typically long enough to collect between 50 and 200 *F. heteroclitus*. Ten adults of each sex were then selected and placed in separate aerated buckets and brought back to the laboratory, typically within two hours for processing. The remaining fish in the traps were sexed and then returned to their habitat. The average weight of male *F. heteroclitus* brought back to

the laboratory was 6.93 g, average length was 8.0 cm; the average weight of females was 8.01 g, average length 8.3 cm.

During spring/summer, *F. heteroclitus* were collected within two days of the full moon in the months of April, May and June. Gonadal development in *F. heteroclitus* has been shown to peak during this time of year in the mid-Atlantic (Taylor et al., 1979). Fall sampling occurred from late October to early November, with all sites sampled within a two-week period.

When available, males of a second species of fish, *C. variegatus* were collected at the same sites as *F. heteroclitus* in order to compare vitellogenin in another species. Male *C. variegatus* have been shown to readily produce vitellogenin when exposed to estrogenic EDCs (Hemmer et al., 2001). If male *C. variegatus* from a sampling site contained elevated levels of plasma vitellogenin, while vitellogenin levels in male *F. heteroclitus* from the same site were significantly lower or below the detection level, this would be evidence that *C. variegatus* was more sensitive than *F. heteroclitus* to the estrogenic EDCs present at the site. Male *C. variegatus* were found in sufficient numbers at three of the same sites established for *F. heteroclitus* including Patapsco River, Fort Armistead and Beaverdam Creek. The same minnow traps were used for *C. variegatus*.

2.3. Parameters measured

Blood samples were taken using heparinized micro-hematocrit capillary tubes, and spun at 11,500 rpm. Plasma vitellogenin was quantified using a direct enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody (MAb), specific for *F. heteroclitus* vitellogenin

(5F8(HL1562)) developed by the Interdisciplinary Center for Biotechnology of the University of Florida, was used in the ELISA. The antibody was tested and found to react specifically with *F. heteroclitus* vitellogenin, and not other male plasma proteins (K. Kroll, pers. comm.).

A brief description of the direct ELISA protocol follows, and is essentially that developed and used by a number of researchers (Denslow, et al., 1999; Bowman et al., 2000). Standards or samples (unknown) in triplicate were first coated (50 μ l) onto 96 well microplates (Dynatech Laboratories, Inc., Chantilly, VA) in phosphate buffered saline with sodium azide (PBSZ). Sample dilutions ranged from 1:500 to 1:25,000. Vitellogenin standards ranged from 0.01 to 1.0 μ g ml⁻¹. Control (containing no vitellogenin) male plasma was added to the standards and processed in the same way as samples. Plated samples and standards were allowed to incubate overnight at 4° C in a humidified chamber, followed by washing with PBSZ. Plates were then blocked, washed again with PBSZ followed by addition of the MAb and incubation again overnight at 4° C. Following this, plates were again washed and a second antibody, biotinylated anti-mouse IgG (Pierce Chemical Company, Rockford, IL), was added followed by washing and addition of streptavidin-alkaline phosphatase (Pierce Chemical Company, Rockford, IL). *p*-Nitrophenyl phosphate (pNPP) disodium (Sigma Chemical Company, St. Louis, MO) was then added to each well after a final wash. The microplates were read at 405 nm on a SPECTRAMax® PLUS (Molecular Devices Corporation, Sunnyvale, CA) spectrophotometer, and the standard curve fit using a quadratic equation. The detection limit for the ELISA was 5 μ g ml⁻¹. Plasma vitellogenin in male *C. variegatus* was analyzed by the University of Florida using the same methodology as *F. heteroclitus*, and a MAb specific for *C. variegatus*.

In addition to measuring plasma vitellogenin, the gonadosomatic index (gonad weight/body weight X 100) or GSI, and the hepatosomatic index (liver weight/body weight X 100) or HSI were calculated. The GSI has been used to assess the impact of EDCs in fish, particularly during the period of gonadal recrudescence (Harries et al., 1997; Jobling et al., 1996). The condition index or CI was calculated as $CI = W/L^3$ according to Mezin and Hale (2000), where W is the weight (g) multiplied by 100, and L is length in cm.

