

1 **Vitellogenesis in male Fundulus heteroclitus (killifish)**

2 **induced by selected estrogenic compounds**

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9
10 **Abstract**

11 The response of male Fundulus heteroclitus to estrogenic compounds was assessed in
12 anticipation of using this species in endocrine disrupter field studies in the Chesapeake Bay.
13 Measurements of plasma vitellogenin, gonadosomatic (GSI) and hepatosomatic (HSI) indices,
14 and an assessment of changes in gonadal histology were made. Of the parameters assessed,
15 vitellogenin was found to be the most sensitive biomarker. Plasma vitellogenin production
16 occurred in a dose-dependant manner in males exposed to 4-nonylphenol, 4-(tert-octyl)phenol,
17 bisphenol-A, and 17 β -estradiol. There was some indication that the effect on GSI may be
18 influenced by the season in which the experiments are carried out. Two time course experiments
19 revealed that vitellogenin is a fairly long-lived biomarker in male F. heteroclitus. There was also
20 evidence that fish from two moderately contaminated areas injected with 4-nonylphenol or
21 bisphenol-A produced less vitellogenin than those collected from a more pristine habitat.
22 Production of vitellogenin in male F. heteroclitus appeared similar to two other species dosed
23 with the same compounds.

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Key Words: endocrine disruption, Fundulus heteroclitus, nonylphenol, octylphenol, bisphenol-A

1. Introduction

The endocrine system consists of various glands which synthesize and secrete hormones that regulate development, growth, metabolism and reproduction. The discovery that certain environmental contaminants including some of the alkylphenols, PAHs and PCBs can adversely affect endocrine function has prompted concern over possible impacts in wildlife and humans, particularly those related to reproduction (Harrison et al., 1997; Colborn et al., 1997).

Much of the research on endocrine disrupters in wildlife has focused on fish (Celius et al., 1999; Jobling and Sumpter, 1993), due in part to a demonstrated sensitivity to endocrine disrupting compounds (EDCs) (Jobling et al., 1996; Gray and Metcalfe, 1997), similarities in their endocrine system to higher vertebrates (Bond, 1979), ease of working with fish, and the fact that wild populations in a number of locations have exhibited apparent endocrine disruption (Harries et al., 1996; Lye et al., 1997). Environmental effects seen to date range from biomarkers of exposure to intersex fish in normally gonochoristic species. In oviparous fish, the egg protein vitellogenin is produced by the liver for incorporation into developing oocytes (Anderson et al., 1996). Although typically associated with females, male fish also possess the hepatic estrogen receptor and can produce vitellogenin if exposed to estrogen or estrogenic compounds (Sumpter and Jobling, 1995). This glycopospholipoprotein has become an important biomarker of exposure to EDCs in male fish (Jobling et al., 1998). EDCs have also been shown to impact the development of ovaries and testes, particularly during the period of gonadal recrudescence.

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2 To date, the majority of laboratory EDC investigations involving fish have focused on freshwater
3 species. This paper provides an assessment of the estrogenic effects of selected EDCs on the
4 estuarine killifish Fundulus heteroclitus. The data presented are from a project to assess
5 reproductive endocrine disruption in F. heteroclitus in the Chesapeake Bay. A subsequent paper
6 will present results from the field study. The goal of the laboratory study was to test the
7 hypothesis that male F. heteroclitus will produce significant amounts of plasma vitellogenin
8 when exposed to estrogenic EDCs, and in amounts similar to other species of fish. The
9 production of vitellogenin is an essential requirement for considering F. heteroclitus as a
10 possible sentinel species for endocrine disruption. A 32 day time course experiment was also
11 carried out to assess the persistence of vitellogenin in male F. heteroclitus, also important when
12 considering this species for use in field studies. The hypothesis that male F. heteroclitus from a
13 contaminated location might be more resistant to the effects of certain EDCs as a result of prior
14 exposure to contaminants than fish from a pristine area, was tested by exposing fish from two
15 contaminated areas to EDCs in the laboratory and then analyzing for the production of
16 vitellogenin. Because F. heteroclitus is a nearshore, nonmigratory fish (Hardy, 1978) this
17 species may provide a means to assess the susceptibility of different populations, or the effects of
18 localized environmental conditions on resident populations. Vitellogenin production in F.
19 heteroclitus in the laboratory was also compared to a second species of fish, the sheepshead
20 minnow (Cyprinodon variegatus).

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22 **2. Materials and methods**

1 Male fish were used exclusively in experiments, and most were collected from a reference site
2 (Beaverdam Creek) located on the Eastern Shore of the Chesapeake Bay. The site is on the
3 western edge of the Blackwater National Wildlife Refuge, with no dwellings or commercial
4 operations within 5 km. Fish were captured using standard minnow traps (Frabill, Inc., Allenton,
5 WI) baited with bread and set from a canoe within the marsh. For the susceptibility experiments,
6 F. heteroclitus were taken from two additional sites. The first was an urban/industrial area on the
7 Patapsco River in the city of Baltimore, Maryland. The second was on the Back River in
8 Baltimore County, located approximately 14 km northeast of the Patapsco River site. The Back
9 River site is 1.7 km downstream of the outfall for the Back River Wastewater Treatment Plant,
10 which discharges approximately 150 million gallons of treated wastewater (secondary treatment)
11 per day to the river. As will be shown, the Patapsco and Back river sites are contaminated with
12 moderate levels of PAHs and PCBs. Fish from the Baltimore sites were also captured using
13 standard minnow traps baited with bread and set from shore. Males were placed in aerated
14 buckets and returned to the laboratory, typically within two hours of collection.

