

**VITELLOGENIN: A BIOMARKER OF EXPOSURE TO
ENVIRONMENTAL ESTROGENS FOR MUMMICHOG (*Fundulus
heteroclitus*) FROM A CREOSOTE-CONTAMINATED SITE?**

A Thesis
Presented to

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The College of William and Mary in Virginia

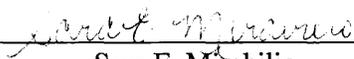
In Partial Fulfillment
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Master of Science

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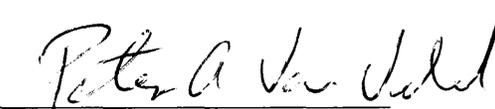
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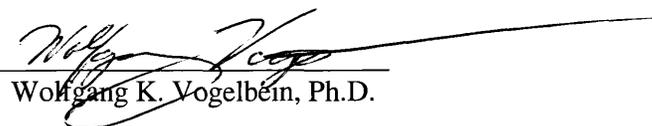
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ABSTRACT. Vitellogenin (VTG) is widely used as a biomarker for environmental estrogens and reproductive disruption in fish. Vitellogenesis is the process by which yolk is formed. The endpoint is most sensitive in male fish where vitellogenesis is an abnormal process. Research examining effects of environmental mixtures of chemicals (e.g. creosote) on vitellogenesis is limited. This study examines plasma VTG expression in both male and female mummichog, *Fundulus heteroclitus*, collected from a creosote-contaminated site and two reference sites in lower Chesapeake Bay, USA, and in wild-caught male reference fish exposed in the laboratory to creosote-contaminated sediment. Further, this study uses tissue somatic indices (gonadosomatic index, hepatosomatic index, and condition factor) as organ-level indicators of reproductive status and creosote exposure. Western blotting with an anti-vitellogenin monoclonal antibody (FV 10-9) was used to identify an abundant (approx. 218 kDa) protein in *F. heteroclitus* plasma samples. Vitellogenin was not observed in any male fish collected from any of the field sites. No site-specific differences were observed in plasma VTG of females from these sites. Overall, fish from the creosote-contaminated site appear resilient and reproductively fit. After seven days of exposure, reference fish exposed to sediment from the creosote-contaminated site suffered extensive mortality (approx. 25%), but displayed no expression of VTG and had no alterations in other measured indices except for increased hepatosomatic index and induction of the biotransformation enzyme, cytochrome P4501A (CYP1A). Lack of response in Atlantic Wood fish could be due to: (1) physiological adaptation of the population from the creosote-contaminated site to creosote exposure (2) species-specific sensitivity (3) creosote not exerting estrogenic effects and regulating vitellogenesis. Thus, while VTG has been used successfully as a practical and reliable screening method for wildlife toxicity, results herein do not support use of this biomarker for *F. heteroclitus* from creosote-contaminated sites. This study does reaffirm constituents of creosote binding to the aryl hydrocarbon receptor resulting in induction of CYP1A. More attention needs to be given to developing alternative biomarkers not sensitive to aryl hydrocarbon-mediated antiestrogens if detection of weak estrogens and reproductive disruption in creosote and other complex mixtures is to be achieved.

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INTRODUCTION

Endocrine Disruption.

In the early 1990's several articles addressed possible impacts of common environmental contaminants on development, reproduction, and endocrine function in humans and wildlife (Colborn *et al.*, 1993; Hileman, 1994; Safe, 1995). Probable endocrine-disrupting chemicals (EDCs) include common herbicides (e.g. Atrazine), fungicides (e.g. hexachlorobenzene), insecticides (e.g. DDT), nematocides (e.g. Aldicarb), as well as various industrial chemicals (e.g. mercury, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalates, and styrenes) (Colborn *et al.*, 1993). Scientific debate and public concern about potential human health effects of EDCs prompted Congress to pass both the Food Quality Protection and Safe Drinking Water Acts in 1996. This legislation mandated the EPA to initiate a program to identify EDCs and human health risks resulting from EDC exposure. The EPA included wildlife health as an endpoint, asserting that wildlife toxicity models can lead to insights into the degree pollutants affect human populations (Ankley *et al.*, 1998). Subsequent field and laboratory wildlife studies have attempted to develop useful and reliable screening methods for identification of EDCs and their potential effects at the individual and population level.

Teleost (bony fish) reproduction is primarily controlled by the reproductive endocrine system (Fig. 1). Neurotransmitters and/or hormones are secreted from the brain, pituitary, and gonads in what has been termed the hypothalamus-pituitary-gonadal (HPG) axis (Thomas and Khan, 1997). In females, the liver also plays a significant role because of its synthesis of vitellogenin (VTG), an egg-yolk-precursor protein (Thomas, 1990). Environmental cues are detected by sense organs that relay information to the hypothalamus. Neurotransmitters are released in response to stimuli, regulating the synthesis and secretion of gonadotropin-releasing hormone (GnRH) or gonadotropin release-inhibiting factors (GRIFs). The binding of GnRH to specific receptors on the gonadotrops of the pituitary activates a phosphoinositol calcium-dependent second messenger pathway. Activation of this pathway triggers the release of two glycoprotein hormones: gonadotropin I and II. Gonadotropins act via receptors at the gonads to stimulate the production and release of steroid hormones into circulating blood (e.g. 17β -estradiol) (Thomas and Khan, 1997). Specific steroid binding proteins bind and transport free

steroid hormones to target cells (e.g. hepatocyte) (Beato, 1989). Steroids enter the cell and bind to specific nuclear receptors (e.g. estrogen receptor) (Smith and Thomas, 1990). Steroidal-bound receptors bind to specific DNA-sequences in the nucleus and thereby activate mRNA synthesis and protein production (e.g. VTG) (Beato, 1989).

EDCs could exert their effects at a variety of sites along the HPG axis (Fig. 1). Sense organs could detect chemicals as noxious, relaying information to the hypothalamus and altering hypothalamic neurotransmitter function. Changes in neurotransmitter secretions would affect pituitary gonadotropin secretion. Alterations in gonadotropin levels would modify steroid hormone production, steroid-regulated feedback mechanisms, and overall gonad function (Thomas, 1990). Some EDCs may directly alter circulating steroid hormone levels by acting as agonists/antagonists and binding to steroid receptors. Other EDCs may affect steroid hormone levels via an indirect activation of steroid receptors by interacting with components of signaling pathways. For example, estrogen receptor activation can be triggered through the binding of epidermal growth factor to its cell membrane receptor. This binding activates signaling cascades involving the MAP kinase pathway and phosphorylation of the estrogen receptor. An EDC could mimic epidermal growth factor, leading to eventual phosphorylation and activation of the estrogen receptor (Crews *et al.*, 2000).

Vitellogenin Biomarker.

Current interest has been directed towards the vitellogenic cycle of fishes as a biomarker of environmental contamination and reproductive disruption (Berndtson and Chen, 1991; Summers *et al.*, 1997; Yamanaka *et al.*, 1998; Tyler *et al.*, 1996, 1999; Kime *et al.*, 1999; Smeets *et al.*, 1999). Vitellogenesis is the process by which yolk is formed (Fig. 2). Vitellogenin is a large molecular weight (approx. 200 kDa) plasma protein containing phosphorus, lipids, carbohydrates, calcium, and iron. Vitellogenin is normally synthesized by the liver in female vertebrates and is regulated by 17β -estradiol (E2) (Nicolas, 1999). A steroid binding protein transports E2 to a hepatocyte (liver cell). Estradiol enters the cell and binds to a nuclear estrogen receptor. The estrogen receptor interacts with another receptor resulting in formation of a homodimer and nuclear localization of the E2-receptor complex. Homodimers bind to a specific estrogen response element (DNA sequence) in the nucleus activating specific gene transcription, mRNA synthesis, and VTG production. Vitellogenin is released into circulating blood, and under gonadotropin promotion, is incorporated into an oocyte by receptor-mediated endocytosis. Following incorporation into the oocyte, VTG is proteolytically cleaved to form the yolk proteins lipovitellin and phosvitin. (Sherwood and Hew, 1994).

Induction of VTG is potentially indicative of exposure to estrogen receptor agonists in oviparous (egg-laying) species, such as teleost fishes. The endpoint is most sensitive in male fish where vitellogenesis is an abnormal process. While male fish possess the gene for VTG synthesis, expression is an inappropriate response triggered by estrogen mimicking chemicals. Reports of VTG expression in male fish begin as early as 1989. Ding *et al.* (1989) unintentionally detected low quantities of VTG in uninduced (by E2) male blue tilapia (*Oreochromis aureus*). Purdom *et al.* (1994) measured plasma VTG in male rainbow trout (*Oncorhynchus mykiss*) from 15 sewage treatment plants across England in response to hermaphroditic fish being observed in areas affected by effluent. In that study, exposure to effluent resulted in 500 to 100,000-fold induction of plasma VTG. In the United States, VTG induction and reduced serum testosterone levels were observed in male carp (*Cyprinus carpio*) collected near the St. Paul, MN, sewage treatment plant (Folmar *et al.*, 1996).

Organ-Level Indicators of Exposure: GSI, HSI, and CF.

There are also valuable organ-level indicators of exposure to environmental stressors and possible EDCs, such as tissue somatic indices. Contaminants could cause atrophy or hypertrophy of organs (e.g. gonads and liver). Environmental stressors could cause changes in feeding and relative condition. Three common indices are: (1) gonadosomatic index (GSI) (2) hepatosomatic index (HSI) (3) condition factor (CF). Gonadosomatic index follows the reproductive cycle of a fish and is the quotient of gonad mass to total mass. This index assumes that an ovary or testis will increase in size with increasing reproductive readiness. Hepatosomatic index provides an indirect measure of reproductive status and general body condition and is the quotient of liver mass to total mass. A more direct measure of well being is CF. Condition factor is the quotient of total mass to cubed total length. The calculation of CF assumes growth to be isometric in mature fish (i.e. an animal is increasing in all dimensions at the same rate) (King, 1995).

Creosote, Polycyclic Aromatic Hydrocarbons as EDCs.

Few field and laboratory wildlife studies have attempted to identify potential EDCs within environmental mixtures of chemicals, such as creosote, or to determine reliable screening methods of effects of such mixtures. Creosote is a complex mixture of chemicals created by high-temperature treatment of beech and other woods (wood creosote), coal (coal-tar creosote), or creosote bush resin (National Safety Council, 1997). Creosote consists of approximately 85 percent polycyclic aromatic hydrocarbons, 10 percent phenolic compounds, and 5 percent

nitrogen-, sulfur-, or oxygen- containing heterocyclic compounds (Mueller *et al.*, 1989). The International Agency for Research on Cancer has determined that coal-tar creosote is a probable carcinogen, citing cases of skin cancer and cancer of the scrotum from chronic, sublethal exposure (National Safety Council, 1997). Research focusing on endocrine-disrupting effects of creosote primarily has been limited to examination of effects of its principal components, such as polycyclic aromatic hydrocarbons.

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic pollutants that are released into the environment in large quantities, mainly due to human activities. Many PAHs are potent carcinogens in mammals and fish (Neff, 1985). Some studies using teleost models have found deleterious effects of PAHs on reproductive function. Sediment PAH concentrations greater than 100 ppm inhibited spawning in English sole (*Parophrys vetulus*) (Collier *et al.*, 1993). Diesel fuel oil and naphthalene both blocked sexual maturation and/or impaired ovarian recrudescence (rejuvenation after latency) in some Atlantic croaker (*Micropogonias undulatus*) (Thomas and Budiantara, 1995). In that study, a majority of oocytes were undeveloped, and widespread oocyte atresia (abnormality) was evident at higher concentrations of the chemicals. In another study, lower percentage hatch and survival were observed in starry flounder (*Platichthys stellatus*) in association with organic contaminants in San Francisco Bay (Spies and Rice, 1988). Exposure of mummichog (*Fundulus heteroclitus*) to benzo(a)pyrene lead to maternal transfer of the toxicant *in vitro* and *in vivo* via association of the toxicant with VTG (Monteverdi and Di Giulio, 2000). Tilghman-Hall and Oris (1991) reported reduced reproductive potential and maternal transfer of anthracene during chronic exposure of fathead minnows (*Pimephales promelas*). Several studies have demonstrated reduction in circulating E2 due to PAH exposure (Pajor *et al.*, 1990; Thomas, 1988, 1990; Johnson *et al.*, 1993; Thomas and Budiantara, 1995; Monteiro *et al.*, 2000). Carp (*Cyprinus carpio*) from Las Vegas Wash and Las Vegas Bay, areas of organochlorine, PAH, and semivolatile industrial compound contamination, had elevated levels of the androgen (male sex hormone) 11-ketotestosterone in females and low concentrations in males (Bevans *et al.*, 1996). Few studies address the mechanisms by which these PAHs alter reproductive fitness and steroid hormone levels.

