

FINAL REPORT
U.S. Department of Energy



Project Title:

**Environmental Analysis of Endocrine Disrupting Effects
from Hydrocarbon Contaminants in the Ecosystem**

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II. Table of Contents

I.	Cover Sheet	1
II.	Table of Contents	2
III.	Executive Summary	3
IV.	Research Objectives	6
	A. Problem Addressed	6
	B. Project Related to Ongoing Research	7
V.	Methods and Results	8
	A. Biotechnology Screening System (<i>in vitro</i> studies)	8
	B. Animal Screening System (<i>in vivo</i> studies)	9
	C. Kinetic and Thermodynamic (<i>in vitro</i>) Studies	12
VI.	Relevance, Impact and Technology Transfer	14
VII.	Project Productivity	15
VIII.	Personnel Supported	15
IX.	Publications	16
X.	Interactions	17
XI.	Transitions	18
XII.	Patents	19
XIII.	Future Work	19
XIV.	Literature Cited	20
XV.	Feedback	21
XVI.	Appendix: Unpublished Manuscripts	

FINAL REPORT

III. EXECUTIVE SUMMARY

The objective of this project, **Environmental Analysis of Endocrine Disrupting Effects from Hydrocarbon Contaminants in the Ecosystem** awarded to the **Center for Bioenvironmental Research at Tulane and Xavier Universities (CBR)**, was to determine how environmental contaminants, namely hydrocarbons, act as hormones or anti-hormones in different species present in aquatic ecosystems. The three major components of the research included:

- a biotechnology based screening system to identify potential hormone mimics and antagonists;
- an animal screening system to identify biomarkers of endocrine effects; and
- a literature review to identify compounds at various DOE sites that are potential endocrine disruptors.

Species of particular interest in this study were those that can serve as sentinel species (e.g., amphibians) and thus provide early warning signals for more widespread impacts on an ecosystem and its wildlife and human inhabitants. The focus of the literature research was to provide an analysis of the contaminants located on or around various Department of Energy (DOE) sites that are or have the potential to function as endocrine disruptors, and to correlate the need for studying endocrine disruptors with programmatic needs of DOE.

The activities employed by this project to determine these impacts included development of biotechnology screens (*in vitro*), animal screens (*in vivo*), other analyses of aquatic ecosystem biomarkers of exposure, and real-time data collection and dissemination through innovative environmental informatics. Results from this study elucidated how chemicals in the

environment, including those from DOE activities, can signal (and alter) the development of a number of species in aquatic ecosystems. These signals can have detrimental impacts not only on an organismal level, but also on community, population, and entire ecosystem levels, including humans. Results obtained from this research project have provided information on endocrine disrupting contaminants for consideration in DOE's risk analyses for determining clean-up levels and priorities at contaminated DOE sites.

The CBR represents the only research on the impacts of environmental endocrine disrupting chemicals for the Environmental Management Science Program. The CBR has more than five years of experience in developing model aquatic ecosystems for evaluating environmental problems relevant to DOE cleanup activities. Using biotechnology screens and biomarkers of exposure, CBR research has demonstrated that chemicals in the environment can signal the development of species in aquatic ecosystems, as well as show detrimental impacts on community, population, and the ecosystem, including human health.

Since environmental endocrine disruptors are effective at concentrations similar to hormones, and can cause havoc at levels a thousand or more times below measurable levels of most current analytical methods, it is imperative to develop methods that are effective at concentrations in the environment. Therefore, research into low levels of environmental contaminants may show that currently accepted cleanup standards are inappropriate for DOE cleanup efforts.

Feedback to the DOE has resulted in new funding for the CBR from the Departments of Defense and Energy and the Office of Naval Research for further research and study of endocrine disruptors and environmental hormones related to biohazards research and management. The CBR is currently working with the Office of Environmental Management in an integrated

research program in support of the long-term stewardship of the DOE complex. Over the past six years, in partnership with the DOE, the CBR has developed a unique natural laboratory for development of new biosensors and biomarker technologies and a program for risk evaluation and communication. Through a three- year DOE cooperative agreement, initiated in September 2000, the CBR will leverage this capacity into development of an integrated research-based program for monitoring technology development, information management, and risk analyses and communication, effectively positioning the DOE to implement its long-term stewardship strategies in the near future.