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16 Experiments were carried out in 10 gallon aquariums housed in Percival environmental
17 chambers (Percival Scientific, Inc., Boone, Iowa), four aquariums to a chamber. Temperature
18 was maintained at 21° C, and chambers were set on a 12 hour light/dark cycle. Each aquarium
19 was fitted with a glass top and an AquaClear Mini® equipped with a biological filter and Cycle
20 Guard® (R.C. Hagen, Inc. Mansfield, MA) activated carbon insert. The aquariums were aerated,
21 and salinity was maintained at 10‰ using Instant Ocean® Sea Salts (Instant Ocean, Aquarium
22 Systems, Inc. Mentor, OH). Fish were randomly assigned to the aquariums and allowed to
23 acclimate at least one week prior to the start of any experiment. All fish were fed daily,

1 alternating between diced frozen shrimp and TetraMin® Tropical flakes (Tetra Sales, USA
2 Blacksburg, VA), at a rate between 3 and 5 percent of body weight.

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4 2.1 Dose/response experiments

5 Each dose/response experiment lasted eight days, and consisted of a control and four doses.
6 Experiments were conducted during fall and early winter. Fish were injected once, with six to
7 eight fish per dose. Four compounds, two of which are alkylphenols, were used to assess the
8 sensitivity of male *F. heteroclitus* to EDCs. 4-Nonylphenol (purity 85%) was purchased from
9 Fluka Chemie (Fluka Chemika, Buchs, Switzerland). 4-(tert-Octyl)phenol (purity 97%) was
10 purchased from the Aldrich Chemical Company, Inc., Milwaukee, WI), as was bisphenol-A
11 (purity 99%+). 17 β -Estradiol (E₂, purity 98%) was purchased from the Sigma Chemical
12 Company (St. Louis, MO). Peanut oil was used as the carrier in all experiments. In the 4-
13 nonylphenol, 4-(tert-octyl)phenol, and bisphenol-A experiments, fish were injected with 0, 10,
14 50, 100, or 150 mg of the chemical/kg of fish. Concentrations used were based on literature
15 values (Christiansen et al., 1998; Arukwe et al., 1998). A stock solution of each chemical was
16 first prepared, typically that which produced the 150 mg/kg dose. The solution was then diluted
17 with peanut oil to produce the 100, 50, and 10 mg/kg doses. In the E₂ experiments, fish were
18 injected with 0, 0.5, 1.0, 5.0, or 10.0 mg/kg. Fish were first anesthetized with a 100 ppm
19 solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma Chemical Company, St. Louis,
20 MO). Between 20 and 50 μ l of the solution was then injected. At the end of each experiment,
21 length and weight were recorded and fish were humanely sacrificed with an overdose of MS-222
22 (500 ppm). A blood sample was immediately collected from the caudal artery of each fish into
23 heparinized micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) and spun at

1 11,500 rpm for 15 minutes in a microcapillary centrifuge (International Equipment Company,
2 Boston, MA) cooled to 4°C. The plasma was then ejected into a 500 µl centrifuge tube
3 (Brinkman Instruments, Inc., Westbury, NY) and frozen at -80° C until analysis. The condition
4 index or CI, was calculated according to Mezin and Hale (2000), as $CI = W/L^3$, where W is the
5 weight (g) multiplied by 100, and L is the total length of the fish³ (cm). Gonads and liver were
6 removed and weighed, and the gonadosomatic index (gonad weight/body weight X 100) and
7 hepatosomatic index (liver weight/body weight X 100) were calculated. Selected testes were
8 preserved in Bouin's Fluid (LabChem, Inc., Pittsburgh, PA) for histologic examination.

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10 2.2 Time course experiments

11 Two experiments, one with 4-nonylphenol the other with E₂ were carried out to assess the
12 persistence of plasma vitellogenin in adult male F. heteroclitus. Fish were injected once with
13 100 mg 4-nonylphenol or 1 mg E₂. Preliminary experiments revealed these concentrations
14 produced easily detectable amounts of vitellogenin. Blood samples were then taken at 0, 2, 4, 8,
15 12, 16, 20, 24, 28, and 32 days post injection. Because of their size, fish could only be sampled
16 once.

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18 2.3 Susceptibility experiments

19 Two additional eight-day experiments were carried out with adult male F. heteroclitus from
20 Patapsco River and Back River. Fish were injected once with 0, 10, 50, 100, or 150 mg/kg of 4-
21 nonylphenol or bisphenol-A. Results were then compared with those from the reference site
22 (Beaverdam Creek) dose/response experiment.

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1 2.4 Interspecies comparison

2 The production of vitellogenin in male F. heteroclitus dosed with EDCs was compared with a
3 second species of fish, Cyprinodon variegatus or sheepshead minnow. C. variegatus can be
4 found in nearshore habitats similar to those used by F. heteroclitus, and are responsive to
5 estrogenic EDCs (Folmar et al., 2000; Hemmer et al., 2001). The experiments with F.
6 heteroclitus and C. variegatus were conducted using 4-nonylphenol or E₂. Fish were dosed once
7 with 4-nonylphenol at concentrations of 0, 10, 50, or 100 mg/kg, or with E₂ at concentrations of
8 0, 0.5, 1.0, or 5.0 mg/kg. Each experiment ran for 21 days, and plasma samples were analyzed
9 for vitellogenin using a direct ELISA.

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11 2.5 Vitellogenin analysis

12 Plasma vitellogenin in F. heteroclitus was the primary biomarker used to assess reproductive
13 endocrine disruption. Vitellogenin was analyzed using a direct enzyme-linked immunosorbent
14 assay (ELISA). The Interdisciplinary Center for Biotechnology Research's (ICBR) Hybridoma
15 and Biomarkers Core at the University of Florida, Gainesville developed the monoclonal
16 antibody (MAb) (5F8 (HL 1562)), which was specific for vitellogenin and not other plasma
17 proteins (K. Kroll, pers. comm.). The University of Florida analyzed C. variegatus plasma
18 samples for vitellogenin.