The Elizabeth River.

An area of high-level creosote contamination and water-soluble creosote derived products is located in the southern branch of the Elizabeth River, VA, at the Atlantic Wood Industries facility. The present Elizabeth River has a deep, central channel fringed by shallows and developed shorelines. The physical nature of the river is such that little flushing of contaminants

occurs (Elizabeth River Project, 2000). The southern branch is distinguished by numerous industries and shipyards, including Atlantic Wood Industries, Inc., an inactive wood-treating facility. The Atlantic Wood site was used predominantly for creosote and pentachlorophenol (PCP) wood preserving, but also to store treated lumber and to dispose wastes from plant operations and the neighboring Norfolk Naval Shipyard. The site was added to the National Priorities List of the most serious, uncontrolled or abandoned hazardous waste sites requiring long-term remediation on February 21, 1990. In 1995, a Remedial Investigation/Feasibility Study of the site indicated that there were areas with high levels of contamination, including creosote products, PAHs, phenols, PCP, and metals, in on-site soils, river sediment, groundwater, and surface water (U.S. EPA, 1999).

The Mummichog.

Abundant in tidemarch habitats like those found along the Elizabeth River shorelines, is a small, euryhaline (able to exist in a wide range of salinities), teleost fish commonly called the mummichog or saltwater killifish. Mummichogs (*Fundulus heteroclitus*) are abundant in saltwater marshes and tidal creeks all along the East Coast of North America (Robins and Ray, 1986). Despite their widespread habitat, *F. heteroclitus* exhibit a restricted home range (Fritz *et al.*, 1975; Lotrich, 1975), and thereby, are good sentinels of local environmental conditions. *Fundulus heteroclitus* are continuous, semilunar spawners, synchronized with the cycle of spring tides in their individual tidemarch habitat (Taylor *et al.*, 1979). Females have asynchronous ovaries (i.e. follicles of all sizes are present at all times) but do exhibit cyclical changes in E2-induced production of VTG between spawning events (Selman and Wallace, 1983). Due to the harsh and ever-changing environment of the marsh, *F. heteroclitus* possess physiological mechanisms that enable them to adjust to drastic heat and salinity changes (Bulger, 1984; Hoffmann *et al.*, 1998; Flemmer *et al.*, 1999; Patrick and Wood, 1999). In addition, several studies have discovered populations acclimated to environmental contaminants as well. For example, embryos and larvae from the highly contaminated New Bedford Harbor, MA, were less sensitive to dioxin-like compounds than reference fish (Nacci *et al.*, 1999). Fish from the chronically polluted Piles Creek, NJ, exhibited greater tolerance to teratogenic effects of high concentrations of methylmercury (Weis *et al.*, 1981). These same fish proved to be less susceptible to behavioral alterations (i.e. prey capture and swimming performance) than fish from reference sites (Zhou *et al.*, 1996).

Studies in the Elizabeth River have found a population of *F. heteroclitus* resistant to creosote-associated acute mortality. Fish inhabiting a creosote-contaminated site adjacent to the

Atlantic Wood facilities appear hardy and seem to be reproducing successfully (Vogelbein unpublished). Research on chronic exposure has focused mainly on histopathological and biochemical changes associated with carcinogenesis. Primary investigations demonstrated that this population of fish exhibits high prevalence of advanced hepatocellular carcinoma (malignant tumors of the liver) and other chronic lesions (tissue abnormalities) (Vogelbein *et al.*, 1990; Fournie and Vogelbein, 1994; Vogelbein and Fournie, 1994; Vogelbein *et al.*, 1997; Vogelbein *et al.*, 1999). Other biomarkers (i.e. glutathione S-transferase, cytochrome P450, and P-glycoprotein) (Van Veld *et al.*, 1991; Van Veld *et al.*, 1992; Van Veld and Westbrook, 1995; Cooper *et al.*, 1999; Vogelbein *et al.*, 1999) demonstrate similarities in protein and enzyme expression comparable to multidrug resistant cell lines. Specifically, these studies suggest that depressed cytochrome P4501A levels and elevated glutathione S-transferase and P-glycoprotein levels within hepatic neoplasms (liver tumors) could be of adaptive significance, affording the Atlantic Wood population resistance to creosote exposure. To date, little research has been directed toward evaluation of reproductive function in this population.

OBJECTIVES

Several specific questions emerge from review of field and laboratory studies focusing on identification of EDCs and their effects on reproductive biology of wildlife: (1) Can VTG be used as a practical and reliable biomarker of creosote exposure and effects in fish? (2) Do chemical components of creosote induce overall classical estrogenic responses (e.g. induce VTG) *in vivo*? (3) Are observed changes in *F. heteroclitus* indicative of species-specific sensitivity? (4) Does tolerance of the Atlantic Wood population of *F. heteroclitus* to creosote-associated toxicity extend to the reproductive system?

To begin consideration of these questions, this study has four specific objectives:

1. To evaluate plasma VTG expression in both male and female *F. heteroclitus* collected from the Atlantic Wood site (creosote-contaminated) and two reference sites in lower Chesapeake Bay.
2. To evaluate plasma VTG expression in wild-caught, male reference *F. heteroclitus* exposed in the laboratory to sediment amended with that from the Atlantic Wood site.
3. To evaluate plasma VTG expression in hatchery-reared, male fathead minnow (*Promelas pimephales*) exposed in the laboratory to sediment amended with that from the Atlantic Wood site.
4. To evaluate reproductive status of all fish samples through the calculation of GSI, HSI, and CF.

Results will specifically assess whether readily metabolized compounds associated with coal-tar creosote, such as PAHs, exert estrogenic effects and regulate VTG. Overall, findings should evaluate use of VTG as a biomarker of exposure in fish from a creosote-contaminated site, and to some extent, of environmentally realistic mixtures and concentrations of xenobiotics as a whole, and provide insight into whole-animal consequences.

MATERIALS AND METHODS

Field Sampling.

Adult *F. heteroclitus* (approx. 6 to 9 cm total length) were collected on 20 June 2000 (three days after the full moon) within two hours of high tide from three sites within lower Chesapeake Bay, USA (Fig. 3). King Creek, located in Gloucester County, VA, feeds into the Mobjack Bay subestuary, and is relatively pristine (Vogelbein *et al.*, 1990). Copper Creek, within Ragged Island Wildlife Management Area, Isle of Wight County, VA, feeds into the James River subestuary, and is relatively free from residential and industrial areas (Virginia Department of Game and Inland Fisheries, 1999). Atlantic Wood, heavily contaminated with PAHs of creosote origin, lies within the southern branch of the Elizabeth River, Portsmouth, VA (Vogelbein *et al.*, 1990). Five female and four male fish were collected from each of six baited minnow traps per site for a total sample size of 54 fish per site. Fish were transported to the laboratory and maintained for 48 hours in separate bait buckets (according to trap number) in flow-through tanks of sand-filtered York River water (approx. 25°C, $S_{25^{\circ}\text{C}} \approx 20$ ppt, $\text{DO} \approx 5.25$ mg/L) to allow fish to stabilize.

Plasma Collection.

Fish were anesthetized with tricaine methanesulfonate (“MS-222”, Argent Chemical Co., Redmond, WA; 150 mg/L). An incision was made above the end of the anal fin, just below the lateral line. Blood was collected into heparinized microhematocrit capillary tubes (Fisher Scientific, Norcross, VA). Samples were chilled on ice for no greater than 20 minutes before centrifugation at 13,500 g for 10 minutes at 4°C in an Autocrit II (Becton, Dickinson, and Co., Parsippany, NJ). Tubes were scored; the plasma layer transferred into 1.5-mL microcentrifuge tubes previously coated with 35 USP heparin (Sigma Chemical Co., St. Louis, MO) and 0.132 TIU aprotinin (ICN Biomedicals, Inc., Aurora, OH) and stored at –80°C until examination for VTG. Fish were killed by decapitation and total length, total wet weight, eviscerated weight, gonad weight, and liver weight measured for each fish. Livers were stored in liquid nitrogen for cytochrome P4501A (CYP1A) analysis.

Mummichog Exposure to Elizabeth River Sediment.

Sixty adult male *F. heteroclitus* (approx. 6 to 9 cm total length) were collected on 4 August 2000 from the King Creek and Atlantic Wood sites. Fish were transported to the laboratory and maintained for one week in flow-through tanks of sand-filtered York River water (approx. 25°C). Surface sediments were collected from each site, sieved through one square-centimeter mesh, homogenized to uniformity, and stored at 0°C until use in the sediment exposure. King Creek sediment was amended on a volume : volume basis with Atlantic Wood sediment to yield 33 percent Atlantic Wood sediment for exposure. Sediments were placed into 38-L aquaria to a depth of two centimeters. King Creek sediment served as the control. Twenty-four hours prior to the introduction of fish, filtered York River water was introduced at a rate near 200 mL/min and maintained throughout the exposure. Twelve fish per site were bled on day zero to establish pre-exposure VTG and CYP1A concentrations and baseline GSI, HSI, and CF measurements. Remaining fish were randomly allocated for a total of 12 fish per tank. Treatments consisted of: (1) Atlantic Wood fish in King Creek sediment (2) Atlantic Wood fish in 33 percent Atlantic Wood sediment (3) King Creek fish in King Creek sediment (4) King Creek fish in 33 percent Atlantic Wood sediment. All exposures were performed in duplicate. Fish were exposed for one week and fed 0.5 g TetraMarine marine flakes (Tetra Sales, Blacksburg, VA) twice daily. Dissolved oxygen and temperature were measured daily and averaged 6.4 mg/L and 25.8°C. Mortalities and general fish health were also noted. On day seven, plasma was collected for examination of VTG; body measurements, for calculation of somatic indices, as described above.

Fathead Minnow Exposure to Elizabeth River Sediment.

Promelas pimephales were exposed to Atlantic Wood sediment to evaluate possible species differences in responsiveness. Eighteen adult male *P. pimephales* (approx. 6 to 7 cm total length; Chesapeake Cultures, Inc., Hayes, VA) were transported to the laboratory and maintained for one week in flow-through tanks of well water (approx. 19°C). King Creek sediment was amended on a volume : volume basis with Atlantic Wood sediment to yield a 20 percent exposure fraction. (The goal of the exposures was to mimic chronic, sublethal exposure. Based on mortality rate of reference *F. heteroclitus* in the sediment exposure described above, a more dilute Atlantic Wood fraction was used for exposure.) King Creek sediment still served as the control. Sediments were placed into 38-L aquaria to a depth of two centimeters. Twenty-four hours prior to the introduction of fish, filtered well water was introduced at a rate near 200 mL/min and maintained throughout the exposure. Nine fish were randomly allocated to either a tank of

contaminated or uncontaminated sediment. Fish were exposed for three weeks and fed 0.5 g TetraMarine marine flakes twice daily. Dissolved oxygen and temperature were measured daily and averaged 6.4 mg/L and 18.5°C. On day 21, plasma was collected for examination of VTG as described above.

Production of VTG Standard.

Adult male *F. heteroclitus* (approx. 7 cm total length) were collected from King Creek and allowed to acclimate for one week in flow-through tanks of York River water (approx. 25°C). After acclimatization, half of the fish were removed, lightly anesthetized in MS-222 (150 mg/L), and injected intraperitoneally with 50 µL 17β-estradiol (0.5 mg E2/mL corn oil; Sigma Chemical Co.). The remaining fish were bled for control plasma. Individuals were injected on day zero, two, and four. Plasma was collected on day seven. Approximately 500 µL of E2-induced and control plasma each were sent to Dr. Nancy D. Denslow at the University of Florida/Interdisciplinary Center for Biotechnology Research for purification of VTG through PerSeptive's BioCAD Workstation (perfusion chromatography). The same was performed with adult male *P. pimephales* (approx. 6 cm total length) to obtain purified VTG from this fish species. Final yields were 0.28 mg/mL and 1.2 mg/mL (50% glycerol). Aliquots were stored at -20°C.

Immunodetection of VTG (Western Blotting).