The Tulane/Xavier partnership is a well-established, effective joint venture between majority and minority universities. It is an extremely complementary relationship with respect to environmental restoration and waste management, with the Xavier University focus on education and undergraduate work, and Tulane University emphasis and experience in graduate education, research, and technology development and transfer. Tulane and Xavier investigators had students, often undergraduates, in their laboratories assisting with this project. In this way the CBR assists research faculty to build and train sufficient qualified personnel to complete research and also create capacity in undergraduate students.

IV. RESEARCH OBJECTIVES

A. Problem Addressed

The objective of this project was to determine how environmental contaminants, namely hydrocarbons, act as hormones or anti-hormones in different species present in aquatic ecosystems. The three major components of the research included:

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The activities employed by this project to determine these impacts included development of biotechnology screens (*in vitro*), animal screens (*in vivo*), other analyses of aquatic ecosystem biomarkers of exposure, and real-time data collection and dissemination through innovative environmental informatics. Results from this study elucidated how chemicals in the environment, including those from DOE activities, can signal (and alter) the development of a number of species in aquatic ecosystems. These signals can have detrimental impacts not only on an organismal level, but also on community, population, and entire ecosystem levels, including humans. Results obtained from this research project have provided information on endocrine disrupting contaminants for consideration in DOE's risk analyses for determining clean-up levels and priorities at contaminated DOE sites.

Previous research within the Center for Bioenvironmental Research at Tulane and Xavier Universities has focused on understanding the effects of environmental agents on human and wildlife health and disease. In particular, this research has focused on how exogenous agents function to mimic or disrupt normal endocrine signaling (i.e. estrogen, thyroid) within various systems covering a wide spectrum from whole animal studies with fish, amphibians and insects to human cancer cell lines. Significant work has focused on the estrogenic and anti-estrogenic action of both synthetic organochlorine chemicals and naturally produced phytochemicals. Recent projects have extended these research activities to include examination of these environmental agents on the symbiotic relationship between nitrogen-fixing rhizobial bacteria and leguminous plants.

In extending this research through the DOE-Environmental Management Science Program (EMSP), the CBR has examined the natural role of flavonoids in the regulation of *Rhizobium*-plant symbiosis and the disruption of this symbiosis by environmental factors as well as the effects that regulation of this symbiosis has on agricultural crop yields and field fertilization requirements. The use of both β -galactosidase and green fluorescence protein reporter gene assays has allowed us to monitor directly not only the effect of these chemicals on gene

expression, but also the actual biological formation of the symbiotic relationship. The examination of this interaction using different species of both *Rhizobium* and host plants allowed the identification of the specificity of individual flavonoids in regulation of symbiosis.

Secondly, the observation that the biosynthesis of flavonoids by plants can be altered by environmental factors was examined using the *Rhizobium* system. The CBR examined how these novel phytochemicals enhance or disrupt symbiosis and gene expression. As a future project, a research study could investigate the action of organochlorine chemicals and other environmental pollutants on this interaction. Overall, these studies have focused on understanding the complex interplay of plant signals and environmental contaminants at the bacterial and plant levels. Information from this research will generate a better understanding of the signals that regulate specificity of symbiosis between specific plant and *Rhizobium* species and how this may be manipulated. In addition to bringing a greater understanding of the general mechanism of symbiosis, this information has implications in crop yield control and offers methods of natural or endogenous crop fertilization. The understanding of the effect of environmental chemicals such as organochlorine on symbiosis will provide insight into possible detrimental effects that the use of specific chemicals have on agricultural crops.