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20 A brief description of the direct ELISA protocol follows, which is essentially that used by
21 Denslow et al. (1999) and Bowman et al. (2000). The standards or sample (unknown) in
22 triplicate were first coated (50 µl) onto 96 well microplates (Dynatech Laboratories, Inc.,
23 Chantilly, VA) in phosphate buffered saline with sodium azide (PBSZ). Samples were diluted

1 depending on the expected concentration of vitellogenin, with dilutions ranging from 1:500 to
2 1:25,000. Vitellogenin standards ranged from 0.01 $\mu\text{g/ml}$ to 1.0 $\mu\text{g/ml}$. Control (containing no
3 vitellogenin) male plasma was added to standards and processed in the same way as samples.
4 Plated samples and standards were allowed to incubate overnight at 4°C in a humidified
5 chamber, followed by washing with PBSZ. Plates were then blocked, washed again with PBSZ
6 followed by addition of the E. heteroclitus-specific MAb and incubation again overnight at 4°C.
7 A second antibody, biotinylated anti-mouse IgG antibody (Pierce Chemical Company, Rockford,
8 IL) was added, followed by streptavidin-alkaline phosphatase (Pierce Chemical Company,
9 Rockford, IL). Finally, p-nitrophenyl phosphate (pNPP) disodium (Sigma Chemical Company,
10 St. Louis, MO), was added to each well, and the microplates were read at 405 nm on a
11 SPECTRAMax® PLUS (Molecular Devices Corporation, Sunnyvale, CA) microplate reader.
12 The standard curve was fit using a quadratic equation. The detection limit for the ELISA was
13 0.005 mg/ml.

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15 2.7 Statistics

16 All data were analyzed using JMP® statistical software (SAS Institute, Inc., Cary, NC).
17 Significant differences between control and treatment groups were determined using analysis of
18 variance (ANOVA). Data to be analyzed were first tested for homogeneity of variance. When
19 necessary, data were transformed using a Box-Cox power transformation (Box and Cox, 1964).
20 Following a significant ANOVA, a Dunnett's Test or Tukey-Kramer HSD was carried out on the
21 original or transformed data. All tests of significance were reported at $P < 0.05$. Means of
22 transformed data are reported with 95% confidence limits. Means of data not requiring
23 transformation are reported with standard error of the mean (SEM).

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3. Results

3.1 Dose/response

The average weight of F. heteroclitus in the dose/response experiments was 4.23 ± 0.09 g, average length was 7.2 ± 0.0 cm. The condition index was a fairly constant 1.1 (Table 1). All doses of 4-nonylphenol resulted in significantly ($P < 0.05$) elevated levels of vitellogenin in F. heteroclitus as compared to the control. At 150 mg/kg, male F. heteroclitus produced a mean plasma vitellogenin concentration of 23.72 ± 4.45 mg/ml. Vitellogenin in male F. heteroclitus injected with 50, 100, or 150 mg/kg 4-nonylphenol was also significantly higher than fish injected with similar amounts of either bisphenol-A or 4-(tert-octyl)phenol. At 150 mg/kg 4-nonylphenol, the concentration of plasma vitellogenin was approximately two orders of magnitude higher than fish injected with the same dose of bisphenol-A, and over an order of magnitude higher than fish injected with 4-(tert-octyl)phenol (Table 1).

Bisphenol-A caused a significant increase in plasma vitellogenin at the 50, 100 and 150 mg/kg doses. The 100 and 150 mg/kg doses of 4-(tert-octyl)phenol caused significant increases compared to controls. Overall, the apparent estrogenic potency of these compounds to male F. heteroclitus was 4-nonylphenol > 4-(tert-octyl)phenol > bisphenol-A. Injection of E_2 resulted in a significant increase in plasma vitellogenin in male F. heteroclitus at all doses compared to the control (Table 1). At 10 mg/kg, fish produced 17.72 ± 2.92 mg of vitellogenin per ml of plasma. At the lowest dose of E_2 (0.5 mg/kg), male F. heteroclitus produced 5.31 ± 1.00 mg/ml.

1 4-Nonylphenol was associated with a slight, but significant increase in the GSI at the 50 and 150
2 mg/kg doses (Table 1). The HSI showed some evidence of an increase, although not significant.
3 Bisphenol-A had no significant effect on the GSI relative to the control at any concentration.
4 The HSI increased numerically although not significantly with dose. Although the 150 mg/kg
5 dose of 4-(tert-octyl)phenol produced a numerically higher GSI, it was not significantly different
6 from that of the control. The mean HSI in fish dosed with 4-(tert-octyl)phenol was numerically
7 higher than controls, however, these differences were not significant. E₂ did not have a
8 significant effect on the GSI.

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10 A qualitative examination of gonadal tissues from the 4-nonylphenol and E₂ eight-day exposures
11 revealed no obvious changes. There were no indications of reductions in cell types or other
12 changes in gonadal structure in exposed compared to control fish. The only significant
13 quantitative difference between control and 150 mg/kg 4-nonylphenol exposed fish was a
14 slightly but significantly higher spermatogenic tissue area in fish dosed with 150 mg/kg 4-
15 nonylphenol (data not shown). There were no significant quantitative differences in gonadal
16 tissues between the control and E₂ exposed fish.