Plasma VTG was quantified similar to that of microsomal CYP1A as described by Van Veld *et al.* (1988) and Van Veld and Westbrook (1995). Plasma samples were kept on ice throughout the procedure. Denatured plasma proteins were size separated on eight percent sodium dodecyl sulfate-polyacrylamide gels and transferred to 20-µm nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA) using a Mini-PROTEAN 3 Cell and Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories). Dr. Charles Rice's monoclonal antibody, FV 10-9 (anti-killifish VTG; undiluted, raw hybridoma supernate), was used as the primary antibody for *F. heteroclitus* analyses. An anti-carp VTG monoclonal antibody ("170115", Cayman Chemical Co., Ann Arbor, MI; 1:400 dil., 5% milk/tris buffered saline) was used for analysis of *P. pimephales* plasma samples. Alkaline phosphatase-conjugated goat anti-mouse Ig (H+L) was used as the secondary antibody (1:200 dil., 5% milk/tris buffered saline; Southern Biotechnology Associates, Inc., Birmingham, AL). Reactive bands were developed and visualized with p-nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (Bio-Rad Laboratories) (Fig. 4).

Color intensity was quantified with a thin-layer chromatography scanner (Shimadzu, Kyoto, Japan) against purified VTG from the corresponding fish species. The standard curve was linear and ranged from 15 to 90 ng VTG. Method detection limit equaled 5.8 ng VTG; the limit of quantitation, 19 ng VTG. Interassay variance was determined as 3.5 mg VTG/mL plasma.

Immunodetection of CYP1A (Western Blotting).

Hepatic microsomal CYP1A was measured in a subset of field fish samples (18 per site for a total of 54 samples) and in all sediment exposure fish samples to monitor exposure level. Determination of CYP1A concentrations followed that described by Van Veld *et al.* (1988) and Van Veld and Westbrook (1995). Samples were kept on ice throughout the procedure. Liver tissues were thawed and homogenized in stabilization buffer (100 mM potassium phosphate, pH 7.4, containing 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride and 20% glycerol) with a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). Homogenates were centrifuged twice at 12,000 g for 10 minutes, with the pellet discarded each time. The resulting supernatant was centrifuged at 100,000 g for 60 minutes. The pellet was resuspended immediately in cold stabilization buffer and stored in liquid nitrogen.

Denatured microsomal proteins were size separated on 12 percent sodium dodecyl sulfate-polyacrylamide gels and transferred to 20- μ m nitrocellulose paper. Dr. John Stegeman's (Woods Hole Oceanographic Institution) monoclonal antibody, "MAb 1-12-3", was used as the primary antibody (1:400 dil., 5% milk/tris buffered saline) and alkaline phosphatase-conjugated goat anti-mouse Ig (H+L) used as the secondary antibody (1:200 dil., 5% milk/tris buffered saline). The label was developed and visualized with p-nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

Color intensity was quantified with a thin-layer chromatography scanner against benzo(a)pyrene-induced *F. heteroclitus* microsomal samples. These microsomal samples were calibrated against hepatic microsomal CYP1A from spot (*Leiostomus xanthurus*) as described by Van Veld *et al.* (1988). The standard curve was linear and ranged from 0.1 to 0.8 pmol CYP1A. Method detection limit equaled 0.0411 pmol CYP1A; the limit of quantitation, 0.137 pmol CYP1A. Interassay variance was determined as 0.00924 pmol CYP1A/ μ g protein.

Calculation of GSI, HSI, and CF.

GSI. The quotient (expressed as a percentage) of gonad mass to total mass (Eq. 1). This assumes that an ovary or testes will increase in size with increasing reproductive readiness.

$$\text{Eq. 1.} \quad \left[\frac{\text{wet mass of the gonad (g)}}{\text{total wet mass of the animal (g)}} \right] \times 100$$

HSI. The quotient (expressed as a percentage) of liver mass to total mass (Eq. 2).

$$\text{Eq. 2.} \quad \left[\frac{\text{wet mass of the liver (g)}}{\text{total wet mass of the animal (g)}} \right] \times 100$$

CF. The quotient of total mass to cubed total length (Eq. 3). This assumes that growth is isometric in mature *F. heteroclitus* (i.e. an animal is increasing in all dimensions at the same rate). This assumption is verified through regression analysis of total length versus calculated CF. Slope should not be significantly different from zero. To correct for elevated condition factors due to varying stages of ovarian development/reproductive status and associated increases in variance, total eviscerated weight was used instead of total mass.

$$\text{Eq. 3.} \quad \left[\frac{\text{total eviscerated mass of the animal (g)}}{[\text{total length of the animal (cm)}]^3} \right] \times 100$$

Statistical Design.

I. Parametric or Nonparametric Statistics. All indices were tested for normality through the Anderson-Darling Test, based on an empirical cumulative distribution function. Equality or homogeneity of variance was assessed using Bartlett's test. An F-test replaced Bartlett's test if there were just two sample groups. If heteroscedasticity was found, data were \log_{10} transformed before analysis since variance seemed to be a function of the mean. The antilog of means and other descriptive statistics are used in discussions to aid in biological interpretation. If data sets included values below detection limits or within the region of less certain quantitation, nonparametric rank tests were utilized.

II. Differences Among Means (Parametric Statistics). The Analysis of Variance (ANOVA) was used to test for differences among sample means. If more than two levels existed, significance was further evaluated through Tukey's method (also called Tukey's HSD or Tukey-Kramer method), a multiple comparison procedure. Tukey's experimentwise error rate was always equal to 0.05. To ensure sufficient replication and to account for within-group variability, both laboratory sediment exposures and the field study were of a hierarchical design. A fully nested ANOVA was first performed to determine whether the "random-effects" nested factor was significant. If significant, no further analyses were performed and significance assessed between levels. If not significant, replicates were collapsed and a one-way ANOVA and Tukey's test performed.

III. Differences Among Means (Nonparametric Statistics). The Kruskal-Wallis test, like Mood's Median test, offers a nonparametric alternative to the one-way ANOVA. It is a single-factor analysis of variance by ranks, testing for the equality of medians for two or more populations. The critical statistic was adjusted for tied ranks. If more than two levels existed, significance was further evaluated through one of two nonparametric multiple comparison tests, Nemenyi or Dunn. The Nemenyi test is a nonparametric Tukey-type multiple comparison but requires equal numbers of data in k groups. The Dunn test uses a modified standard error to account for unequal sample sizes. The experimentwise error rate was maximally equal to 0.05.

IV. Correlation Analysis. Correlation analysis was performed to see whether HSI (i.e. change in liver size) was indirectly related to increases/decreases in biotransformation enzyme level associated with the detoxification of chemical compounds (in contrast to VTG production). To allow for computation with data at detection limits or within the region of lesser uncertainty, the nonparametric Pearson's product moment correlation coefficient was used to establish whether or not the two variables were correlated.

RESULTS

Field Study.

Male and female *F. heteroclitus* were collected from the Atlantic Wood site (creosote-contaminated) and two reference sites in lower Chesapeake Bay to evaluate possible differences in VTG that may be related to contaminant exposure. Analysis indicated that Atlantic Wood female plasma VTG concentrations were not different from those of reference site fish (Nested ANOVA: $F = 1.099$, $df = 2,15$, $p = 0.358$). Males showed no evidence of VTG (Fig. 5).

Tissue somatic indices were also used as organ-level indicators of exposure to creosote and reproductive disruption. Atlantic Wood fish had general body conditions as good as those from both reference sites. Condition factor did differ between reference sites (Tukey's: $df = 159$, $K = 3$, $p = 0.05$) (Fig. 6). If examined by sex, similar trends were observed in female body conditions (Nested ANOVA: $F = 5.669$, $df = 2,15$, $p = 0.015$). In contrast, male body conditions did not differ between any site (One-way ANOVA: $F = 1.03$, $df = 2,69$, $p = 0.364$).

Gonadosomatic index was only examined by sex between sites. Atlantic Wood females had greater relative gonad size than those of reference site fish (Tukey's: $df = 87$, $K = 3$, $p = 0.05$), with ovaries seeming particularly ripe. Testes of Atlantic Wood males were comparable in relative size to those of King Creek fish. However, Ragged Island mean GSI was significantly lower than that of either the Atlantic Wood or King Creek sites (Nemenyi: $n = 72$, $K = 3$, $p = 0.03$) (Fig. 7). Hepatosomatic index differed among all three sites (Tukey's: $df = 159$, $K = 3$, $p = 0.05$). If examined by sex, Atlantic Wood female fish tended to have the greatest relative liver sizes, followed by King Creek and then Ragged Island fish (Nested ANOVA: $F = 33.723$, $df = 2,15$, $p < 0.001$). Mean HSI of Atlantic Wood males was greater than that of Ragged Island, but not that of King Creek males (Tukey's: $df = 69$, $K = 3$, $p = 0.05$) (Fig. 8). Fish lengths did vary, with King Creek fish generally smaller than those of the other two sites (Kruskal-Wallis: $H_c = 32.29$, $df = 2$, $p < 0.001$). If examined by sex, King Creek total lengths still differed (females, Kruskal-Wallis: $H_c = 16.78$, $df = 2$, $p < 0.001$; males, Nested ANOVA: $F = 4.982$, $df = 2,15$, $p = 0.022$). The smaller size of King Creek fish did not appear to be associated with changes in sexual maturation or reproductive readiness when compared to the other sites.

In summary, analyses of indices (Table A.1) indicate that the Atlantic Wood population of *F. heteroclitus* is resilient and reproductively fit when compared to reference fish. Vitellogenin was not observed in any male fish and no site-specific differences were determined in female plasma VTG concentrations. Overall, body conditions were as good as those from both reference sites. Relative gonad size was comparable to or greater than those of reference site fish. Atlantic Wood fish did tend to have slightly larger relative liver sizes.

Cytochrome P4501A concentration was measured to monitor exposure level. Atlantic Wood CYP1A concentrations were significantly elevated relative to those of King Creek, but not elevated compared to Ragged Island concentrations (Nemenyi: $n = 172$, $K = 3$, $p = 0.03$) (Fig. 9). If examined by sex, female Atlantic Wood CYP1A concentrations were approximately five times greater than those of King Creek, but again, were equivalent to those of Ragged Island females ($n = 90$, $p = 0.05$). Male Atlantic Wood CYP1A concentrations were much greater than those of either reference site ($n = 72$, $p = 0.05$) (Fig. 10). Comparisons of CYP1A within each site by sex suggest depression of CYP1A in females. Atlantic Wood males had higher (approximately nine times) CYP1A concentrations than females (Kruskal-Wallis: $H_c = 12.02$, $df = 1$, $p = 0.001$). King Creek male CYP1A concentrations were three times female concentrations ($H_c = 6.65$, $p = 0.010$). Ragged Island fish showed no sex differences in CYP1A concentrations ($H_c = 0.00$, $p > 0.999$). Correlation analysis indicated a minor positive trend between male CYP1A concentrations and (\log_{10}) HSI (Pearson's product moment correlation: $r = 0.411$, $p = 0.046$) (Fig. 11). Correlation analysis for all samples, as well as for females only, determined no significant trend between liver size and CYP1A level ($r = -0.025$, $p = 0.858$; $r = -0.225$, $p = 0.232$).

Laboratory Exposures to Elizabeth River Sediment.

Mummichog. Wild-caught, male reference *F. heteroclitus* were exposed in the laboratory to Atlantic Wood sediment to evaluate plasma VTG expression in male reference fish in response to creosote exposure. No VTG was detected in any of the fish exposed to Atlantic Wood sediment. Gonadosomatic index and CF were comparable between treatments (Nested ANOVA: $df = 3,4$; $F = 0.537$, $p = 0.682$; $F = 5.049$, $p = 0.076$). King Creek control fish did tend to have the greatest relative body conditions; Atlantic Wood fish in reference (King Creek) sediment, the lowest. Atlantic Wood and King Creek fish exposed to Atlantic Wood sediment had similar body conditions. Relative liver sizes did differ with exposure. Mean HSI of King Creek fish exposed to Atlantic Wood sediment was greater relative to that of King Creek and Atlantic Wood fish

maintained in control sediment, but not to that of Atlantic Wood fish maintained in Atlantic Wood sediment (Tukey's: $df = 60$, $K = 4$, $p = 0.05$) (Fig. 12).

In summary, analyses of indices (Table A.2) indicate parallel trends in reference fish exposed to creosote-contaminated sediment relative to those found in Atlantic Wood fish examined from the field. No VTG was detected in any of the male fish exposed to Atlantic Wood sediment. Body conditions and relative gonad sizes were comparable between sediment treatments. Reference fish exposed to Atlantic Wood sediment did tend to have larger relative liver sizes. Overall, fish remained active and fed throughout the exposure. There were no mortalities in any treatments with Atlantic Wood fish. One mortality was observed on day two, and another on day six, from King Creek fish in reference sediment. King Creek fish exposed to 33 percent Atlantic Wood sediment suffered extensive mortalities (approx. 25%); one on day three, one on day four, three on day five, and another on day seven. Four of these came from one tank, decreasing overall experiment sample size from twelve to eight fish per tank.