B. Project Related to Ongoing Research

Many synthetic chemicals in the environment possess estrogenic activity. Synthetic chemicals with estrogenic activity include bisphenol-A, a polycarbonate by-product, biodegradation products of alkylphenol ethoxylate surfactants such as octylphenol, and the insecticides DDT and its metabolites and endosulfan. The activity of these chemicals has been reportedly mediated by their interaction with the intracellular estrogen receptor (ER) indicating that they may mimic the actions of estradiol at the molecular level. Such environmental chemicals are classified as “endocrine-disrupters” based on reports of feminization of male fish in some rivers in the United Kingdom, altered sex ratios of seagulls and turtles and decreased phallus size of alligators living in Lake Apopka, Florida.

Phytochemicals are another class of chemicals with reported estrogenic activity. The flavonoids represent a family of plant-produced chemicals that function to deter herbivores, protect the plant from fungal and bacterial pathogens, and initiate the symbiosis with *Rhizobium* bacteria. Recent research in CBR laboratories and others has demonstrated that many flavonoid phytochemicals can mimic or disrupt aspects of the human endocrine system as well. Specifically these studies have shown that certain flavonoid phytochemicals can act as the endogenous human estrogen, 17- β -estradiol. Additionally, we have shown that some flavonoids can act as anti-estrogens blocking the activity of 17- β -estradiol similar to tamoxifen which is the drug used clinically to treat breast cancer.

Recently a number of epidemiological studies have demonstrated that populations with diets high in soy have a decreased risk of breast and prostate cancer. Studies have also shown that flavonoid chemicals can prevent cancer or carcinogenesis in animal models and may also function to prevent osteoporosis and cardiovascular disease. The suggestion that the high flavonoid content of soy may function to prevent cancer and disease is bolstered by the observation that the predominant flavonoid chemicals found in soy, namely genistein and daidzein, can affect estrogen signaling and prevent cancer in animal models.

Recent research in collaboration with the U.S. Department of Agriculture has suggested that phytochemicals other than genistein and daidzein might also be considered as biologically active flavonoids. The levels and types of flavonoids found in plants are dependent upon the growth conditions and environmental factors to which the plant has been exposed. Specifically, stress conditions such as bacterial or fungal infection, heat or cold shock as well as exposure to certain environmental pollutants or organochlorines can alter biosynthesis of flavonoids in plants.

We must also consider that the hormone-like effects of these chemicals may extend to other biological systems such as the *Rhizobium*-plant symbiosis as well. *Rhizobium* represents a genus of nitrogen fixing soil bacteria that participate in a symbiotic relationship with host plants, particularly legumes. The symbiosis between the two species relies on bacterial colonization of host plant root hairs and formation of root-nodules that provide a controlled environment for the bacteria, of which the metabolic by-products provide a nitrogen source for the host plant. Symbiosis occurs as a result of a complex signaling between host plant and bacteria, and then the core is directed by plant recruitment of bacteria to root nodules through the plant release of small molecule polyphenolic compounds known as flavonoids. Flavonoids function to activate expression of specific gene cassettes within bacteria known as *nod* (nodulation) genes. The activation of the *nod* genes results in a bacteria differentiation that promotes infection of plant root hairs. The root hair infection then initiates through both plant and bacteria the formation of nodules. The interaction between certain species of host plants and species of *Rhizobium* is determined by both the specific flavonoid signals generated from the plant, the infection of the root hair, and subsequent nodule formation in the plant.

In contrast to the beneficial health effects of soy foods and flavonoid phytochemicals, therefore, a number of organochlorine pesticides and herbicides have been shown to possess estrogenic effects. Due to their bioaccumulation, bio-magnification and persistence in the environment, these chemicals are thought to pose potential hazard to wildlife and human health and fertility. They may also be associated with an increased risk of certain cancers. The role of environmental estrogens in human and animal health must therefore be placed in the context of species differences of endogenous hormonal systems and the relative exposure to all hormonally active compounds. The innovative aspect of this CBR research is its study of the impacts of environmental endocrine disrupting chemicals in model aquatic ecosystems. Collaborative CBR research studies for the US Departments of Agriculture and Defense have investigated related environmental signals and their modulation of endogenous cell signaling pathways and steroid hormone systems.