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18 3.2 Interspecies comparison

19 The results of the 4-nonylphenol dose/response experiment with F. heteroclitus and C. variegatus
20 can be seen in Figure 1a. Both species produced similar amounts of vitellogenin at all doses
21 except 100 mg/kg. At this dose, C. variegatus produced a significantly higher amount of
22 vitellogenin. The results of the E₂ dose/response experiment with F. heteroclitus and C.

1 variegatus can be seen in Figure 1b. Production of vitellogenin in the two species was not
2 significantly different at any dose.

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4 3.3 Time course experiments

5 The results of the 32 day time course experiments with 4-nonylphenol and E₂ are shown in
6 Figure 2. The average length of F. heteroclitus was 7.4 ± 0.1 cm, average weight was $4.30 \pm$
7 0.06 g. In both experiments, plasma vitellogenin concentrations were significantly higher on all
8 days sampled compared with Day 0. There was a rapid and significant increase in plasma
9 vitellogenin in F. heteroclitus during the first 12 days of the 4-nonylphenol time course
10 experiment (Figure 2a). By Day 2, vitellogenin had increased to 1.20 ± 0.65 mg/ml. Plasma
11 vitellogenin peaked on Day 12 (7.68 ± 2.19 mg/kg) and then gradually decreased. Vitellogenin
12 was detectable in F. heteroclitus plasma throughout the remainder of the experiment, and on Day
13 32, the mean plasma vitellogenin concentration was 2.22 ± 0.53 mg/ml, well above the detection
14 limit of 0.005 mg/ml. The results of the time course experiment with E₂ are shown in Figure 2b.
15 Injection of E₂ at a concentration of 1 mg/kg also resulted in a rapid and significant rise in
16 plasma vitellogenin in F. heteroclitus, peaking at Day 8 (5.90 ± 1.37 mg/ml), followed by a
17 gradual decline.

18

19 3.4 Susceptibility

20 The results of the susceptibility experiments are shown in Figure 3. Male F. heteroclitus from
21 the reference site (Beaverdam Creek) dosed with 50, 100 or 150 mg/kg 4-nonylphenol (Figure
22 3a) produced significantly higher levels of plasma vitellogenin than fish from either Back River
23 or Patapsco River. The setup in all three experiments was the same for these eight-day

1 exposures. The results from the bisphenol-A susceptibility experiment were not as clear. While
2 F. heteroclitus from Beaverdam Creek produced numerically higher levels of vitellogenin than
3 fish from Patapsco River or Back River at 50, 100, and 150 mg/kg, vitellogenin production in the
4 Beaverdam Creek fish was significantly higher only at the 50 and 100 mg/kg bisphenol-A doses
5 (Figure 3b).

6

7 **Discussion**

8 The primary goal of this research was to determine if male F. heteroclitus respond to selected
9 EDCs by producing significant amounts of plasma vitellogenin, and was done in anticipation of
10 using this species in a field study in the Chesapeake Bay. In the laboratory, male F. heteroclitus
11 produced significant amounts of plasma vitellogenin when dosed with 4-nonylphenol, 4-(tert-
12 octyl)phenol, or bisphenol-A. Excluding the natural hormone 17 β -estradiol, F. heteroclitus
13 appeared to respond most strongly to 4-nonylphenol. The highest dose of 4-nonylphenol
14 resulted in an average plasma vitellogenin concentration of 23.72 ± 4.45 mg/ml, higher than that
15 typically found in reproductive female F. heteroclitus (unpublished data). The results presented
16 here appear to be the first published work on plasma vitellogenin production in male F.
17 heteroclitus, and demonstrates that male F. heteroclitus are responsive to the EDCs tested. Rice
18 and Xiang (2000) assessed the production of plasma vitellogenin in a closely related species, F.
19 grandis, however, in addition to 4-nonylphenol, fish were fed Aroclor 1254, tributyltin, and 3-
20 methylcholanthrene in the diet. There was also some indication that the food source (fish meal)
21 may have been contaminated with estrogens, making an assessment of effect difficult. McArdle
22 et al. (2000) investigated the effects of sewage effluent on male F. heteroclitus, however, no

1 effect was seen on hepatic vitellogenin levels as measured in density units; plasma vitellogenin
2 was not measured.

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4 Plasma vitellogenin production in male F. heteroclitus can also be compared with other species
5 dosed in a similar manner, where that information is available. In the current work, an initial
6 experiment with male F. heteroclitus and sheepshead minnows revealed similar amounts of
7 vitellogenin in both species when dosed with either 4-nonylphenol or 17 β -estradiol.

8 Christiansen et al. (1998) found that immature rainbow trout dosed with 50 mg/kg 4-nonylphenol
9 produced between 0.05 and 1.0 mg/ml vitellogenin after six days, similar to that in male F.
10 heteroclitus (1.52 \pm 0.90 mg/ml). Two investigations involving bisphenol-A were found.

11 Lindholst et al. (2001) found that male juvenile rainbow trout injected with 35 mg/kg bisphenol-
12 A produced 0.004 mg/ml vitellogenin after seven days. Christiansen et al. (1998) found that
13 immature rainbow trout injected with 50 mg/kg bisphenol-A produced between 0.005-0.9 mg/ml
14 after nine days. In the current study, male F. heteroclitus injected with 50mg/kg bisphenol-A
15 produced an average 0.17 mg/ml plasma vitellogenin, higher than that found by Lindholst et al.
16 (2001), but in the range found by Christiansen et al. (1998). Rainbow trout injected with a
17 concentration of 0.5 mg/kg E₂ produced between 5-10 mg/kg plasma vitellogenin, also close to
18 that produced by F. heteroclitus. From the results presented, male F. heteroclitus appear to
19 produce plasma vitellogenin in amounts comparable to sheepshead minnows and possibly
20 rainbow trout, when dosed in a similar manner with the same EDCs. Although intraperitoneal
21 injections are not the most environmentally relevant exposure, they do provide a means to
22 determine if a species responds to compounds of interest. A comparison of plasma vitellogenin
23 production with other fish begins to address the question of sensitivity to EDCs between species.