Corresponding to such a lethal exposure was high level induction of CYP1A. Induction of CYP1A in King Creek fish exposed to Atlantic Wood sediment was strikingly apparent (approximately 19.5 times greater than the median for Atlantic Wood fish in Atlantic Wood sediment; 41 times greater than the median for King Creek fish in King Creek sediment), and differed from all other fish/treatment combinations (Dunn: $n = 63$, $K = 4$, $p = 0.04$) (Fig. 13). Correlation analysis of HSI versus CYP1A for all samples illustrated a positive trend between liver size and biotransformation enzyme level (Pearson's product moment correlation: $r = 0.440$, $p = 0.001$). Correlation analysis of HSI versus CYP1A for exclusively King Creek fish showed a stronger trend ($r = 0.578$, $p = 0.001$).

In addition, 12 fish per site were bled on day zero of the sediment exposure to establish pre-exposure VTG and CYP1A concentrations and baseline GSI, HSI, and CF measurements. There was little variation of CF between day 0 and day 7 Atlantic Wood control fish (One-way ANOVA: $F = 1.62$, $df = 2,25$, $p = 0.218$). Some alterations were evident between day zero and day seven controls from each site for GSI, HSI, and CYP1A. While relative gonad size differed between day seven replicates, values from both day seven replicates were indistinguishable from those of day zero (Tukey's: $df = 25$, $K = 3$, $p = 0.05$). Day seven control Atlantic Wood fish had greater relative liver sizes than those of day zero fish (One-way ANOVA: $F = 7.56$, $df = 1,26$, $p = 0.011$). Conversely, day zero CYP1A concentrations were greater than those of day seven fish (Kruskal-Wallis: $H = 8.00$, $df = 1$, $p = 0.005$). Approximately the same was determined for control King Creek fish (those exposed to King Creek sediment). There was no difference of CF between day zero and day seven fish (One-way ANOVA: $F = 0.76$, $df = 1,26$, $p = 0.390$).

Relative gonad sizes for day seven fish were slightly lower than those of day zero fish ($F = 4.50$, $p = 0.044$). Again, day seven relative liver sizes were greater than those of day zero fish ($F = 23.98$, $p < 0.001$); day zero CYP1A greater than day seven (Kruskal-Wallis: $H_c = 6.76$, $df = 1$, $p = 0.009$). Total length did differ significantly (One-way ANOVA: $F = 5.18$, $df = 1,26$, $p = 0.031$), but again, did not appear to be associated with changes in sexual maturation or reproductive readiness.

Fathead Minnow. *Promelas pimephales* were exposed to Atlantic Wood sediment to evaluate responsiveness of alternate species to creosote exposure. As with *F. heteroclitus*, no plasma VTG was detected in any of the male fish exposed to Atlantic Wood sediment. There were no mortalities in any treatments. Fish exposed to Atlantic Wood sediment demonstrated considerable induction of CYP1A (approximately 45 times greater than the median of control samples) (Kruskal-Wallis: $H_c = 12.92$, $df = 1$, $p < 0.001$).

DISCUSSION

Male and female *F. heteroclitus* were sampled from two reference and a creosote-contaminated (Atlantic Wood) site in lower Chesapeake Bay to evaluate possible differences in VTG that may be related to contaminant exposure. Vitellogenin was not observed in any male fish collected from any of the sites. No site-specific differences were observed in plasma VTG of females from these sites. Lack of response could be attributed to: (1) physiological adaptations of the Atlantic Wood population to creosote exposure (2) species-specific sensitivity to hepatotoxicity (3) readily metabolized compounds associated with coal-tar creosote, such as PAHs, not exerting estrogenic effects and regulating VTG.

Atlantic Wood fish exhibit depressed CYP1A and elevated glutathione S-transferase and P-glycoprotein relative to exposed reference fish (Van Veld *et al.*, 1991; Van Veld *et al.*, 1992; Van Veld and Westbrook, 1995; Cooper *et al.*, 1999; Vogelbein *et al.*, 1999), enabling them to resist creosote-associated acute mortality, and possibly, to remain reproductively fit. Research suggests that PAHs require metabolic transformation before they can produce endocrine-disrupting effects (Bulger *et al.*, 1985; Thomas and Smith, 1993; Anderson *et al.*, 1996). Modulations in protein and enzyme expression endogenous to the Atlantic Wood population could therefore help these fish to maintain “normal” reproductive function in a contaminated environment. Depressed CYP1A levels would decrease metabolic transformation of possible EDCs. Elevated glutathione S-transferase and P-glycoprotein levels would increase clearance of possible EDCs from the body. Combined, this change in protein profile would decrease/inhibit the ability of EDCs to affect the reproductive endocrine system.

Analyses of indices indicate comparable CF, GSI, and HSI and maintenance of reproductive function of the Atlantic Wood population. Results are also in agreement with a variety of studies that suggest lower total P450 content and lesser enzymatic rates of several monooxygenases in reproductively active females, which is paralleled in CYP1A expression. Some researchers suggest that (indirectly) P450 protein translation may be suppressed by translation of E2-induced VTG (Pajor *et al.*, 1990; Gray *et al.*, 1991). Comparisons of CYP1A within sites by sex indicate that male concentrations were greater than those of females at the Atlantic Wood and King Creek sites, supporting (E2) suppression of CYP1A in females at these

sites. (Interestingly, Ragged Island fish displayed no sex differences in CYP1A. Lesser mean GSI and HSI for Ragged Island fish suggest a possible difference in reproductive stage when compared to that of Atlantic Wood and King Creek fish. If E2 levels of Ragged Island females were not as high as that of females from the other sites, there would be less possible depression of CYP1A concentrations. Comparable CYP1A levels might also stem from changes in habitat and/or diet. However, Ragged Island fish had smaller relative body conditions. Thus, it is more likely that there might be a greater influx of xenobiotics into the tidal Ragged Island wildlife refuge from nearby heavily industrialized areas of the James River, increasing CYP1A level.) Overall, results from the field study suggest a possible lack of response of vitellogenesis to creosote associated contaminants as a result of physiological adaptations of the Atlantic Wood population to creosote exposure.

Results from the *F. heteroclitus* laboratory sediment exposure do not support physiological adaptations of the Atlantic Wood population to creosote exposure as the mechanism for maintenance of reproductive function. Rather, analyses of indices suggest species-specific sensitivities or readily metabolized compounds associated with coal-tar creosote, not regulating *F. heteroclitus* vitellogenesis. No VTG was detected in any of the male fish exposed to Atlantic Wood sediment. It is possible that exposure levels were inadequate to induce a response. However, exposure was sufficient to cause mortality. Further, in surviving reference fish exposed to Atlantic Wood sediment there was marked induction of CYP1A, significantly different from all other fish/treatment combinations. Thus, the lack of VTG induction cannot be for lack of exposure. Differences are either due to species-specific sensitivity to hepatotoxicity or whole creosote not eliciting estrogenic responses.

Promelas pimephales were exposed to Atlantic Wood sediment to evaluate responsiveness of an alternate species. Once more, no plasma VTG was detected in any of the male fish exposed to Atlantic Wood sediment. There was a 45-fold induction of CYP1A, indicating adequate exposure. In this research, production of VTG standards resulted in final yield of E2-induced, purified *F. heteroclitus* VTG which was an order of magnitude less than that from an equal volume of *P. pimephales* plasma. Differences in VTG production suggest that while both male *F. heteroclitus* and *P. pimephales* are capable of E2-induced VTG induction, *F. heteroclitus* may be less sensitive to alterations compared to other fish species. However, Nichols *et al.* (1999) also found no detectable plasma VTG in male *P. pimephales* following a three-week exposure to effluents from a central Michigan wastewater treatment plant. Overall, findings more strongly suggest that observations are not due to species-specific sensitivity or to exposure time, but rather to the nature of the chemical constituents of creosote itself.

Research focusing on creosote- or PAH-induced reproductive disruption is limited. Embryotoxicity of petroleum creosote (via culture medium) was observed in mouse embryos exposed to concentrations greater than 33 µg/mL (Iyer *et al.*, 1992). In a follow-up study, petroleum creosote was not found to be teratogenic in mouse fetuses (Iyer *et al.*, 1993). One hundred percent of Pacific herring (*Clupea pallasii*) embryos adhering to creosote-treated wood failed to complete development (Vines *et al.*, 2000). In that study, nearly 50 percent of embryos exposed to water-soluble creosote-derived compounds also failed to hatch. Survivors exhibited a 93 percent reduction in heart rate and alteration in larval movement. More recently, whole creosote was found to bind to the mouse estrogen receptor, approximating 165 mg/L of E2 equivalents (Fielden *et al.*, 2000). The compounds responsible for this binding were not identified. Drawing conclusions about the lack of response of the reproductive endocrine system, especially vitellogenesis, to creosote exposure is therefore difficult. Review of the effects of its principal component, PAHs, sheds little additional insight into the mechanism of action.

Some studies have found deleterious effects of PAHs on reproductive function and vitellogenesis, while others report minimal or no effects. Several researchers have observed a decrease in female plasma VTG levels in several fish species exposed to PAH contamination (Chen *et al.*, 1986; Singh, 1989; Thomas, 1990). Pereira *et al.* (1992) and Anderson *et al.* (1996) in their studies of winter flounder (*Pleuronectes americanus*) and rainbow trout (*Oncorhynchus mykiss*) both describe increases in female plasma VTG under low contaminated conditions, but decreases if concentrations were high. Bevans *et al.* (1996) detected VTG in blood of male carp (*C. carpio*) from Las Vegas Wash and Las Vegas Bay. No VTG induction was detected in retene-exposed brown trout (*Salmo trutta*) (Sherry *et al.*, 1999). Dietary PAH exposure did not affect germ cell development or ovarian growth in female flounder (*P. flesus*) (Monteiro *et al.*, 2000). No significant alteration of gonadal maturation or VTG levels was observed in starry flounder (*P. stellatus*) from clean and contaminated sites in San Francisco Bay (Spies *et al.*, 1990). Hansson *et al.* (1982) were unable to detect any impairment of pituitary function in rainbow trout (*Salmo gairdneri*) exposed to PAHs.

The mechanism of action that enables Atlantic Wood fish to maintain reproductive readiness in a highly contaminated site and display no induction/reduction of VTG remains undetermined. Results herein do not show strong support for an estrogenic response to creosote exposure, as VTG was not observed in any male fish and female plasma VTG concentrations were comparable. Further, virtually no difference was observed in reproductive readiness between Atlantic Wood fish and those of the two reference sites of either sex or of male reference fish exposed to creosote-contaminated sediment. Some researchers support an aryl hydrocarbon-

mediated antiestrogenic activity (Kharat and Saatcioglu, 1996; Klinge, 1999; Nicolas, 1999; Navas and Segner, 2000). This study did not specifically address antiestrogenic activity, as reference females were not exposed to creosote-contaminated sediment. If chemical tolerance of Atlantic Wood fish does extend to the reproductive system, antiestrogenic effects would not be observed in wild-caught Atlantic Wood females. Some cyclic hydrocarbons bind to the androgen receptor as well as the estrogen receptor (Danzo, 1997). This study used no indicators of exposure to an (anti-) androgen other than GSI. While there might be competitive ligand binding for the androgen receptor, it is difficult to distinguish between agonist or antagonist bound ligand. In addition, the P450 family has a wide range of reactions, but the catalytic mechanism is similar for all, depending on the latitude for substrate binding. This allows for much overlap of activities with few substrates exclusive to one protein. Many organic compounds can induce hepatic monooxygenases, which could result in increased steroid catabolism and clearance from circulation (Stegeman and Hahn, 1994). Furthermore, mixtures of EDCs often behave differently compared to their individual behavior. It then becomes necessary to determine whether their actions are additive or synergistic. Lack of observable creosote-induced effects on reproductive function, especially vitellogenesis, might stem from synergistic actions of its chemical constituents.

Last, overall variance of female plasma VTG levels was greater between fish within a sample than between replicates. A partial source of error might be the variable nature of western blot results. Interassay variance was considerable, equaling 3.5 mg VTG/mL plasma. Variance might also arise from differences caused by asynchronous ovaries and cyclical spawning. In other applications, differences in VTG induction might occur from varying sensitivities to hepatotoxicity. Cytochrome P4501A increased with chemical exposure, as did the standard deviations of untransformed data, causing heterogeneity of variance. Within a sample of fish, some specimens will always respond dramatically, others, not at all. Xenobiotic-induced VTG induction will be subject to this differential sensitivity. It is recommended that in testing effects or comparing fish from different natural sampling sites, the average of several samples (replicates) would be a better basis for statistical comparison than a single sample of many fish.