V. METHODS AND RESULTS

A. Biotechnology Screening System (*in vitro* studies)

The objective of this research was to determine the ability of PAHs identified in Bayou Trepagnier (Louisiana), an area that has been heavily contaminated since the 1920s, to function as hormone mimics or antagonists at environmentally relevant concentrations. Three biotechnology screens were *developed* and tested: 1) the Yeast Expression System (YES); 2) an *in vitro* screen for thyroid hormone disruption; and 3) an *in vitro* screen for the Arthropod ecdysone receptor. These screens allow us to test the ability of PAHs and other environmental hormones or hormone receptors of humans, amphibians, and invertebrates (e.g., shrimp). Yeast

genetically engineered to produce human estrogen, progesterone, glucocorticoid, or thyroid hormone receptors was exposed to polyaromatic hydrocarbons known to occur in Bayou Trepagnier. The ability of the chemicals to interact with hormone receptors was determined by colorimetric assay. Development of all these screens is completed and all have demonstrated success in identifying numerous compounds, including PAHs, which interact with these important receptors.

B. Animal Screening System (*in vivo* studies)

Animal screen studies have been funded through this grant and include the effects of environmental hormones on: 1) the African clawed frog (*Xenopus laevis*), 2) the leopard frog (*Rana pipiens*), and 3) grass shrimp (*Palaeomonetes* spp.). Frogs were chosen as a species of concern since unusual frequencies of frogs with morphological abnormalities have been observed recently in the western parts of North America; environmental hormones are capable of disrupting reproductive physiology in amphibians; amphibians are often the most susceptible species for adverse environmental impacts and, thus, can serve as sentinel species for ecosystem inhabitants; and many are threatened and endangered. Grass shrimp was chosen as a species of concern due to its economic and ecological value as well as the many industrial impacts on its aquatic habitats.

Since frog metamorphosis is primarily controlled by thyroid hormones, we used metamorphosis as a model for environmental hormone interaction with the thyroid axis. We exposed premetamorphic tadpoles of the African clawed and leopard frogs to a variety of environmental hormones and measured the growth and rate of metamorphosis. For both frog species, metamorphosis was affected (either delaying or accelerating metamorphosis) by these environmental chemicals. For the African clawed frog, in particular, biomarker analyses suggest that the endocrine neuroimmune systems could be adversely impacted by various hydrocarbons and organometals.

The impact of PAH contamination on grass shrimp was investigated in a contaminated bayou and in the laboratory. Grass shrimp caught in the bayou showed a contaminant-gradient increase in heat shock protein 63 and cytochrome P450 1A (as measured by ECOD metabolism). Grass shrimp exposed to contaminated sediment in the laboratory showed a trend for elevated ECOD metabolism; however individual variation was too large for statistical significance. Heat shock proteins were also not significantly elevated in laboratory-exposed shrimp. This lack of significant increase in these two biomarkers could be due to the short exposure regime.

Experimental methods and principal results of the research in this component follow.

Biomarker Analysis

One study was carried out to determine whether PAHs and PCBs can interact with the Arthropod steroid hormone system. Ecdysteroid molting hormones control growth, molting, and reproduction in Arthropods. A spike in 20-OH ecdysone (20 HE) triggers the molt cycle in crustaceans, and earlier studies have shown that PAHs can affect this molt cycle in several crab species. However, the mechanism of this molt cycle interaction is unknown. Both PAHs and PCBs interact with other nuclear receptors; however, nothing is known about their ability to interact with the invertebrate Ecdysone Receptor (EcR). Four PAHs, benzo[a]pyrene,

benzo[b]fluoranthene, pyrene and chrysene, and the commercial PCB mixture, Aroclor 1254, were used to determine the ability of these classes of compounds to induce ecdysone-dependent reporter gene expression, and to modify the proliferation and differentiation response of the ecdysteroid-responsive Cl. 8+ cell line. The four PAHs were each able to enhance the ecdysteroid response in both the reporter gene and the cell proliferation assays only when given in conjunction with ecdysteroids. Aroclor 1254 had no effect in either system, either alone or in conjunction with ecdysteroids. These studies show that although the PAHs alone do not activate ecdysteroid-dependent gene expression or cell differentiation, they are able to enhance the effect of ecdysteroids, presumably through a non-receptor mediated process. This mechanism may explain the effects on molting that have been reported after low-level crude oil exposures in crustaceans.