Gonadal tissues from male and female *F. heteroclitus* collected in Fall 1998 and Spring 1999 were assessed for gonadal anomalies. Tissues were fixed in Bouin's Fluid (Fisher Scientific, Pittsburgh, PA), dehydrated with ethanol, cleared with chloroform (J.T. Baker, Inc., Phillipsburg, NJ), and embedded in Paraplast® Plus (Oxford Labware, St. Louis, MO). Embedded tissues were transversely sectioned at 3 to 5 µm and stained with hematoxylin and eosin (Fisher Scientific, Pittsburgh, PA). Tissue sections were taken from the center of the gonad. Microscopic (Leica DMR, Wetzlar, Germany) examination (25x to 200x) of tissues from males was carried out to determine the presence of primary oocytes or oviducts in the testes (ovotestes), vacuolization of spermatogenic tissues, and thickening of interstitial tissues, all effects seen in impacted populations of wild fish (Jobling et al., 1998; Lye et al., 1998). Measurement of the cross sectional area of the gonad, spermatogenic tissue, efferent ducts, and spermatids/spermatozoa were made using Image-Pro® Plus (Media Cybernetics, Silver Spring, MD) digitizing software. It has been shown that spermatogenic lobule diameters in the common carp (*Cyprinus carpio*) are reduced by exposure to 4-(tert-pentyl)phenol (Gimeno et al., 1998). In *F. heteroclitus*, diameters of the spermatogenic tubules were measured to assess differences that might exist in fish between sites.

In female *F. heteroclitus*, oocytes were classified as to the stage of development (primary, early vitellogenic, late vitellogenic, mature, or atretic) according to the rating system described by Taylor and DiMichele (1980). All oocytes in a cross section were counted, and the oocytes in each stage were compared between sites and seasons. The ratio of male to female *F. heteroclitus* found in the traps at each sampling site was also recorded.

Finally, *F. heteroclitus* tissues from Spring 1999 were analyzed by the Environmental Quality Laboratory of the U.S. Department of Agriculture (Beltsville, MD) for 4-nonylphenol, nonylphenol monoethoxylate (NP1EO), and nonylphenol diethoxylate (NP2EO). Pooled whole fish samples were extracted using accelerated solvent extraction (ASE), followed by cleanup with aminopropylsilica. Extracts were derivatized with pentafluorobenzoyl chloride and analyzed by GC/NCI-MS in the SIM mode.

2.4. Statistics

All data were analyzed using JMP® statistical software (SAS Institute, Cary, NC). Data were first tested for normality (Shapiro-Wilks). When data were found to be normally distributed (e.g., GSI in male and female *F. heteroclitus* in Spring 1999 and 2000), an ANOVA was run followed by pairwise comparisons (Tukey-Kramer HSD). Data that did not meet the condition of normality (e.g., HSI in male and female *F. heteroclitus* in Spring 2000) were first ranked, followed by an ANOVA and pairwise comparisons on the ranked data. Some vitellogenin data sets, including all of the samples from male *F. heteroclitus*, and fall samples from female *F. heteroclitus* contained many nondetects. This data was analyzed using the methodology found

in Zar (1999). Data were first ranked from low to high; ties (e.g., nondetects) received the same rank number, and the ANOVA run on the ranked data. Vitellogenin in female *F. heteroclitus* collected during the spring were all above the detection limit, but did not meet the conditions of normality. Data were first ranked before the ANOVA and pairwise comparisons were made. Finally, a contingency table analysis was used to assess variations in the male/female ratio between sites. Significance was reported at $p < 0.05$.

A number of studies (Collier et al., 1998; Anderson et al., 1996; Thomas, 1988) have shown that contaminants can negatively impact endocrine function, particularly in terms of gonadal development. In the current study, a number of nonparametric Spearman's rank correlation coefficients were generated to assess whether there was an association between reported sediment contamination and selected parameters in *F. heteroclitus*. To do this, published data on contaminant levels in sediments adjacent to the sampling sites were assembled from readily available sources (Ashley and Baker, 1999; EPA, 2000). Sediment levels for 12 PAHs, five PCBs, and two trace elements (cadmium and chromium) were found in the literature for six of the sites where *F. heteroclitus* were collected. The sums of the inventoried contaminants by class (i.e., PAHsum, PCBsum) along with the two trace elements were used as indicators of the relative level of contamination between sites. Nonparametric correlation coefficients were generated to look at correlations between GSI, vitellogenin, fish weight and length, and the contaminants. An analysis was also carried out to group the data from those sites that had higher reported levels of contaminants versus those areas with lower contaminant levels to see if vitellogenin, GSI or HSI might vary by reported level of contamination.

3. Results

3.1. Vitellogenin

Results of the plasma vitellogenin analysis in male *F. heteroclitus* are shown in Figure 2. There were no significant differences between years within a season for male *F. heteroclitus*, so the data were combined. Approximately 14 % of the males tested had plasma vitellogenin levels above the detection limit. The highest concentration of vitellogenin detected in a male *F. heteroclitus* during this study was 580 $\mu\text{g ml}^{-1}$ at the Choptank River site (agricultural area). An ANOVA on the ranked male *F. heteroclitus* vitellogenin data revealed no significant differences between sites during the fall ($p = 0.6297$) or spring ($p = 0.1616$) samplings, and there were no significant differences in vitellogenin concentration in male *F. heteroclitus* between any of the sites over the course of the study ($p = 0.5412$). There were also no differences in vitellogenin concentration in males ($p = 0.1072$) or the percent detections ($p = 0.2092$) between seasons. Finally, vitellogenin in males *F. heteroclitus* was not significantly correlated with either fish weight ($p = 0.4396$) or length ($p = 0.1927$).