1
2 Vitellogenin was found to be a fairly long-lived biomarker in male F. heteroclitus. Vitellogenin
3 was easily detectable throughout the two time course experiments. On Day 32 of the 4-
4 nonylphenol exposure, the concentration of vitellogenin was well above the detection limit. The
5 current work supports the findings of Elliot et al. (1979) that plasma vitellogenin is a fairly long-
6 lived biomarker in male fish, and should be useful in assessing exposure of male F. heteroclitus
7 to estrogenic EDCs in the field. Interestingly, production of plasma vitellogenin in male F.
8 heteroclitus injected with 4-nonylphenol in the time course experiment peaked at Day 12, while
9 in E₂-exposed fish, the maximum vitellogenin concentration occurred on Day 8. The quicker
10 response of F. heteroclitus to E₂ may have been a function of the higher binding affinity of E₂ to
11 the estrogen receptor compared with 4-nonylphenol, as noted by White et al. (1994).

12
13 Although F. heteroclitus responded to the estrogenic EDCs by producing vitellogenin, the
14 dose/response experiments had little or no effect on the GSI, in contrast to what has been found
15 by others (Mills et al., 2001; Harries et al., 1997). Jobling et al. (1996) noted, however, that the
16 degree of testicular inhibition in rainbow trout was strongly affected by timing of exposure, as
17 did Billard et al. (1981), and that fish undergoing seasonal gonadal regression suffered no ill
18 effects on gonadal structure after being exposed to high concentrations of natural estrogens. The
19 same was found in the current study. The dose/response experiments were carried out during
20 late fall and early winter, the period of gonadal regression in F. heteroclitus. Because the
21 experiments were carried out during the period of natural gonadal regression and only lasted
22 eight days, it is perhaps not surprising there was little effect on the GSI. In contrast, all
23 compounds tested significantly altered vitellogenin production. In an initial screening

1 experiment, male F. heteroclitus were exposed to E₂ during the period of gonadal recrudescence.
2 The effect on the GSI in this spring exposure versus fall/winter exposure is shown in Figure 4.
3 Exposure in spring produced a downward trend in the GSI, similar to that found in other
4 recrudescing fish (Jobling et al., 1996; Harries et al., 1997), indicating the timing of exposure in
5 male F. heteroclitus is probably important in terms of gonadal effects.

6
7 The susceptibility experiments begin to test the hypothesis that fish with prior exposure to
8 contaminants may be less susceptible to EDCs, at least in terms of vitellogenin production. A
9 number of authors (Stegeman, 1978; Elskus and Stegeman, 1989; Van Veld et al., 1990;
10 Armknecht et al., 1998) have shown that fish living in environments contaminated with PAHs
11 and PCBs frequently have elevated or altered levels of the Phase I (cytochrome P450
12 monooxygenases) or Phase II (conjugating enzymes) enzymes which can aid in the
13 detoxification and excretion of lipophilic chemicals (Goksoyr and Forlin, 1992). The results
14 from the current work suggest that male F. heteroclitus taken from the Patapsco River and Back
15 River sites produced less vitellogenin when dosed with 4-nonyphenol and perhaps bisphenol-A,
16 than fish from the more pristine Beaverdam Creek reference site. If the Phase I and/or Phase II
17 enzyme systems were induced in F. heteroclitus from these areas, higher biotransformation and
18 excretion rates of the EDCs might result in less of the compound being available for endocrine
19 disruption. A number of studies have shown elevated levels of Phase I or Phase II enzymes in F.
20 heteroclitus from contaminated environments. For example, Stegeman (1978) found that levels
21 of P450 were significantly higher in F. heteroclitus in a marsh contaminated by an oil spill, than
22 those from a reference marsh. Prince and Cooper (1995) found elevated levels of P450 as
23 measured by ethoxyresorufin O-deethylase (EROD) activity in fish from a New Jersey marsh

1 contaminated with PAHs, PCBs and dioxins. Armknecht et al. (1998) found higher levels of
2 glutathione S-transferases (Phase II enzyme) in F. heteroclitus in an area of the Elizabeth River
3 in Virginia, heavily contaminated with creosote compared to fish from a more pristine area.
4 Working with Atlantic salmon (Salmo salar), Arukwe et al. (2000) found that 4-nonylphenol is
5 metabolized through both Phase I and Phase II pathways. If the same is true for F. heteroclitus,
6 elevated levels of these enzymes in fish from the Patapsco and Back river sites could have
7 resulted in higher rates of metabolism of 4-nonylphenol and bisphenol-A, and ultimately less
8 production of vitellogenin.

9
10 Although an assessment of sediment contamination at F. heteroclitus collection sites was not
11 feasible in this study, there is enough information available in the literature to estimate the
12 general level of contamination at these sites. Table 2 contains information on sediment
13 contamination adjacent to the sites where F. heteroclitus were collected. Data in the table are
14 from Baker et al. (1997) and from the Environmental Protection Agency (EPA, 2000).
15 Contaminants were chosen for inclusion based on data availability for all three sites. From Table
16 2, it can be seen that PAH sediment concentrations near the Patapsco and Back River sites were
17 1-2 orders of magnitude higher than at the Beaverdam Creek reference site. Higher levels of
18 contaminants at the Patapsco and Back river sites would support the hypothesis that F.
19 heteroclitus from these areas have elevated levels of P450.