In a second study, the impact of polycyclic aromatic hydrocarbon (PAH) and lead contamination on benthic community structure and grass shrimp (*Palaemonetes* spp.) biochemical markers was investigated in Bayou Trepagnier. The benthic community had decreased species richness as well as decreased numbers of individuals along a contamination gradient. Grass shrimp collected in the field showed a contaminant-gradient increase in heat shock protein 63 and cytochrome P450 1A (as measured by ECOD metabolism). Grass shrimp had elevated ECOD metabolism when exposed in the laboratory to sediments from the most contaminated site. However, individual variation was too large for statistically significant changes. In addition, heat shock protein levels were not significantly elevated in laboratory-exposed shrimp. Benthic community structure and wild-caught grass shrimp are clearly impacted in this bayou.

Developmental exposure

This study was undertaken to determine if anthracene and estradiol alter sex differentiation and reproductive success in medaka (*Oryzias latipes*). Polyaromatic hydrocarbons (PAHs) impair fish reproduction and can antagonize estradiol action *in vitro*. To test the hypothesis that PAH exposure could alter reproductive success by disrupting estrogen action, medaka (*Oryzias latipes*) were exposed for 2 or 8 weeks post-hatch to 0, 12, and 20 ug/L anthracene & 0.27 ug/L estradiol-17β (E2). At the end of the exposure, fish were transferred to clean water, grown to sexual maturity (14 weeks), and placed in mating pairs. Eggs were collected, counted, and scored for percentage fertilization (mating success). After seven days of mating, adults were sacrificed and blood samples were taken for analysis of vitellogenin concentration. Eight-week exposure to E2 resulted in 100% females, while the two-week exposure resulted in more than 50% females. Exposure to 20 ug/L anthracene for eight weeks reduced mating success by half, but co-exposure to E2 prevented the effect. Exposure to anthracene for two weeks did not alter mating success, but did reduce the proportion of E2-induced sex reversal in co-exposed fish. Anthracene did not prevent the E2 effect in fish co-exposed for eight weeks. In conclusion, anthracene disrupts estrogen action, since anthracene-induced effects on mating success are prevented by E2, while E2-induced effects on sex ratio are prevented by anthracene.

A second project studied potential mechanisms of thyroid disruption in the interaction of organochlorine compounds with human thyroid receptor β1, thyroid hormone binding globulin, and transthyretin. Organochlorine compounds, particularly PCBs, are known to alter serum thyroid hormone levels in humans. Hydroxylated organochlorines have relatively high affinities

for the serum transport protein transthyretin, but the ability of these compounds to interact with human thyroid receptor is unknown. Using a baculovirus expression system in insect cells (Sf9 cells), recombinant human thyroid receptor β (hTR β) was produced. In competitive binding experiments, the recombinant receptor had the expected relative affinity for thyroid hormones and their analogs. In competitive inhibition experiments with PCBs, hydroxylated PCBs (OH-PCBs), DDT and its metabolites, and several organochlorine herbicides, only the OH-PCBs competed for binding. The affinity of hTR β for OH-PCBs was 10,000-fold lower ($K_i = 20\text{-}50\ \mu\text{M}$) than its affinity for thyroid hormone (T_3 , $K_i = 10\ \text{nM}$). Because their relative affinity for the receptor was low, the ability of OH-PCBs to interact with the serum transport proteins, transthyretin and thyroid binding globulin was tested. With the exception of one compound, the OH-PCBs had the same affinity ($K_i = 10\text{-}80\ \text{nM}$) for transthyretin as thyroid hormone (T_4). Only two of the OH-PCBs bound thyroid binding globulin ($K_i = 3\text{-}7\ \mu\text{M}$), but with a 100-fold lower affinity than thyroid hormone (T_4). Hydroxylated PCBs have relatively low affinities for human thyroid receptor *in vitro*, but they have a thyroid hormone-like affinity for the serum transport protein transthyretin. Based on these results, OH-PCBs *in vivo* are more likely to compete for binding to serum transport proteins than for binding to the thyroid receptor.