As mentioned, sites were also classified as being either contaminated (e.g., Fort Armistead, Back River, Patapsco River, and Annapolis) or uncontaminated (e.g., Beaverdam Creek and Point Lookout), based on the available sediment chemical contaminant data. An analysis of the ranked data indicated no difference in male *F. heteroclitus* vitellogenin concentration between contaminated and uncontaminated sites during the course of the study ($p = 0.3217$).

For this study, male *C. variegatus* were also collected when available, at the same sites as *F. heteroclitus*, in order to compare vitellogenin levels in males of another species. As noted, if *C. variegatus* had elevated levels of vitellogenin, but *F. heteroclitus* had lower or levels below the assay detection limits, this would be evidence not only for the presence of estrogenic EDCs in the environment, but also a greater sensitivity of *C. variegatus* to the EDCs present. However, only 5 % of *C. variegatus* plasma samples contained detectable levels of vitellogenin (data not shown), less than that for male *F. heteroclitus*. There were also no significant differences in plasma vitellogenin between sites ($p = 0.2016$) or seasons ($p = 0.0779$) for male *C. variegatus*. Finally, there were no significant differences in plasma vitellogenin between male *F. heteroclitus* and male *C. variegatus* at the sites where both species were sampled ($p = 0.3302$).

Plasma vitellogenin detected in female *F. heteroclitus* during the spring collections is shown in Figure 3. As expected, plasma vitellogenin in females was higher during the reproductive periods. In Spring 1999, an analysis of the ranked vitellogenin levels in female *F. heteroclitus* showed significant differences between sites ($p < 0.001$), and the pairwise comparisons indicated significantly lower vitellogenin in females collected from Fort Armistead, Patapsco River and Choptank River compared to Beaverdam Creek. In Spring 2000, ($p < 0.001$) however, females from Back River and Choptank River had significantly higher vitellogenin levels than females from Beaverdam Creek. Vitellogenin levels in female *F. heteroclitus* collected during the fall were low. The average concentration was $40 \pm 10 \mu\text{g ml}^{-1}$ (mean \pm SEM, data not shown). Fifty-four percent of fall plasma samples from female *F. heteroclitus* were below the ELISA detection limit for vitellogenin. ANOVAs on the ranked data indicated no significant

1 difference in the concentrations of vitellogenin in female *F. heteroclitus* between sites in Fall
2 1998 or Fall 1999.

3 3.2. Gonadosomatic index

4 There were significant differences in the male GSIs in Spring 1999 ($p < 0.0001$) and Spring
5 2000 ($p < 0.0001$) as shown in Table 1. In Spring 1999 male *F. heteroclitus* from Back River,
6 Fort Armistead, Patapsco River and Choptank River had significantly lower GSIs than males
7 from Beaverdam Creek. In Spring 2000, however, males from Annapolis and Patapsco River
8 had GSIs significantly higher than those from Beaverdam Creek. In Fall 1998 and 1999, there
9 were no significant differences in the male GSIs between the reference and other sites (data not
10 shown).

11 GSIs also varied by site for female *F. heteroclitus* in both Spring 1999 ($p < 0.0001$) and Spring
12 2000 ($p < 0.0001$). In Spring 1999, female *F. heteroclitus* from Fort Armistead had GSIs
13 significantly lower than females from Beaverdam Creek (Table 1). In Spring 2000, females
14 from Fort Armistead again had significantly lower GSIs than females from Beaverdam Creek;
15 females from Back River had GSIs significantly higher.

16 3.3 Hepatosomatic and condition indices

17 In Spring 1999, there were no significant differences ($p > 0.05$) in male *F. heteroclitus* HSIs
18 between sites (Table 1). In Spring 2000 ($p < 0.001$), an analysis of the ranked data indicated that
19 HSIs in males from Back River and Annapolis were significantly higher than males from

Beaverdam Creek. In Spring 1999 ($p < 0.001$), Fort Armistead males had a significantly lower CI; the same was true in Spring 2000.

An analysis of the ranked data from Spring 1999 indicated that none of the HSIs in female *F. heteroclitus* were significantly different from the Beaverdam Creek reference site. An analysis of the ranked Spring 2000 ($p < 0.0001$), data indicated that females from two sites - Back River and Annapolis had significantly higher HSIs than fish from Beaverdam Creek. Female CIs varied by site in Spring 1999 ($p < 0.0001$) with Back River females having higher CIs than those from Beaverdam Creek reference site. In Spring 2000 ($p < 0.0001$), female *F. heteroclitus* from Fort Armistead had significantly lower CIs than those from Beaverdam Creek.