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21 **5. Conclusions**

22 The results presented here indicate that male Fundulus heteroclitus respond to a number of EDCs
23 including 4-nonylphenol, bisphenol-A, and 4(tert-octyl)phenol, as well as the endogenous

1 hormone E₂, by producing the egg protein vitellogenin. The production of plasma vitellogenin in
2 male F. heteroclitus injected with 4-nonylphenol or bisphenol-A was comparable to rainbow
3 trout (Christiansen et al., 1998) and sheepshead minnows dosed in a similar manner. Production
4 of plasma vitellogenin in male F. heteroclitus was a better indicator of exposure to estrogenic
5 compounds than the GSI, which appears to be influenced by season. The time course
6 experiments revealed that once produced, vitellogenin remains detectable in male F. heteroclitus
7 for an extended period of time, important for its use as a biomarker in this species. After 32
8 days, the level of vitellogenin was still well within the detection limit of the ELISA.

9
10 There may be variations in the susceptibility to estrogenic compounds among different
11 populations of male F. heteroclitus. Males from two moderately contaminated areas appeared
12 less susceptible to 4-nonylphenol and possibly bisphenol-A than those from a more pristine area,
13 as measured by production of plasma vitellogenin. If these differences are real, F. heteroclitus
14 living in moderately contaminated areas may be somewhat resistant to the estrogenic effects of
15 certain EDCs. Additional studies would be needed, however, to confirm this possibility.

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3

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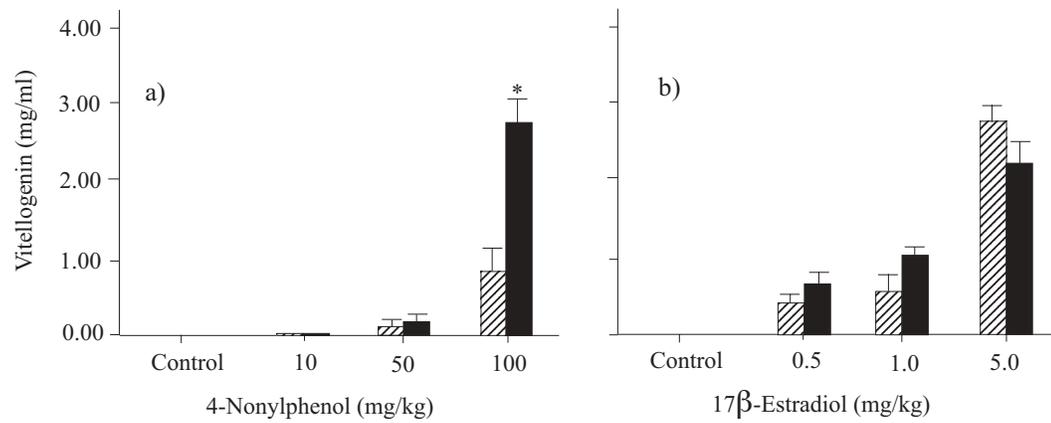


Figure 1. Vitellogenin production in *Fundulus heteroclitus* (▨) and *Cyprinodon variegatus* (■) dosed with 4-nonylphenol (a) or 17β-estradiol (b). Twenty-one day exposure. Error bars represent SEM. Asterisk indicates significant ($P < 0.05$) difference between species. $n = 3-4$.

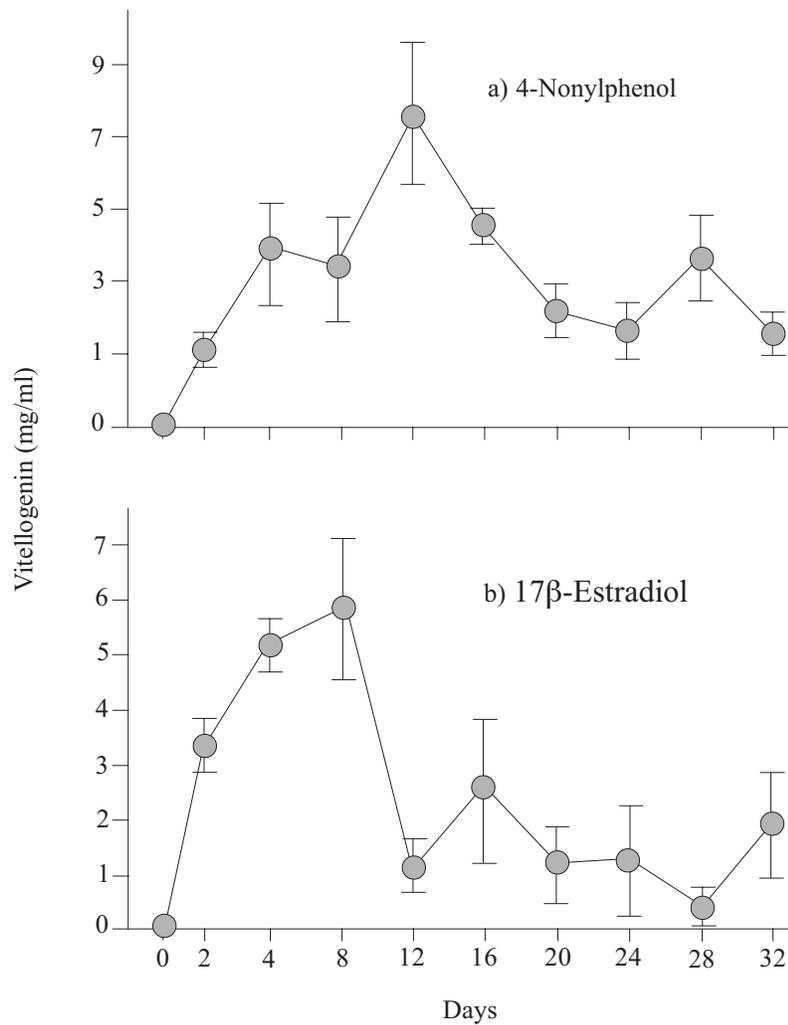


Figure 2. Results of 4-nonylphenol and 17β-estradiol time course experiments. Vitellogenin produced in male *Fundulus heteroclitus* from single injection of 4-nonylphenol (100 mg/kg) or 17β-estradiol (1mg/kg). Error bars represent SEM. Production of vitellogenin at all days in both experiments was significantly ($P < 0.05$) different from Day 0. $n = 4-6$.