Frog Metamorphosis

One project studied the alteration of leopard frog (*Rana pipiens*) metamorphosis by the herbicide acetochlor. Based on the geographic correlation between the use of the pre-emergent herbicide acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide] and the natural range of Northern leopard frogs (*Rana pipiens*), we investigated the effects of acetochlor (ACETO) on frog metamorphosis. The interaction of ACETO with thyroid hormone (T_3) and corticosterone (CORT), hormones that regulate natural metamorphosis was examined. ACETO, T_3 , and CORT were administered via immersion. Growth, developmental stage, and onset of metamorphic climax (forelimb emergence, FLE) were measured. Three hypotheses were examined: 1) ACETO may alter metamorphosis. Premetamorphic tadpoles with low endogenous T_3 were exposed to ACETO $\pm 10^{-9}\ \text{M}$ T_3 for 7 days. 67% of tadpoles exposed to ACETO + T_3 attained FLE, while 0% of T_3 treated animals did. A second hypothesis was considered: 2) ACETO mimics T_3 action at the thyroid receptor (TR). Tadpoles were pre-treated with T_3 for 3 days to induce TR expression, then treated for 7 days with vehicle (DMSO), T_3 , or ACETO $\pm T_3$. ACETO treatment after T_3 priming did not accelerate FLE, suggesting that ACETO does not interact directly with the TR. Co-treatment with ACETO + T_3 after T_3 priming accelerated FLE relative to tadpoles primed with T_3 , then treated with T_3 .

Because the ACETO + T_3 acceleration of FLE appeared similar to the effect of CORT, we examined a third hypothesis: 3) ACETO may interact with CORT to accelerate FLE. Premetamorphic tadpoles were exposed to various doses of ACETO $\pm T_3$ in the presence or absence of $10^{-7}\ \text{M}$ CORT. CORT inhibited growth and hindlimb development and delayed FLE. ACETO never inhibited growth or hindlimb development, but ACETO did counteract the effects of CORT when T_3 was present. ACETO consistently accelerated T_3 -induced metamorphosis, apparently interacting with T_3 via a non-TR mediated mechanism.

Environmental estrogens are capable of disrupting reproductive physiology in amphibians. Recently, the endogenous estrogen, estradiol-17 β (E2), has been shown to delay frog metamorphosis. A study investigated the morphological and biochemical effects of

environmental estrogens on frog metamorphosis. Since frog metamorphosis is primarily controlled by thyroid hormones, we used metamorphosis as a model for environmental estrogen interaction with the thyroid axis. Premetamorphic tadpoles of the African clawed frog, *Xenopus laevis*, were exposed to steroidal and environmental estrogens and measured growth, rate of metamorphosis, thyroid hormone receptor (TR) expression, and vitellogenin expression. Thyroid hormone auto-induces TR expression, so alterations in TR concentration indicate interaction with the thyroid axis. Estradiol and estrogen mimicking chemicals induce vitellogenin (egg yolk protein) expression, so Vg expression indicates interaction with the estrogen receptor. It was determined that steroidal estrogens consistently delayed spontaneous and thyroid hormone induced metamorphosis when given during early premetamorphosis.