3.4 Residues of 4-nonylphenol in *F. heteroclitus*

Although 4-nonylphenol (23 ng g^{-1}) detected in the blanks prevented an assessment in fish tissue, the blanks were free of the monoethoxylate (NP1EO) and diethoxylate (NP2EO). Values for NP1EO in the tissues ranged from 6 to 25 ng g^{-1} and were low compared to results obtained for other species of fish (C.A. Rice and J. Loyo-Rosales, personal communication); NP2EO was not found in any of the fish tissues analyzed.

3.5 Correlations with sediment contaminants

In Spring 1999, there was a significant negative correlation between reported concentrations of PAHs and PCBs in adjacent sediments and GSI in male *F. heteroclitus* (Table 2). The strongest was between sediment PAHs and GSI in Spring 1999 ($p < 0.05$). In Spring 2000, there were no

1 significant negative correlations between PAHs or PCBs and GSI in male *F. heteroclitus* (Table
2).

3
4 The spring correlations for female *F. heteroclitus* are also shown in Table 2. A significant
5 negative correlation existed between GSI and sediment PAHs ($p < 0.01$), in Spring 1999, and
6 also in Spring 2000 ($p < 0.001$). Likewise, there was a significant negative correlation between
7 vitellogenin and PAHs in both Spring 1999 ($p < 0.0001$) and Spring 2000 ($p < 0.05$). There
8 were no significant correlations with PCBs or the two metals for either year.

9 10 3.6 Histology

11 An examination of testicular tissue in *F. heteroclitus* from both Fall 1998 and Spring 1999
12 revealed only one gross anomaly in the fish examined (approximately 60 fish); severely
13 degraded testes in a fish from the Patapsco River site in Spring 1999. No ovotestes were found
14 in any *F. heteroclitus*. In Fall 1998 there were no significant differences between the reference
15 and other sites in terms of the relative contribution of spermatogenic tissue or efferent ducts to
16 total cross sectional area, or the average spermatogenic tubule diameter. In Spring 1999, male *F.*
17 *heteroclitus* from Fort Armistead, Choptank River and Patapsco River had a significantly ($p <$
18 0.0001) lower percentage of spermatogenic tissue (ranked data) compared to Beaverdam Creek
19 fish, and a correspondingly higher proportion of efferent ducts (data not shown). There were no
20 differences between sites in terms of the relative amount of spermatids/spermatozoa or the
21 spermatogenic tubule diameter in Spring 1999.

In Fall 1998, primary oocytes dominated the cross sections for female *F. heteroclitus*, followed by early and late vitellogenic oocytes. No mature oocytes were found in any of the cross sections examined from fish collected in Fall 1998. In Fall 1998 and Spring 1999, there were no significant differences in oocyte types in the cross sections at any site compared with the reference site.

3.7 Male/female ratios

The results of the contingency table analyses are shown in Table 3. Asterisks identify those sites where the ratio of male to female fish was significantly different from the expected (50:50). Four sites had significantly different male to female ratios at least once during the study. One of these, Point Lookout, was a reference site. The Annapolis site was the only location sampled where *F. heteroclitus* females consistently and significantly outnumbered males (Table 3).

4. Discussion

4.1. Plasma vitellogenin

Plasma vitellogenin levels in the majority of male *F. heteroclitus* (86 %) were below the detection limit. The Back River and Fort Armistead sites are located near STPs. The Back River site is approximately 1.7 km downstream of the Back River Sewage Treatment Plant; the Fort Armistead site is approximately 4 km downstream of the Patapsco Sewage Treatment Plant. Although relatively close to STPs, there was no indication that the concentration of plasma vitellogenin was higher in males at these sites compared to the reference sites. This would

1 appear to indicate that the environmental concentration of estrogenic EDCs were too low at the
2 sites to elicit a response in male *F. heteroclitus*. At the downstream locations where the fish
3 were sampled, dilution and biodegradation of estrogenic EDCs that occurs as noted by Williams
4 et al. (2003) may have played a role. In addition, Kirby et al. (2004) noted that an upgrade of an
5 STP in the United Kingdom to secondary treatment was associated with lower levels of
6 estrogenic EDCs and effects in fish. The Back River and Patapsco River STPs both have
7 secondary treatment of wastewater.

8
9 There was also no evidence of seasonal changes in the concentration of vitellogenin in male *F.*
10 *heteroclitus*. Kirby et al. (2004) and Kleinkauf et al. (2004) have shown strong seasonal
11 differences in vitellogenin concentration in male flounder (*Platichthys flesus*) in several
12 estuaries in the UK, and are thought to be related to the migration patterns of this species. Male
13 flounder move into the estuaries from marine waters in the summer and generally have relatively
14 low levels of vitellogenin (Kleinkauf et al., 2004). However, vitellogenin in males sampled in
15 the estuaries in late winter tend to be significantly higher, presumably due to exposure to
16 estrogenic compounds over the six month period the fish are resident in the estuaries (Kirby et
17 al., 2004). Because *F. heteroclitus* is a nonmigratory species (Bigelow and Schroeder, 1953), it
18 is perhaps not surprising that seasonal effects on vitellogenin concentration in males were not
19 observed.