This research also used tissue-level tools to evaluate reproductive disruption. HSI was the most variable, and thus, informative. Relative liver size tended to be greater in Atlantic Wood fish than in reference fish. Reference fish exposed to Atlantic Wood sediment also exhibited increases in HSI. Several probable causes of increased liver size are: (1) swelling of fish tissues (2) increased production of proteins synthesized in the liver, thus increasing total hepatic RNA and HSI (3) increased carbohydrates in the diet.

Exposure to contaminants often leads to either hypertrophy or atrophy of fish tissues. Studies have discovered that the Atlantic Wood population of fish has high prevalence of advanced hepatocellular carcinoma and other chronic lesions (Vogelbein *et al.*, 1990; Fournie and Vogelbein, 1994; Vogelbein and Fournie, 1994; Vogelbein *et al.*, 1997; Vogelbein *et al.*, 1999). Abnormal cellular growth would increase liver mass. Chronic lesions would cause swelling of the tissue and increasing liver size.

Increased detoxification enzyme activity can also (indirectly) increase liver size (Dixon *et al.*, 1987; Stegeman and Hahn, 1994). Production of biotransformation enzymes would be preceded by upregulation of mRNA, thus increasing total hepatic RNA and HSI. Correlation analysis of both field and sediment exposure male data supports this concept. In general, males tended to have increasing CYP1A levels in parallel with increasing HSI. Analysis of female data found no significant trend between HSI and CYP1A, suggesting other increases in total RNA, such as mounting VTG production.

Results from the laboratory exposure (*F. heteroclitus*) suggest dietary alterations of HSI as well. Regardless of site, day seven controls had greater mean HSI than that of corresponding day zero controls. Causes of liver enlargement could be due to increases in carbohydrates in the diet (Dixon and Hilton, 1981). TetraMarine marine flakes are approximately 46 percent protein, with the bulk filler carbohydrates such as brown rice, oatmeal, and wheat flour. Possibly, minor increases in HSI were associated with dietary changes of wild-caught fish specimens during the laboratory exposure. Overall, alterations in HSI seem to primarily stem from increases in production of biotransformation enzymes.

Some changes in CYP1A during the sediment exposures might have been due to an external influence as well. In both sites, day seven CYP1A levels were lower than corresponding day zero controls. Differences probably arise from holding both fish types in a cleaner environment during the sediment exposure. Atlantic Wood fish were maintained in only 33 percent Atlantic Wood sediment. King Creek fish were kept in King Creek sediment, but without import of naturally occurring PAHs and other xenobiotics. Again, CYP1A induction in reference fish exposed to Atlantic Wood sediment was so marked that such minor fluctuations would have little effect on determination of the occurrence of contaminant-induced CYP1A.

In summary, while VTG has been used successfully as a practical and reliable screening method for wildlife toxicity, its use in assessment of *in vivo* creosote-associated toxicity is questionable. Lack of induction or change in VTG level does not support use of this biomarker for *F. heteroclitus* from creosote-contaminated sites. Cytochrome P4501A and HSI continue to be reliable biomarkers of creosote (and PAH) contamination. Further studies need to explore

development of alternative biomarkers not sensitive to aryl hydrocarbon- mediated antiestrogens if detection of weak estrogens in creosote and other complex mixtures is to be achieved. Additional research is needed to better explain the mechanism by which PAHs effect vitellogenesis and alter steroid hormone levels. Last, a broader range of organisms needs to be investigated.

Table A.1. Summary statistics for field-collected *Fundulus heteroclitus* separated by indices, site and sex.

	Mean	Median	S.E.	S.D.	N
VITELLOGENIN (mg/mL plasma)					
<i>FEMALES:</i>					
Atlantic Wood	4.0	4.0	0.23	1.3	30
King Creek	3.5	3.6	0.26	1.4	30
Ragged Island	3.2	3.1	0.22	1.2	30
GONADOSOMATIC INDEX (%)					
<i>FEMALES:</i>					
Atlantic Wood	6.60	5.70	0.465	2.55	30
King Creek	2.88	2.84	0.150	0.821	30
Ragged Island	1.90	1.80	0.106	0.578	30
<i>MALES:</i>					
Atlantic Wood	1.84	1.75	0.0856	0.419	24
King Creek	1.83	1.77	0.108	0.530	24
Ragged Island	1.17	1.07	0.113	0.551	24
<i>TOTAL:</i>					
Atlantic Wood	4.49	4.28	0.416	3.05	54
King Creek	2.41	2.26	0.119	0.875	54
Ragged Island	1.58	1.48	0.0910	0.669	54
HEPATOSOMATIC INDEX (%)					
<i>FEMALES:</i>					
Atlantic Wood	3.19	3.29	0.100	0.549	30
King Creek	2.54	2.43	0.103	0.564	30
Ragged Island	1.86	1.83	0.0654	0.358	30
<i>MALES:</i>					
Atlantic Wood	1.94	1.80	0.0792	0.388	24
King Creek	1.74	1.58	0.0827	0.405	24
Ragged Island	1.61	1.63	0.0883	0.433	24
<i>TOTAL:</i>					
Atlantic Wood	2.64	2.60	0.107	0.790	54
King Creek	2.19	2.11	0.0868	0.638	54
Ragged Island	1.75	1.74	0.0557	0.409	54

APPENDIX A

Summary Statistics Tables

Table A.1. Summary statistics for field-collected *Fundulus heteroclitus* separated by indices, site and sex (*cont.*)

	Mean	Median	S.E.	S.D.	N
CONDITION FACTOR					
<i>FEMALES:</i>					
Atlantic Wood	1.2	1.2	0.013	0.070	30
King Creek	1.2	1.2	0.015	0.080	30
Ragged Island	1.1	1.1	0.014	0.076	30
<i>MALES:</i>					
Atlantic Wood	1.3	1.3	0.017	0.085	24
King Creek	1.2	1.3	0.010	0.050	24
Ragged Island	1.2	1.2	0.023	0.11	24
<i>TOTAL:</i>					
Atlantic Wood	1.2	1.2	0.012	0.086	54
King Creek	1.2	1.2	0.0094	0.069	54
Ragged Island	1.2	1.2	0.015	0.11	54
CYTOCHROME P4501A (fmol/μg protein)					
<i>FEMALES:</i>					
Atlantic Wood	9.48	7.81	2.25	7.11	10
King Creek	2.52	1.71	0.645	2.04	10
Ragged Island	10.2	7.22	2.14	6.78	10
<i>MALES:</i>					
Atlantic Wood	79.9	69.1	13.5	38.1	8
King Creek	5.69	5.02	1.07	3.02	8
Ragged Island	9.69	7.71	1.81	5.12	8
<i>TOTAL:</i>					
Atlantic Wood	40.8	19.6	10.3	43.8	18
King Creek	3.93	2.91	0.692	2.93	18
Ragged Island	9.95	7.22	1.40	5.93	18

* All six traps were pooled for each site to achieve one summary statistic/indices/site.

** The antilog of means and other descriptive statistics are used here to aid in biological interpretation.

Table A.2. Summary statistics for *Fundulus heteroclitus* from day seven of the laboratory sediment exposure, separated by indices and treatment.

Fish Type/Sediment Type	Mean	Median	S.E.	S.D.	N
VITELLOGENIN (mg/mL plasma)					
Atlantic Wood/King Creek	n/a	n/a	n/a	n/a	16
King Creek/King Creek	n/a	n/a	n/a	n/a	16
Atlantic Wood/Atlantic Wood	n/a	n/a	n/a	n/a	16
King Creek/Atlantic Wood	n/a	n/a	n/a	n/a	16
GONADOSOMATIC INDEX (%)					
Atlantic Wood/King Creek	0.734	0.689	0.0718	0.287	16
King Creek/King Creek	0.926	0.933	0.0564	0.226	16
Atlantic Wood/Atlantic Wood	0.960	0.770	0.119	0.478	16
King Creek/Atlantic Wood	0.810	0.822	0.0751	0.301	16
HEPATOSOMATIC INDEX (%)					
Atlantic Wood/King Creek	1.84	1.81	0.147	0.589	16
King Creek/King Creek	1.89	2.00	0.117	0.468	16
Atlantic Wood/Atlantic Wood	2.31	2.19	0.187	0.750	16
King Creek/Atlantic Wood	2.62	2.57	0.205	0.819	16
CONDITION FACTOR					
Atlantic Wood/King Creek	1.0	1.0	0.019	0.078	16
King Creek/King Creek	1.2	1.2	0.022	0.089	16
Atlantic Wood/Atlantic Wood	1.1	1.1	0.016	0.064	16
King Creek/Atlantic Wood	1.1	1.1	0.020	0.078	16
CYTOCHROME P4501A (fmol/μg protein)					
Atlantic Wood/King Creek	22.3	21.4	3.73	14.4	15†
King Creek/King Creek	9.36	10.3	1.53	6.14	16
Atlantic Wood/Atlantic Wood	26.5	26.7	4.91	18.4	14†
King Creek/Atlantic Wood	507	426	55.4	215	15†

† - Values below detection limits were excluded from summary statistic computations.

* Replicate tanks were pooled for each sediment exposure treatment to achieve one summary statistic/indices/site.

** The antilog of means and other descriptive statistics are used here to aid in biological interpretation.

APPENDIX B

List of Abbreviations

Abbrev.	Formal Name
ANOVA:	Analysis of Variance
CF:	Condition Factor
CYP1A:	Cytochrome P4501A
DDT:	dichlorodiphenyltrichloroethane
DNA:	Deoxyribonucleic Acid
DO:	Dissolved Oxygen
E2:	17 β -estradiol
EDCs:	Endocrine-Disrupting Chemicals
EPA:	Environmental Protection Agency
GnRH:	Gonadotropin-Releasing Hormone
GRIFs:	Gonadotropin Release-Inhibiting Factors
GSI:	Gonadosomatic Index
HPG:	Hypothalamus-Pituitary-Gonadal
HSI:	Hepatosomatic Index
mRNA:	Messenger Ribonucleic Acid
MS-222:	tricaine methanesulfonate
PAHs:	Polycyclic Aromatic Hydrocarbons
PCP:	pentachlorophenol
S:	Salinity
TIU:	Trypsin Inhibiting Unit
USP:	United States Pharmacopeial Unit
VTG:	Vitellogenin

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Figure 1. Schematic representation of the reproductive endocrine system of a female teleost (bony fish) (modified from Thomas, 1990). Neurotransmitters and/or hormones are secreted from the brain, pituitary, and gonads in what has been termed the hypothalamus-pituitary-gonadal (HPG) axis (Thomas and Khan, 1997). In females, the liver plays a significant role because of its synthesis of vitellogenin (VTG), an egg-yolk-precursor protein. Endocrine-disrupting chemicals (EDCs) can interfere with (1) neurotransmitter release in response to stimuli detected by sense organs, altering the synthesis and secretion of gonadotropin-releasing hormone (GnRH) or gonadotropin release-inhibiting factors (GRIFs) by the hypothalamus. (2) binding of GnRH to specific receptors on the gonadotrops of the pituitary, modulating activation of a phosphoinositol calcium-dependent second messenger pathway. (3) release of two glycoprotein hormones: gonadotropin (GtH) I and II. (4) action of gonadotropins via receptors at the gonads to stimulate the production of steroid hormones. (5) binding of steroid hormones to nuclear receptors in target tissue, altering mRNA and protein synthesis. Normal feedback mechanisms are primarily controlled by steroid hormones [in females, 17β -estradiol (E2)]. “R” = receptor; “SBP” = steroid binding protein; “P” = phosvitin; “L” = lipovitellin; “+” = positive feedback; “-“ = negative feedback.