E2 treatment did not alter TR expression during spontaneous or thyroid hormone induced metamorphosis, but did induce Vg synthesis. *o,p'*DDD, a metabolite of DDT, appeared to inhibit thyroid-hormone induced metamorphosis at high (1 μ M) concentrations, but this inhibition was due to toxicity since tadpoles died after ten days of exposure. *o,p'*DDD did not alter the rate of spontaneous metamorphosis, but did cause tadpoles to produce Vg. Higher concentrations of *o,p'*DDD elicited a greater Vg response. 3,3',4,4'-tetrachlorobiphenyl (PCB 77) did not change the rate of metamorphosis, nor did it alter TR or Vg expression. This work is the first to show that estradiol not only delays metamorphosis, but induces Vg synthesis in tadpoles. Because TR expression does not change with E2 exposure, E2 probably does not delay metamorphosis by interacting directly with thyroid hormone signaling, but by some other mechanism. At sublethal doses, *o,p'*DDD mimics the Vg-inducing effects of E2, but does not mimic the inhibitory effect of E2 on metamorphosis. Surprisingly, PCB 77, a compound known to interact with thyroid signaling in mammals, has absolutely no effect on tadpole metamorphosis. This work highlights not only the variation in sensitivity among species, but also the ability of environmental estrogens to mimic some, but not all the actions of estrogen.

C. Kinetic and Thermodynamic (*in vitro*) Studies

Kinetic exclusion assays were developed on the KinExATM immunoassay instrument to quantify the thermodynamics and kinetics of binding reactions between the human estrogen receptor and individual compounds that compete with 17- β -estradiol for the hormone binding site on the receptor. Three different covalent conjugates of 17- β -estradiol with bovine serum albumin (BSA) were surveyed for their ability to capture and permit the quantification of free, unliganded estrogen receptor in the KinExA: the 17-hemisuccinate; the 6-(O-carboxymethyl)oxime; and the 3-(O-carboxymethyl)oxime derivatives. Each β -estradiol BSA conjugate was adsorption coated onto polymethylmethacrylate beads and deposited in a packed bed above the porous screen in the observation/flow cell of the spectrofluorimeter. The level of fluorescence observed from the beadpack was used to quantify the number of receptor molecules captured from the solution. Standard curves of instrument response as a function of the estrogen receptor concentration were linear up to 10 nM.

A new method to quantify protein-ligand binding interactions in homogeneous solution was developed on a beta unit of the KinExATM immunoassay instrument. As part of an effort to develop, validate, and exploit new methodologies to facilitate *in vitro* kinetic and thermodynamic studies of protein-protein interactions in the blood coagulation cascade, a series of kinetic exclusion assays were devised to determine equilibrium binding and association/dissociation

rate constants for high affinity protein-ligand binding interactions. These assays were developed on a *beta* unit of the KinExATM immunoassay instrument. The KinExA is a flow spectrofluorimeter designed to achieve the rapid separation and quantification of free, unbound protein present in reaction mixtures of free protein, free ligand, and protein-ligand complexes. Briefly, the KinExA is comprised of a capillary flow/observation cell fitted with a microporous screen through which various solutions are drawn under negative pressure. Uniform particles larger than the average pore size of the screen are adsorption coated with the ligand and deposited above the screen in a packed bed. The immobilized ligand is merely a tool used to capture and retain a portion of the free, uncomplexed protein present in homogeneous solution reaction mixtures. The time of exposure of each reaction mixture to the immobilized ligand is kept sufficiently brief so as to insure that negligible dissociation of soluble protein-ligand complexes can occur. Those protein molecules in solution that are already complexed with the soluble ligand are thus kinetically excluded from binding to the ligand on the immobile phase (hence the term, 'kinetic exclusion assay'). The amount of free protein thus captured is quantified using a fluorescently-labeled antibody directed against the protein of interest.

Following the initial development of kinetic exclusion binding assays on the KinExA, it was of interest to assess the accuracy of the thermodynamic and kinetic parameters thus obtained. Toward that end, another model experimental system was assembled that would permit the same rate and equilibrium constants to be determined instrumentally on a minimum of three instruments. The model system was comprised of an anti-fluorescein monoclonal antibody and bovine serum albumin (BSA) covalently labeled *via* its single reduced cysteine residue with fluorescein maleimide. When the antibody bound to the fluorescein-BSA conjugate, the fluorescein fluorescence was dramatically quenched. Thus binding between the two reagents in solution was monitored by stopped flow spectrofluorimetry using an OLIS 1000TM configured for fluorescence studies. Binding of the soluble reagents was also studied in the KinExA where beads adsorption coated with BSA bearing multiple equivalents of fluorescein isothiocyanate served as the immobilized ligand. Finally, the binding of anti-fluorescein to fluorescein-BSA was studied by surface plasmon resonance in a BIAcore 2000TM using fluorescein-BSA covalently coupled to dextran polymers as the immobilized phase.