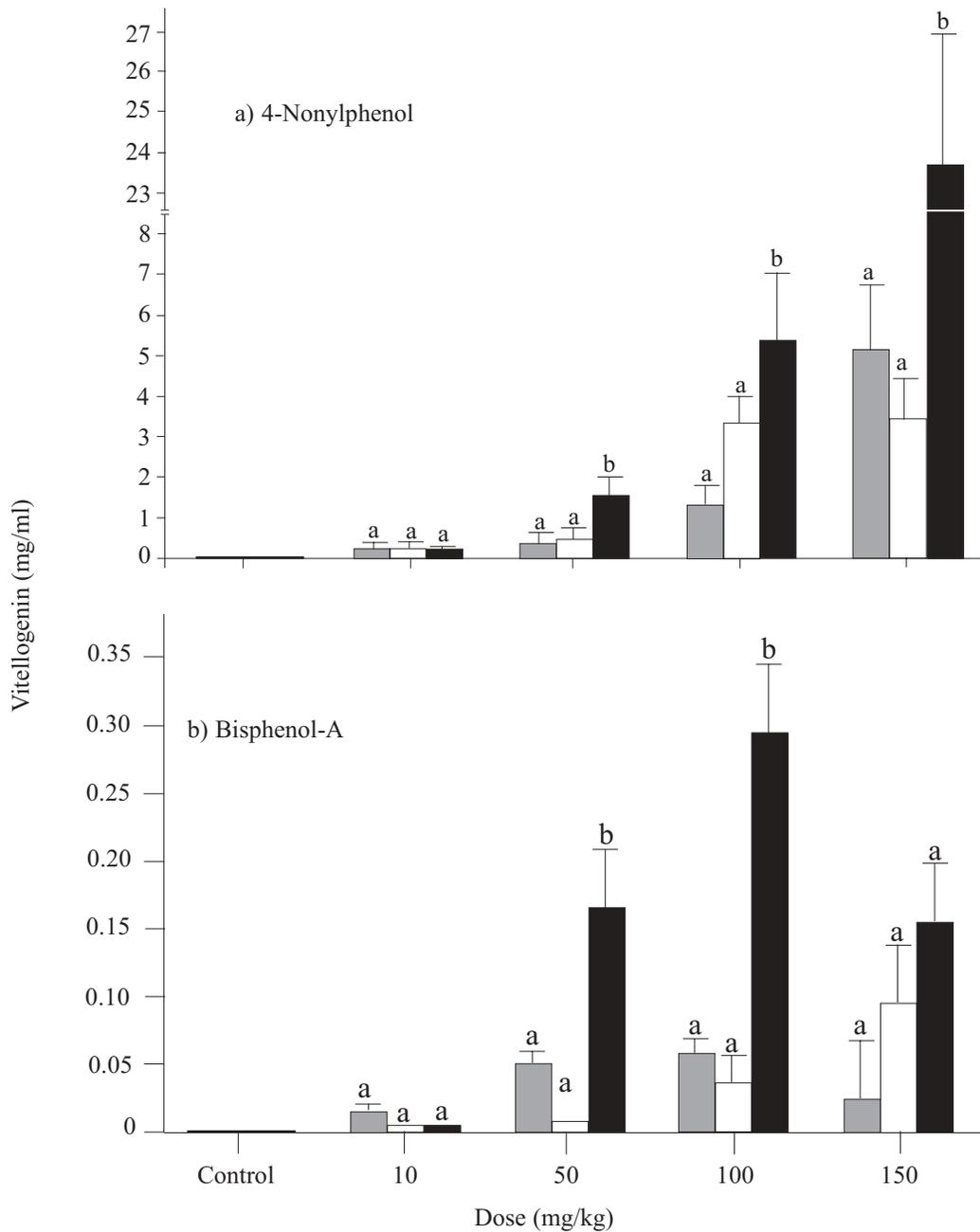


Figure 3. Vitellogenin production in *Fundulus heteroclitus* from Beaverdam Creek (■), Back River (□), or Patapsco River (▒) dosed with either 4-nonylphenol or bisphenol-A. Different letters represent sites within a dose that were significantly ($P < 0.05$) different. Error bars represent 95% confidence limits. $n = 6$.