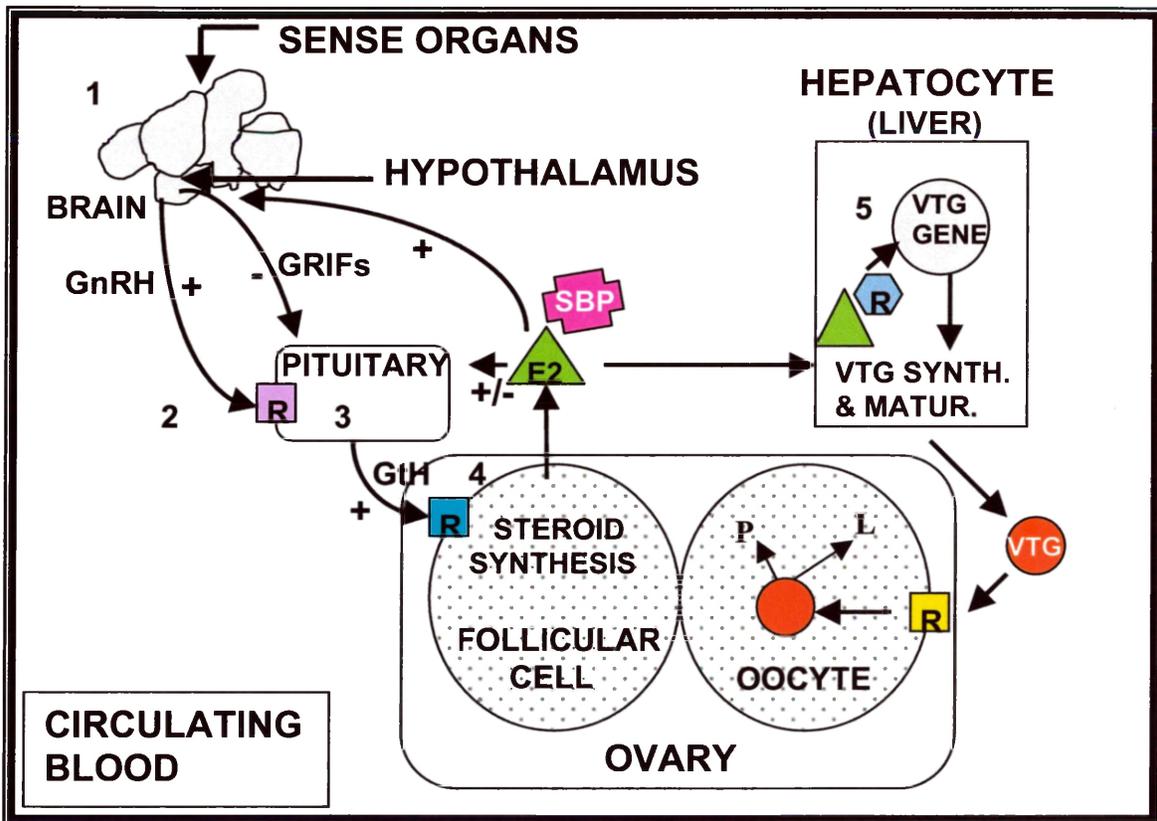


Figure 2. Schematic representation of the vitellogenic cycle of a female teleost (modified from Sherwood and Hew, 1994). Vitellogenesis is the process by which maturing oocytes accumulate yolk. Vitellogenin (VTG) is normally synthesized in the liver and is regulated by 17β -estradiol (E2). (1) Free steroid (e.g. E2) is transported in circulating blood by steroid binding proteins (SBP) and transported to target cells (e.g. hepatocyte). (2) E2 enters the cell and binds to an estrogen receptor (ER). (3) A pair (homodimer) of hormonal-bound receptors binds to specific DNA sequences in the nucleus, activating mRNA synthesis and protein production [e.g. VTG]. (4) Under gonadotropin promotion, VTG is incorporated by receptor (R)-mediated endocytosis into oocytes. (5) VTG is proteolytically cleaved to form the yolk proteins lipovitellin and phosvitin. (6) Male fish possess the gene for VTG synthesis, but expression is an inappropriate response triggered by xenobiotics (X) mimicking E2 and binding to the ER. This binding stimulates VTG synthesis. Because male fish do not possess oocytes, VTG continues to travel in circulating blood.

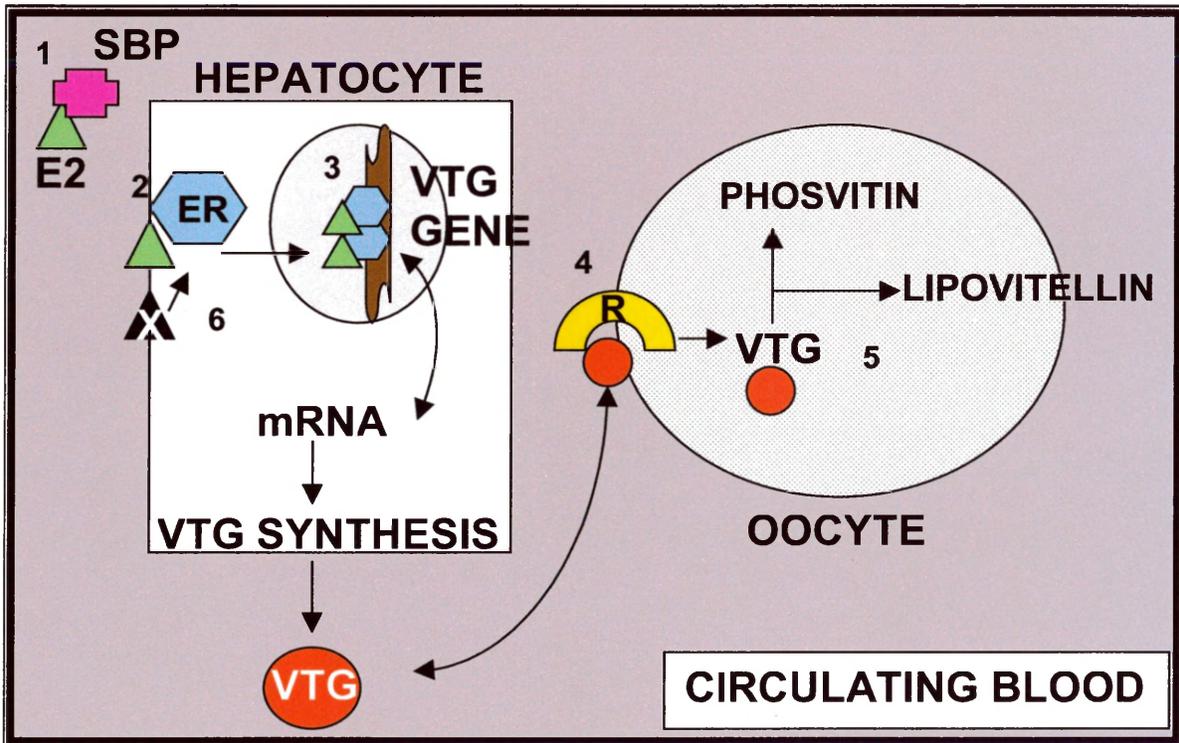


Figure 3. Collection sites for *Fundulus heteroclitus* in lower Chesapeake Bay, USA. Site “1” – King Creek (reference site 1); Site “2” – Copper Creek on Ragged Island (reference site 2); Site “3” – Atlantic Wood (creosote-contaminated site).

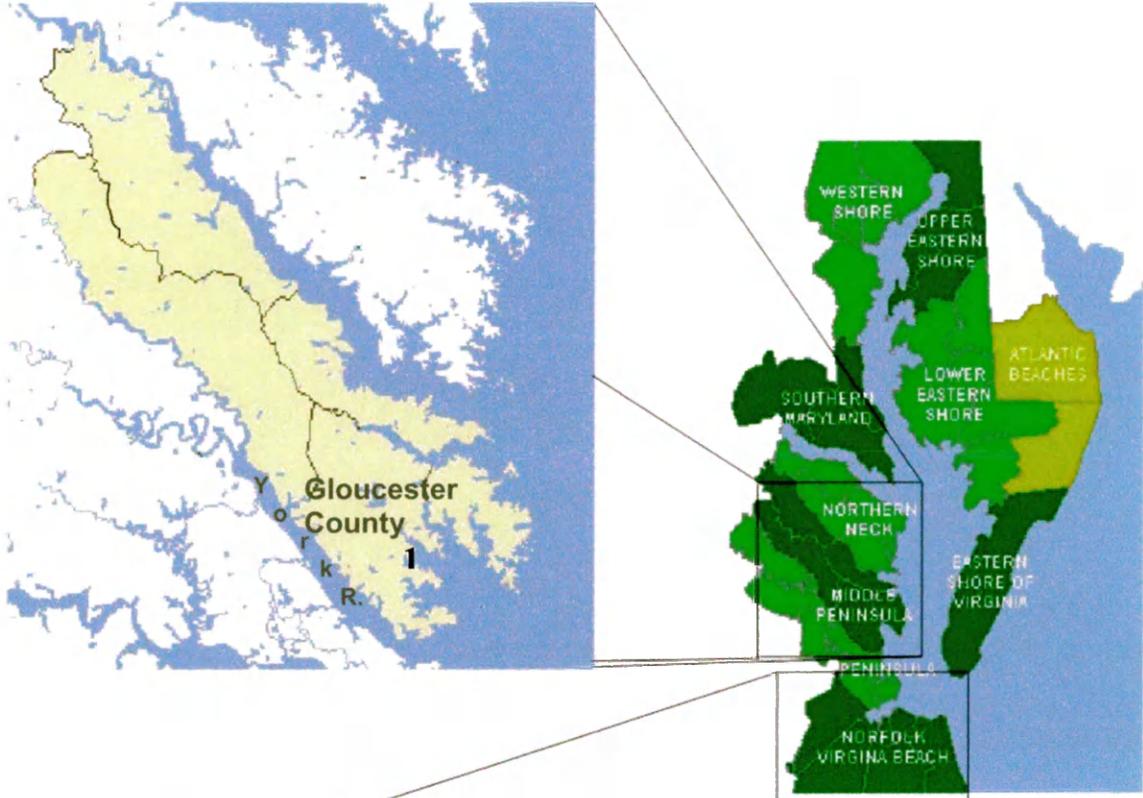


Figure 4. Western blot of vitellogenin (VTG) from *Fundulus heteroclitus* plasma. Monoclonal antibody FV 10-9 (C. Rice, Clemson University) and goat anti-mouse Ig (H+L)-alkaline phosphatase conjugate were used for identification of an immunoreactive band at approximately 218 kilodaltons (kDa). Kaleidoscope prestained standards are in lane 7. The blue band is myosin (218 kDa), followed by β -galactosidase, magenta (131 kDa), bovine serum albumin, green (86 kDa), carbonic anhydrase, violet (43.8 kDa), and soybean trypsin inhibitor, orange (33 kDa). Lanes 1-5 are of purified *F. heteroclitus* VTG. Concentrations are in increasing order (6, 12, 24, 48, and 96 ng VTG). Lanes 6 and 8 are empty. Lanes 9 and 10 are female control plasma (1:50 and 1:100 dil. respectively). Lanes 11 and 12 are estradiol-induced male plasma diluted 1:50 and 1:100. Lane 13 is a representative field female sample at 1:25 dilution. Lanes 14 and 15 are undiluted representative male field samples. All dilutions were with sodium dodecyl sulfate reducing buffer. No banding was identified in lanes containing male plasma. One microliter was loaded for all fish plasma samples.

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

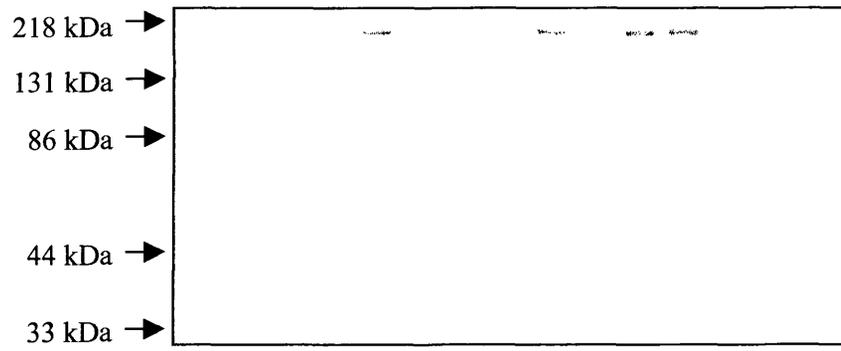


Figure 5. Western blot of vitellogenin (VTG) within plasma from field-collected Atlantic Wood fish. Lanes 1, 3, 5, 12, and 14 are female samples diluted 1:75. Lanes 2, 4, 6, and 13 are male samples undiluted. Standards are in lanes 7-11 and are purified *Fundulus heteroclitus* VTG. Concentrations are in increasing order (15, 30, 50, 70, and 90 ng VTG). Lane 15 is the interassay pool (IAP) sample (estradiol-induced male plasma diluted 1:200). All dilutions were with sodium dodecyl sulfate reducing buffer. No banding was identified in lanes containing male plasma. One microliter was loaded for all fish plasma samples.