20
21 The lack of significant differences in vitellogenin found in this study among sites was true not
22 only for male *F. heteroclitus*, but also for male *C. variegatus*. Higher or more frequent
23 detections of vitellogenin in male *C. variegatus* would have been evidence for a greater

1 sensitivity to estrogenic EDCs present compared to *F. heteroclitus*. Although the concentration
2 of estrogenic EDCs was not quantified in the water column, the results would seem to indicate
3 that the levels of these estrogens were too low to elicit a significant response in either male *F.*
4 *heteroclitus* or male *C. variegatus*.

5
6 The lack of significant differences in plasma vitellogenin in male fish between sites is similar to
7 that found in a few other studies in the U.S. No indication of elevated vitellogenin was found in
8 male carp from the Mississippi River (Parks et al., 1997). Vitellogenin in male mosquitofish
9 (*Gambusia affinis*) downstream of a large STP near Birmingham, Alabama was also not elevated
10 (Angus et al., 2002). Goodbred et al. (1997) investigated reproductive endocrine disruption in
11 carp at 25 sites and two impoundments in the U.S. Overall, only 16 % of male carp collected
12 had vitellogenin levels above the detection limit, similar to the 14 % in male *F. heteroclitus* in
13 the current study. In their study, Goodbred et al. (1997) also detected vitellogenin in male carp
14 at several minimally contaminated locations. In the current study, vitellogenin was occasionally
15 detected in male *F. heteroclitus* at the reference sites. Goodbred et al. (1997) concluded that
16 male carp appear to have low background levels of vitellogenin naturally; the same may also be
17 true for *F. heteroclitus*.

18
19 Scott et al. (2006) and Scott et al. (2007) found a correlation between fish size (weight) and
20 vitellogenin in male cod (*Gadus morhua*) and male dab (*Limanda limanda*), respectively. They
21 hypothesized that these two oceanodromous species accumulate estrogenic EDCs over time
22 through the food chain, and that larger males have accumulated higher amounts, resulting in
23 higher vitellogenin levels. In male *F. heteroclitus*, however, there was no correlation between

vitellogenin and size (weight and length) of the fish. Given the relatively short life span of *F. heteroclitus*, estimated at a maximum of 4-5 years by Kneib and Stiven (1978), along with the smaller size and different feeding habit of this species, the results are perhaps not surprising.

4.2 GSI and contaminants

An analysis of the data did reveal some significant associations between selected parameters in *F. heteroclitus* and reported sediment contaminant concentrations during the spring samplings. In female *F. heteroclitus*, there was a significant negative correlation between the GSI and PAHs in both Spring 1999 and Spring 2000. In male *F. heteroclitus*, there was a significant negative correlation between GSI and sediment PAHs in Spring 1999. A number of studies have shown that contaminants can negatively impact gonadal development in fish. Thomas (1988) found that benzo[a]pyrene, in the diet of female Atlantic croaker (*Micropogonias undulatus*), resulted in significantly lower GSIs. Inhibited gonadal development in adult English sole has been correlated with exposure to PAHs in sediments (Collier et al., 1998). From the current work, it appears there may be an association between PAHs and GSIs in *F. heteroclitus*, particularly in females, although additional work is needed to assess whether PAHs or some other chemicals or environmental parameters were responsible.

4.3 Plasma vitellogenin and contaminants

The PAH β -naphthoflavone has been found to depress vitellogenin synthesis in the liver of juvenile rainbow trout (Anderson et al., 1996). Pereira et al. (1992) detected lower vitellogenin levels in female winter flounder (*Pleuronectes americanus*) in an area of Boston Harbor contaminated with PAHs and other contaminants. Vitellogenin production in female *F.*

heteroclitus was also negatively correlated with sediment PAHs in the spring of both years.

Evidence for antiestrogenic and antiandrogenic effects in *F. heteroclitus* appeared to be stronger in Spring 1999 than in Spring 2000. The reason for this is unknown. However, a drought in the region reached its peak in Spring 1999, and along with higher temperatures encountered around the time of sampling could have physiologically stressed the fish.

4.4 Male/female ratios

Hansen et al. (1998) stated that a preponderance of female fish in some waterways is of concern to environmental regulators. In the current study, there was no clear pattern in deviations from a 50:50 distribution of males to females between sites. A significant deviation from the expected ratio occurred at one of the reference sites (Point Lookout). The only site where there were consistently greater and significant numbers of females than males was Annapolis. However, Annapolis did not produce a greater detection of plasma vitellogenin in males, nor did any of the fish from the site contain ovotestes or other obvious gonadal anomalies.