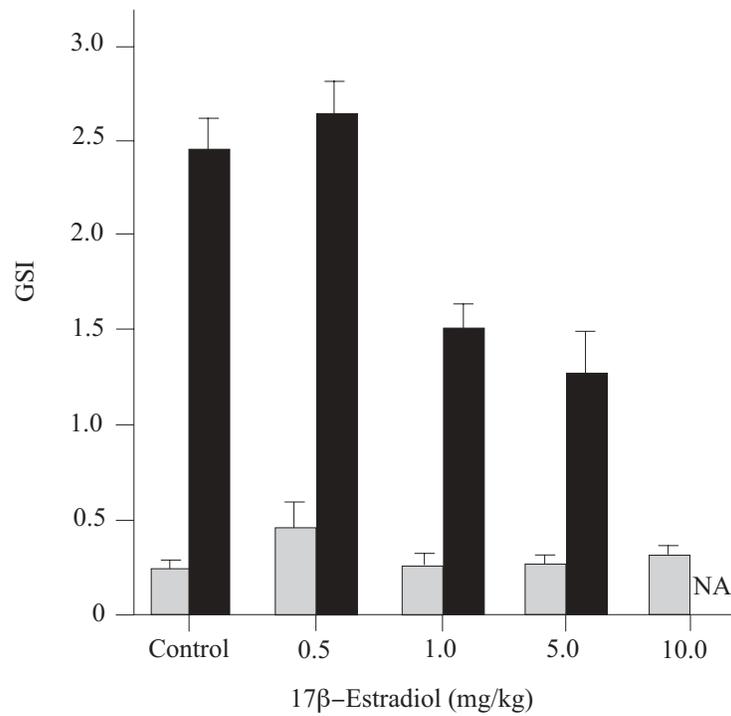


Figure 4. Change in GSI in male *Fundulus heteroclitus* during spring (■) or fall/winter (□) exposure to 17β-estradiol. Error bars represent SEM; NA, data not available. n = 4-6.

Table 1. Measurements of vitellogenin, GSI, and HSI from the dose/response experiments.

Compound	Control	Dose (mg/kg)			
		10	50	100	150
4-Nonylphenol					
Vtg (mg/ml)	0.00 ± 0.00	0.02 ± 0.00*	1.52 ± 0.90*	5.22 ± 1.94*	23.72 ± 4.45*
GSI	0.17 ± 0.01	NA	0.24 ± 0.02*	0.21 ± 0.01	0.24 ± 0.01*
HSI	1.38 ± 0.18	NA	1.35 ± 0.19	1.46 ± 0.11	1.57 ± 0.12
CI	1.1	1.0	1.1	1.1	1.1
n	8	8	7	7	7
Bisphenol-A					
Vtg (mg/ml)	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.05*	0.29 ± 0.06*	0.15 ± 0.04*
GSI	0.19 ± 0.03	0.20 ± 0.01	0.19 ± 0.02	0.21 ± 0.02	0.19 ± 0.00
HSI	0.99 ± 0.14	1.16 ± 0.11	1.18 ± 0.09	1.24 ± 0.14	1.76 ± 0.47
CI	1.0	1.1	1.1	1.1	1.1
n	6	7	6	7	7
4-(<u>tert</u>-Octyl)phenol					
Vtg (mg/ml)	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.32 ± 0.11*	0.89 ± 0.12*
GSI	0.19 ± 0.02	0.19 ± 0.02	0.19 ± 0.02	0.20 ± 0.01	0.40 ± 0.20
HSI	0.99 ± 0.14	1.23 ± 0.17	1.42 ± 0.15	1.30 ± 0.16	1.32 ± 0.08
CI	1.0	1.1	1.2	1.1	1.1
n	6	7	7	7	7
17β-Estradiol					
Vtg (mg/ml)	0.00 ± 0.00	5.31 ± 1.00*	4.52 ± 0.63*	7.96 ± 1.12*	17.72 ± 2.92*
GSI	0.19 ± 0.02	0.39 ± 0.16	0.23 ± 0.01	0.23 ± 0.02	0.25 ± 0.01
HSI	1.36 ± 0.07	0.98 ± 0.06	0.81 ± 0.08*	1.71 ± 0.17	1.77 ± 0.12
CI	1.0	1.1	1.1	1.1	1.2
n	6	7	7	7	7

Note: Asterisks indicate significant ($P < 0.05$) difference from control. Values are ±SEM for bisphenol-A and 17β-estradiol, 95% confidence limits for 4-nonylphenol and 4-(tert-octyl)phenol.

Abbreviations: Vtg, vitellogenin; GSI, gonadosomatic index (gonad wt./body weight X 100); HSI, hepatosomatic index (liver wt./ body weight X 100); CI, condition index (weight X 100/length³)

Table 2. Selected PAH and PCB sediment concentrations near sampling sites.

PAH (ng/g dry weight)	Site		
	Back River	Patapsco River	Beaverdam Creek
Acenaphthene	16 (a) 33.91 (b)	47.42 (b)	1.40 (a)
Anthracene	88.12 (b)	44 (a) 114.24 (b)	2.88 (a)
Benzo[<i>a</i>] pyrene	153 (a) 446.88 (b)	966 (a) 538.32 (b)	13.70 (a)
Benz[<i>a</i>] anthracene	281 (a) 356.84 (b)	283 (a) 413.61 (b)	9.83 (a)
Fluoranthene	498 (a) 846.22 (b)	1,500 (a) 889.59 (b)	24.2 (a)
Fluorene	64 (a) 54.76 (b)	29 (a) 56.62 (b)	3.38 (a)
Phenanthrene	315 (a) 316.04 (b)	269 (a) 517.10 (b)	18.20 (a)
Pyrene	515 (a) 857.89 (b)	1,250 (a) 873.09 (b)	30.80 (a)
PCB (ng/g dry wt.)			
PCB 18	5.05 (b)	1.328 (b)	BDL (a)
PCB 44	11.27 (b)	2.139 (b)	BDL (a)
PCB 52	10.97 (b)	2.316 (b)	BDL (a)
PCB 180	11.68 (b)	8.373 (b)	BDL (a)
PCB 206	0.23 (b)	3.123 (b)	BDL (a)

a. EPA, 2000; b. Baker et al., 1997; BDL, below detection level

Approximate distance from contaminant site to actual *F. heteroclitus* sampling site: Patapsco River, 1.6km; Back River, 2.4km; Beaverdam Creek, 8km