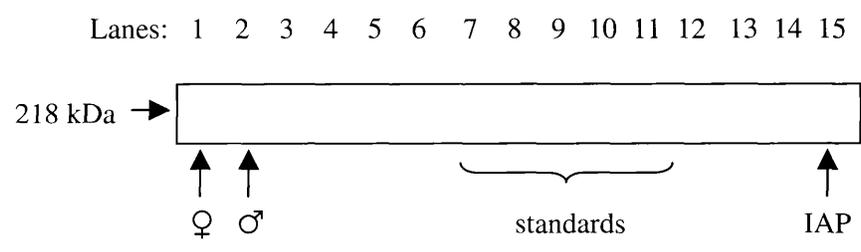


Figure 6. Multiple comparison of condition factor for all field-collected *Fundulus heteroclitus*. King Creek fish (Type “A”, $\bar{u} = 1.24$) differ from Ragged Island fish (Type “B”, $\bar{u} = 1.17$). Atlantic Wood could not be differentiated from either site (Type “AB”, $\bar{u} = 1.20$). (Tukey’s: $df = 159$, $K = 3$, $p = 0.05$). (error bars represent one standard deviation; horizontal lines unite indistinguishable means).

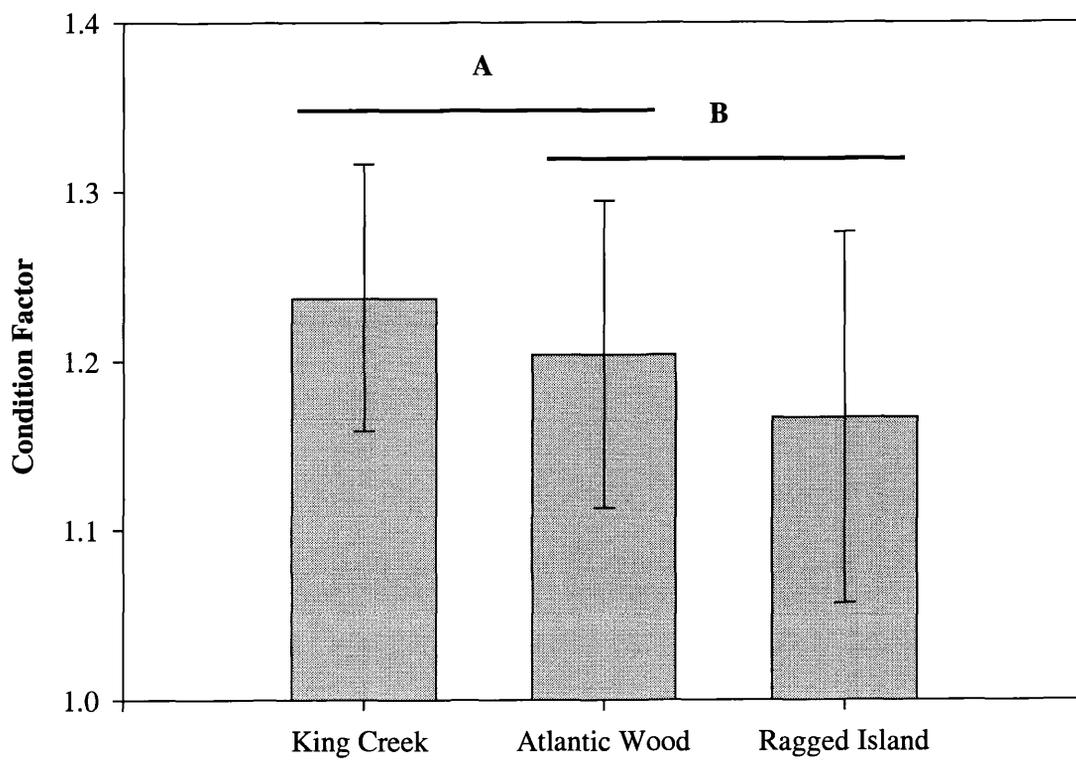


Figure 7. Nonparametric multiple comparison of gonadosomatic index (GSI) for male, field-collected *Fundulus heteroclitus*. Atlantic Wood and King Creek fish (Type “A”, $\bar{u}_{\text{rank}} = 45.4$; $\bar{u}_{\text{rank}} = 44.7$) clearly differ from those of Ragged Island (Type “B”, $\bar{u}_{\text{rank}} = 19.4$). (Nemenyi: $n = 72$, $K = 3$, $p = 0.03$). (horizontal lines unite indistinguishable mean ranks; bars represent medians; dots represent average rank).

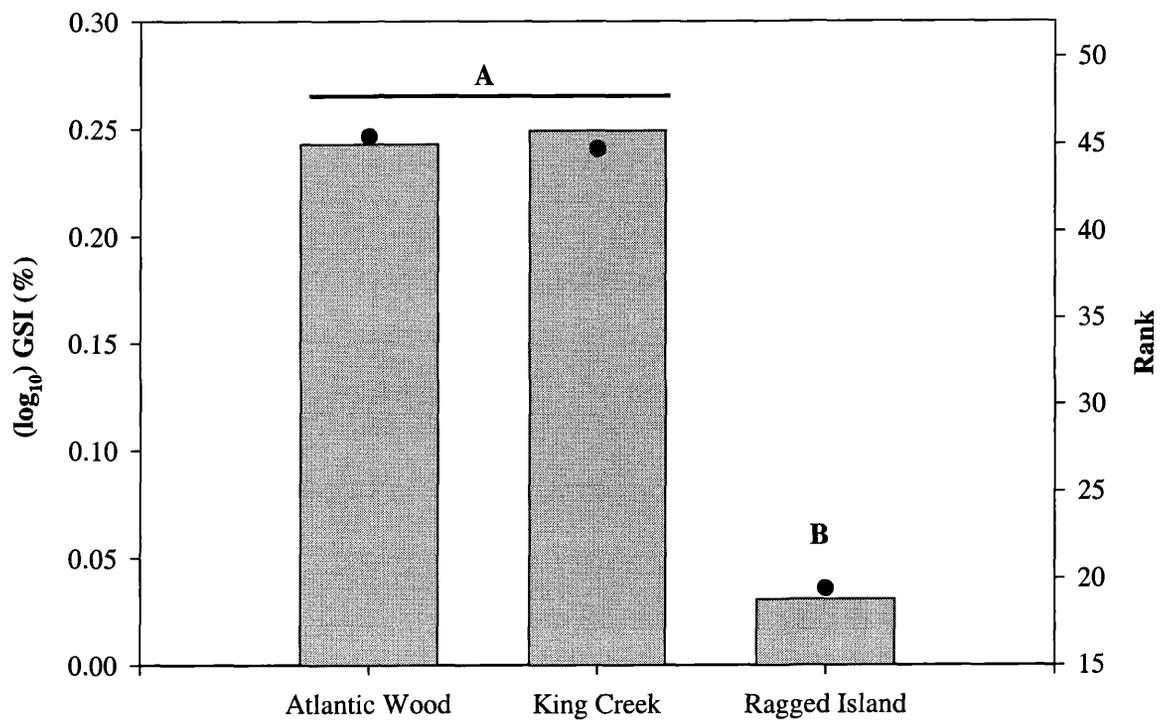


Figure 8. Multiple comparison of hepatosomatic index (HSI) for male, field-collected *Fundulus heteroclitus*. Atlantic Wood fish (Type “A”, $\bar{u} = 0.402$) differ from Ragged Island fish (Type “B”, $\bar{u} = 0.23$). King Creek fish could not be differentiated from those of either site (Type “AB”, $\bar{u} = 0.322$). (Tukey’s: $df = 69$, $K = 3$, $p = 0.05$). (error bars represent one standard deviation; horizontal lines unite indistinguishable means).

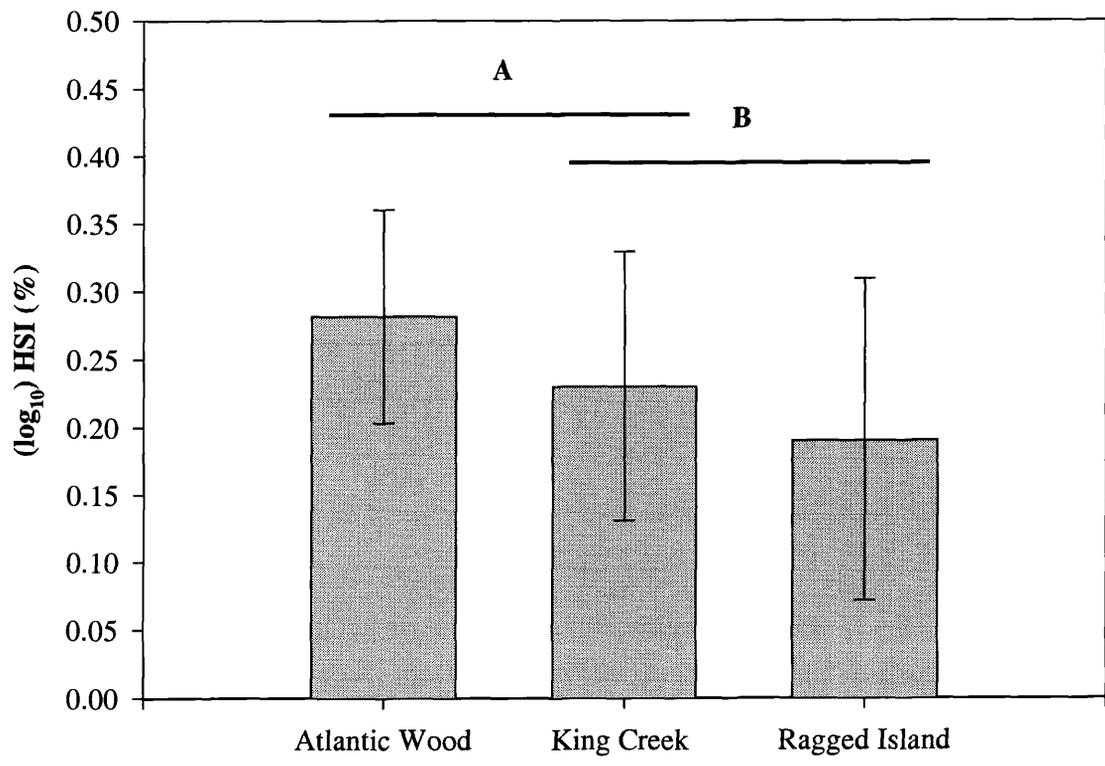


Figure 9. Nonparametric multiple comparison of cytochrome P4501A (CYP1A) from all field-collected *Fundulus heteroclitus*. The Atlantic Wood and Ragged Island populations, both Type “A”, ($\bar{u}_{\text{rank}} = 37.6$, $\bar{u}_{\text{rank}} = 30.3$) differ from the King Creek population, Type “B” ($\bar{u}_{\text{rank}} = 14.7$). (Nemenyi: $n = 172$, $K = 3$, $p = 0.03$). (horizontal lines unite indistinguishable mean ranks; bars represent medians; dots represent average rank).

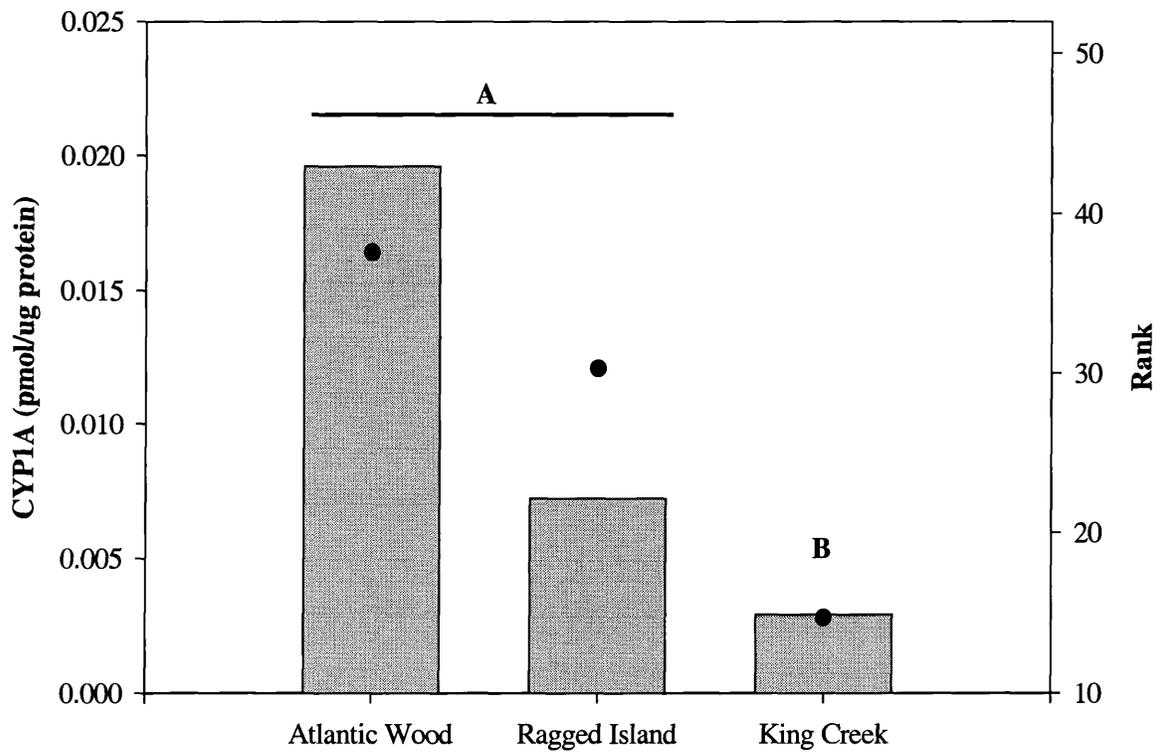


Figure 10. Nonparametric multiple comparison of cytochrome P4501A (CYP1A) from male, field-collected *Fundulus heteroclitus*. Atlantic Wood fish (Type “B”, $\bar{u}_{\text{rank}} = 20.5$) greatly differ from both those of Ragged Island and King Creek (Type “A”, $\bar{u}_{\text{rank}} = 10.5$, $\bar{u}_{\text{rank}} = 6.5$). (Nemenyi: $n = 72$, $K = 3$, $p = 0.05$). (horizontal lines unite indistinguishable mean ranks; bars represent medians; dots represent average rank).