Given the level of effects seen in this study, it is perhaps not surprising that the male/female ratio was not revealing. If endocrine disruption in an area was so severe that *F. heteroclitus* originally programmed to be male ultimately became female, a spectrum of endocrine-related effects might be expected, including intersex fish. The results of the analysis indicating significant, possibly natural variability in the ratio of males to females between sites and years supports the general findings of this study.

Conclusions

The goal of this study was to survey the killifish *Fundulus heteroclitus* at sites around the Chesapeake for indicators of reproductive endocrine disruption. There were no significant differences in concentrations of plasma vitellogenin in male *F. heteroclitus* between sites in either the spring or fall samplings or between contaminated and uncontaminated sites. An analysis of plasma vitellogenin in males of a second species (*Cyprinodon variegatus*), indicated no difference in the concentration of vitellogenin compared with male *F. heteroclitus* at sites where both species were sampled. No ovotestes were seen in the gonads of *F. heteroclitus* from Fall 1998 or Spring 1999. There were some indications of antiestrogenic and antiandrogenic effects, particularly in Spring 1999. Lower GSIs and plasma vitellogenin levels in female *F. heteroclitus* were negatively correlated with reported sediment PAH concentrations in both Spring 1999 and Spring 2000. In conclusion, overt reproductive endocrine disruption in the killifish *F. heteroclitus* at the sites sampled in the Chesapeake Bay appeared minimal. Additional work is needed, however, to further assess endocrine disruption in this and other species in estuaries such as the Chesapeake Bay, incorporating factors such as land use and size of the receiving waters, to better understand the effects of EDCs in coastal and marine waters of the US.

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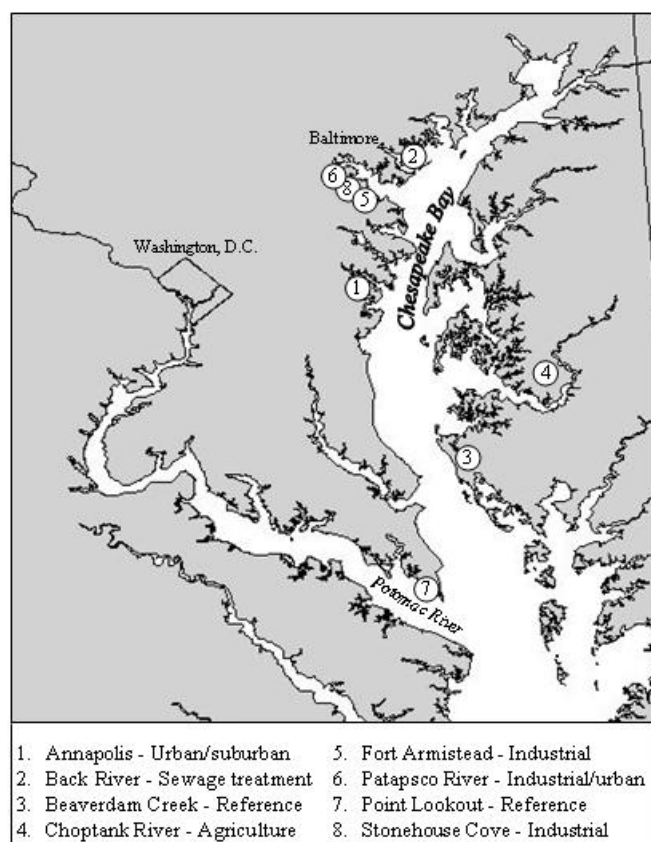


Figure 1. Collection sites for *F. heteroclitus*.