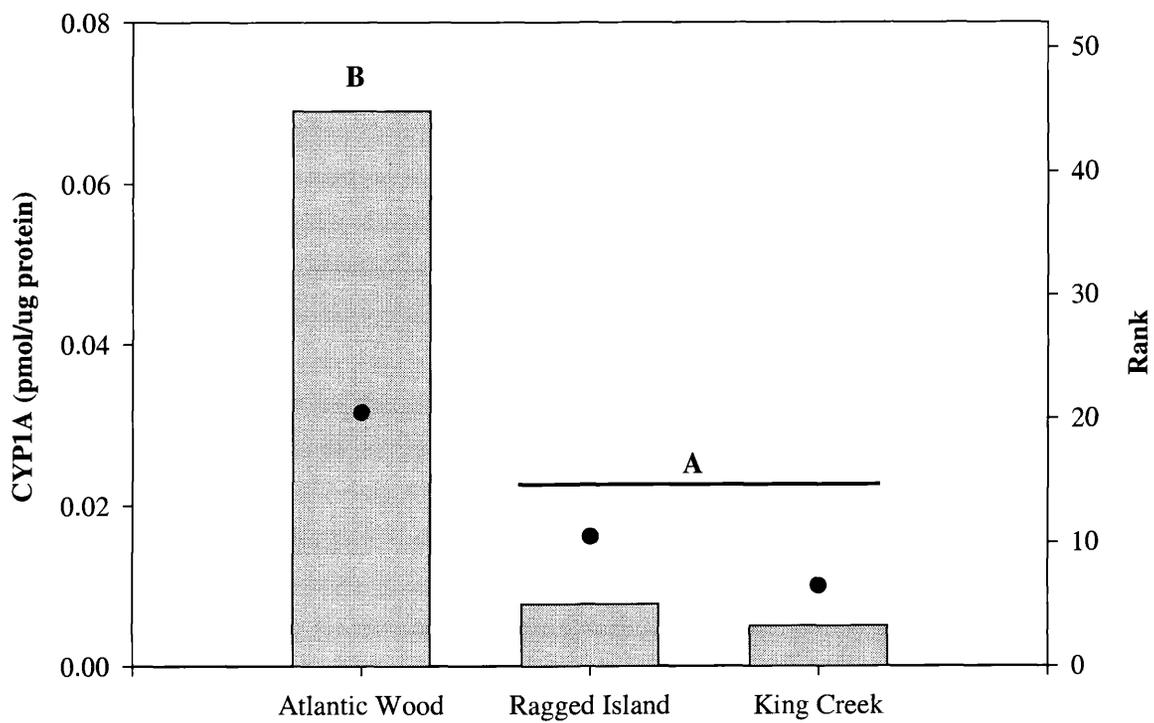


Figure 11. Scatterplot of hepatosomatic index (HSI) versus cytochrome P4501A (CYP1A) for all field-collected *Fundulus heteroclitus*. Correlation analysis indicates a minor positive trend between male CYP1A concentrations and (\log_{10}) HSI (Pearson's product moment correlation: $r = 0.411$, $p = 0.046$). Analysis of all samples, as well as for females only, determined no significant trend between liver size and CYP1A level ($r = -0.025$, $p = 0.858$; $r = -0.225$, $p = 0.232$).

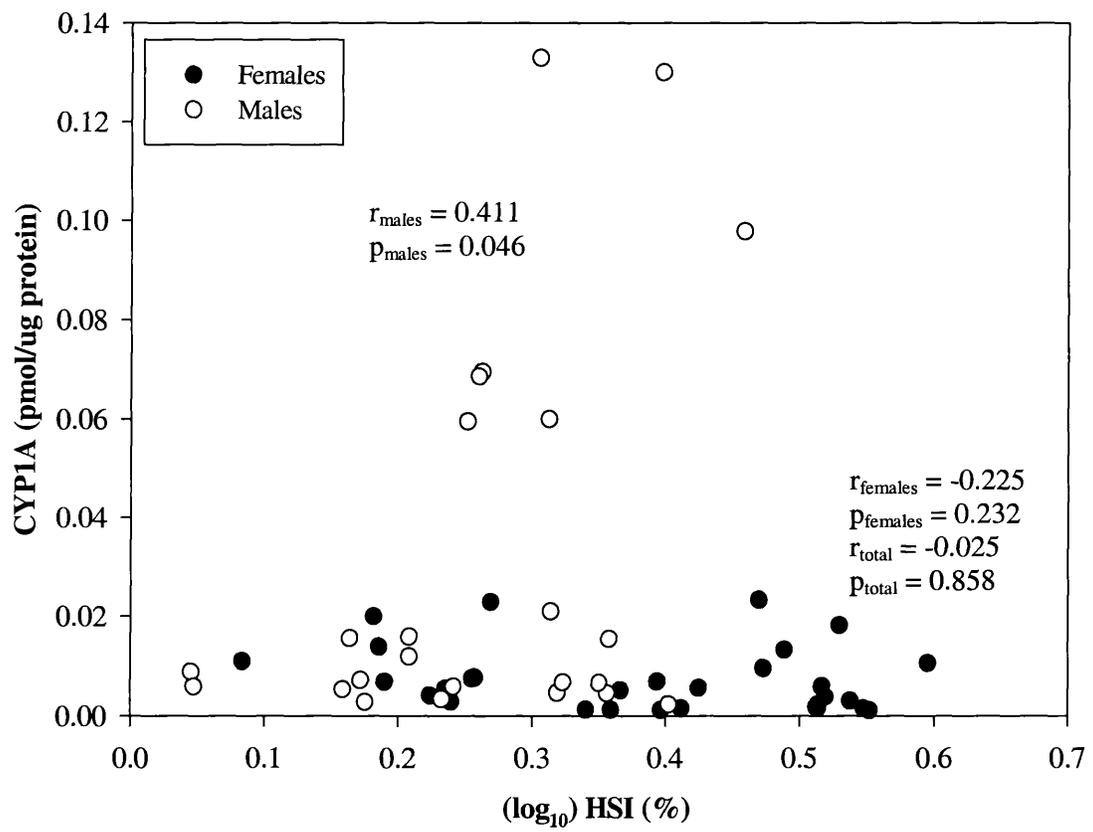


Figure 12. Multiple comparison of hepatosomatic index (HSI) for *Fundulus heteroclitus* from day seven of the sediment exposure. King Creek fish exposed to 33 percent Atlantic Wood (33%-KC, Type “A”, $\bar{u} = 2.62$) clearly differ from King Creek fish and Atlantic Wood fish in control sediment (0%-KC and 0%-AW, Type “B”, $\bar{u} = 1.88$, $\bar{u} = 1.84$). Atlantic Wood fish in 33 percent Atlantic Wood sediment (33%-AW) could not be differentiated from those of other exposure treatments (Type “AB”, $\bar{u} = 2.31$). (Tukey’s: $df = 60$, $K = 4$, $p = 0.05$). (error bars represent one standard deviation; horizontal lines unite indistinguishable means).

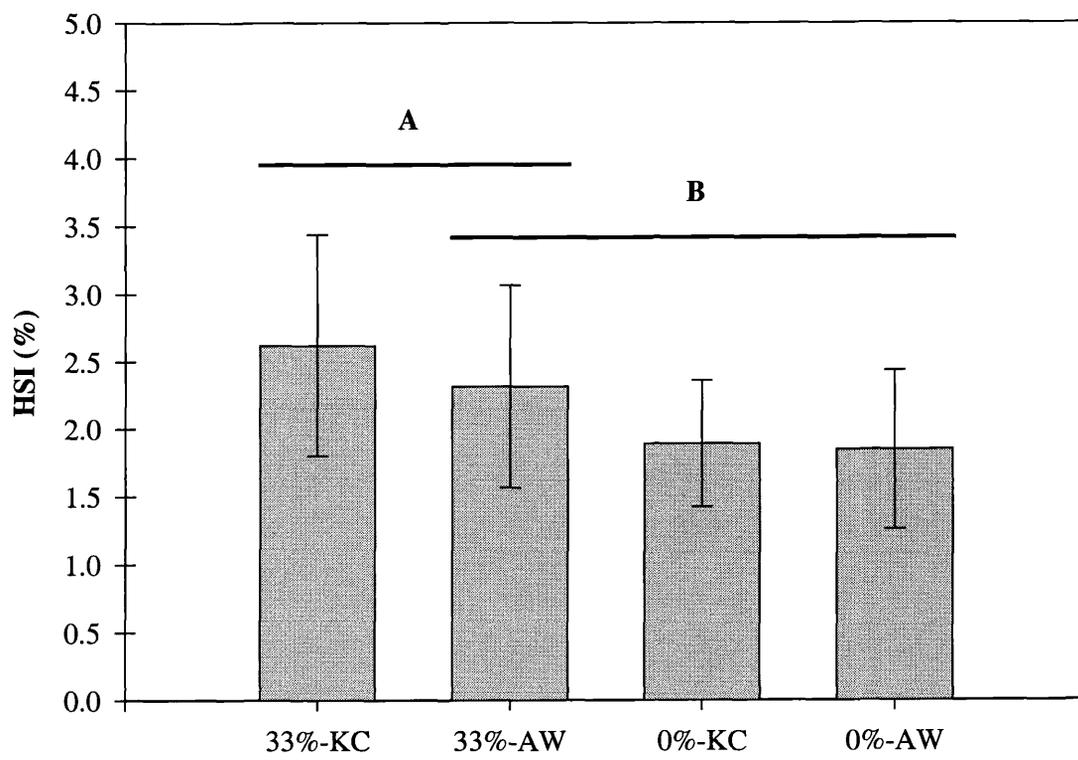
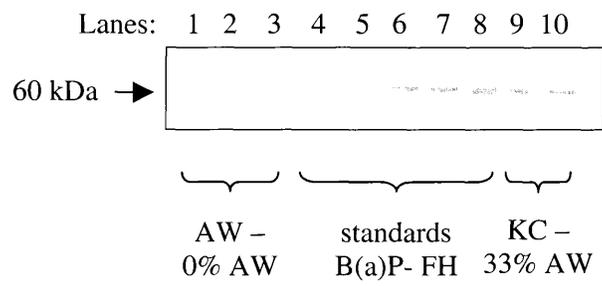


Figure 13. Western blot of cytochrome P4501A (CYP1A) within hepatic microsomes isolated from *Fundulus heteroclitus* from day seven of the sediment exposure. Monoclonal antibody “MAb 1-12-3” (J. Stegeman, Woods Hole Oceanographic Institution) and goat anti-mouse Ig (H+L)-alkaline phosphatase conjugate were used as primary and secondary antibodies respectively. Lanes 1, 2, and 3 contain undiluted (12 µg protein blotted) microsomal samples from Atlantic Wood (AW) fish in control sediment (0% AW). Lanes 4-8 are of benzo(a)pyrene-induced *F. heteroclitus* [B(a)P-FH] microsomes. Standard concentrations are in increasing order (0.1, 0.2, 0.4, 0.6, and 0.8 pmol CYP1A). Lanes 9 and 10 are representative King Creek (KC) fish exposed to 33 percent Atlantic Wood (33% AW) sediment (1:4 dilution, sodium dodecyl sulfate reducing buffer, 1.5 µg protein blotted; undiluted, 1.0 µg protein blotted). Exposed King Creek fish displayed marked increase in color intensities when compared to that of Atlantic Wood fish. All fish were male.



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