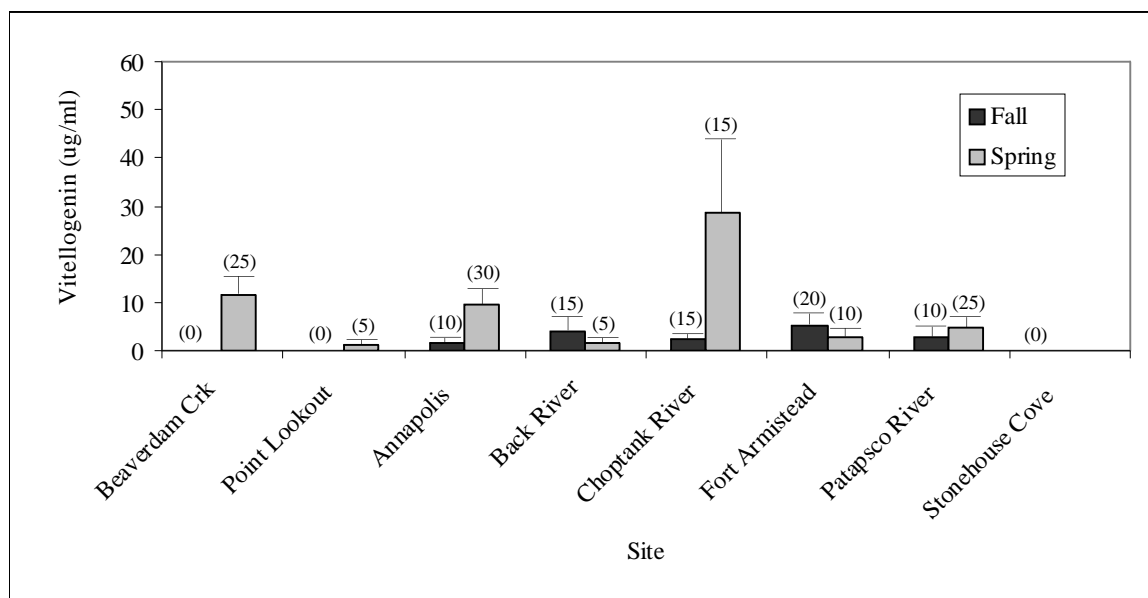


Figure 2. Vitellogenin detected in male *F. heteroclitus*. Values are the mean, error bars represent SEM. Values in parentheses represent the percentage of detections at the site during the season. n = 10-20.

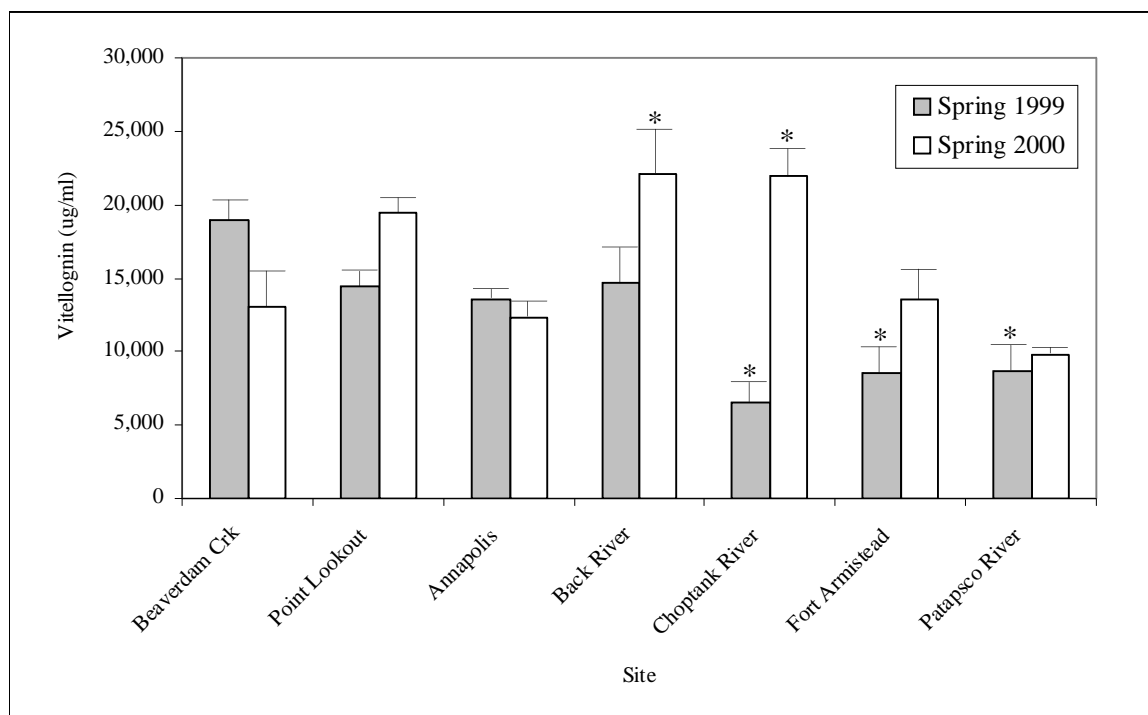


Figure 3. Vitellogenin detected in female *F. heteroclitus*. Values are the mean, error bars represent the SEM. Asterisks indicate those sites significantly different from Beaverdam Creek within a spring sampling, n = 10-20.

Table 1. Spring GSI, HSI and CI measurements made in *F. heteroclitus*.

	Site						
	Beaverdam Creek	Annapolis	Back River	Choptank River	Fort Armistead	Patapsco River	Point Lookout
Male							
				<u>GSI</u>			
Spring 1999	3.5 (± 0.1)	3.0 (± 0.4)	2.3* (± 0.3)	1.9* (± 0.1)	1.9* (± 0.3)	1.8* (± 0.5)	—
Spring 2000	2.4 (± 0.2)	3.3* (± 0.2)	2.3 (± 0.1)	1.9 (± 0.1)	2.3 (± 0.2)	3.1* (± 0.2)	2.7 (± 0.1)
				<u>HSI</u>			
Spring 1999	1.5 (± 0.1)	1.9 (± 0.2)	1.8 (± 0.1)	1.5 (± 0.2)	1.6 (± 0.2)	1.5 (± 0.1)	—
Spring 2000	1.6 (± 0.1)	2.2* (± 0.1)	2.4* (± 0.1)	1.8 (± 0.1)	1.7 (± 0.1)	2.1 (± 0.2)	1.8 (± 0.1)
				<u>CI</u>			
Spring 1999	1.3 (<0.1)	1.3 (<0.1)	1.3 (<0.1)	1.3 (<0.1)	1.2* (<0.1)	1.3 (<0.1)	—
Spring 2000	1.3 (<0.1)	1.3 (<0.1)	1.3 (<0.1)	1.3 (<0.1)	1.1* (<0.1)	1.3 (<0.1)	1.4 (<0.1)
Female							
				<u>GSI</u>			
Spring 1999	11.6 (± 0.9)	6.7 (± 1.5)	13.7 (± 1.1)	6.6 (± 2.0)	3.9* (± 0.6)	9.6 (± 1.8)	—
Spring 2000	10.5 (± 1.0)	7.4 (± 0.8)	13.9* (± 0.5)	7.8 (± 0.7)	6.2* (± 0.3)	9.5 (± 1.1)	9.5 (± 0.5)
				<u>HSI</u>			
Spring 1999	2.4 (± 0.1)	2.5 (± 0.2)	3.4 (± 0.2)	2.0 (± 0.2)	2.0 (± 0.2)	2.2 (± 0.2)	—
Spring 2000	2.5 (± 0.1)	3.4* (± 0.1)	4.5* (± 0.1)	3.0 (± 0.2)	3.0 (± 0.2)	3.2 (± 0.3)	2.6 (± 0.1)
				<u>CI</u>			
Spring 1999	1.3 (<0.1)	1.3 (<0.1)	1.5* (<0.1)	1.3 (<0.1)	1.2 (± 0.1)	1.2 (<0.1)	—
Spring 2000	1.4 (<0.1)	1.4 (<0.1)	1.5 (<0.1)	1.3 (<0.1)	1.2* (<0.1)	1.3 (<0.1)	1.4 (<0.1)

Asterisks indicate those sites significantly different from the Beaverdam Creek reference site at $p < 0.05$. Values in parentheses are SEM. GSI, gonadosomatic index; HSI, hepatosomatic index; CI, condition index; —, no data available. $n = 5-10$.

Table 2. Spearman rank coefficients for *F. heteroclitus*.

Variable 1	Variable 2	Spearman's ρ
<u>Spring 1999 - Male</u>		
GSI	PAHsum	-0.3927*
GSI	PCBsum	-0.3248*
GSI	Cadmium	-0.3267*
<u>Spring 2000 - Male</u>		
GSI	PAHsum	0.1734
GSI	PCBsum	-0.1215
<u>Spring 1999 - Female</u>		
GSI	PAHsum	-0.4147*
GSI	PCBsum	-0.1631
Vtg	PAHsum	-0.5095*
Vtg	PCBsum	-0.227
<u>Spring 2000 - Female</u>		
GSI	PAHsum	-0.4454*
GSI	PCBsum	0.0423
Vtg	PAHsum	-0.3267*
Vtg	PCBsum	0.123

*Correlation was significantly different ($\alpha = 0.05$).

Values generated by running nonparametric correlations between reported contaminant levels versus the GSI and Vtg. PAHsum includes 12 polycyclic aromatic hydrocarbons; PCBsum, 5 polychlorinated biphenyls.

Table 3. Comparison of *F. heteroclitus* sex ratios at the sampling sites.

Site	Collection	<i>n</i>	Chi Square
Annapolis	Fall 1999	645	6.316*
Annapolis	Spring 1999	217	40.974*
Annapolis	Spring 2000	548	29.843*
Back River	Fall 1998, 1999	470	4.110*
Back River	Spring 1999	107	3.223
Back River	Spring 2000	42	1.757
Beaverdam Creek	Fall 1999	236	0.034
Beaverdam Creek	Spring 1999, 2000	453	0.535
Choptank River	Fall 1998, 1999	308	0.526
Choptank River	Spring 1999, 2000	468	0.107
Fort Armistead	Fall 1998, 1999	420	3.753
Fort Armistead	Spring 1999	379	6.916*
Fort Armistead	Spring 2000	183	0.011
Patapsco River	Fall 1998, 1999	504	0.063
Patapsco River	Spring 1999, 2000	366	0.137
Point Lookout	Fall 1998, Spring 2000	778	13.462*

*Significantly different ($\alpha = 0.05$) from 50:50 ratio of males to females.

Years within a season were combined when tests revealed no significant difference.