Preliminary studies were conducted to determine whether the KinExA could be exploited to study protein-DNA binding interactions. The principle behind this assay is to capture and retain a DNA binding protein on the beads in the observation cell of the instrument regardless of whether DNA is bound to the protein of interest or not. The experimental question was whether any DNA that was captured collaterally with the binding protein could subsequently be quantified to provide a measure of the concentration of protein-DNA complex captured from solution. Accordingly, the ability of the KinExA to quantify DNA was investigated using organic compounds whose fluorescence was enhanced upon intercalation into double stranded DNA. Different amounts of calf thymus DNA covalently conjugated to insoluble fibrous cellulose (Sigma Chemical Co.) were physically trapped within the interstitial spaces of a typical microcolumn beadpack in the observation cell of the KinExA. The beadpacks containing different quantities of trapped DNA were then exposed to fluorescent DNA intercalators that were expected to develop a fluorescent signal that could be quantified in the instrument. A standard curve of instrument response as a function of the total amount of DNA present in the beadpack was a linear function up to 1.0 ng of DNA per beadpack. It is anticipated that the

sensitivity of this assay for DNA captured on the beads will be sufficient to study the thermodynamics and kinetics of estrogen receptor-DNA binding interactions.

VI. RELEVANCE, IMPACT AND TECHNOLOGY TRANSFER

The Center for Bioenvironmental Research (CBR) at Tulane and Xavier Universities represents the only research on the impacts of environmental endocrine disrupting chemicals for the Environmental Management Science Program. The CBR has more than five years of experience in developing model aquatic ecosystems for evaluating environmental problems relevant to DOE cleanup activities. Using biotechnology screens and biomarkers of exposure, CBR research has demonstrated that chemicals in the environment can signal the development of species in aquatic ecosystems, as well as show detrimental impacts on community, population, and the ecosystem, including human health.

Since environmental endocrine disruptors are effective at concentrations similar to hormones, and can cause havoc at levels a thousand or more times below measurable levels of most current analytical methods, it is imperative to develop methods that are effective at concentrations in the environment. Therefore, research into low levels of environmental contaminants may show that currently accepted cleanup standards are inappropriate for DOE cleanup efforts.

Research progress to develop kinetic exclusion assays to quantify the kinetics and thermodynamics of binding reactions between the estrogen receptor and environmental pollutants has focused primarily on the development and validation of semi-automated assays to quantify high affinity binding interactions in general. Initial assay development was accomplished using well-characterized model systems. Subsequent studies have been conducted to determine whether the same approach could be exploited to study binding interactions between the estrogen receptor and DNA in the presence of different hydrocarbon contaminants. These studies generated sufficient preliminary results to support research funding by the National Institute for Environmental Health Sciences and the Department of Defense. For technology transfer, see Section XI. These kinetic exclusion assays bridge the gap between fundamental research and applied technology development.

This project has studied the mechanisms in which environmental factors interact with the hormonal systems of different species, including humans. This field of environmental signaling has been judged by the National Research Council, the USEPA, and the NIEHS as one of the most important new areas in environmental science with a variety of impacts. Although the endocrine disrupting effects of contaminants such as dioxin and PCBs have begun to be well characterized in both animals and humans, little is known about the capacities of other hydrocarbon contaminants to act as endocrine disrupters. Projected risks include decreased sperm counts, and breast disease. Promising approaches to the problem of characterizing endocrine disruption caused by environmental contaminants are being studied at the CBR through multiple sources of funding.

The CBR is uniquely situated to conduct such a complex research plan of larger scale trials since we have established ongoing multidisciplinary projects which span engineering, public health, medicine, ecology in both basic and applied research. Tulane and Xavier are innovators among universities in facilitating the mechanisms that allow scientists from multiple disciplines to work together in resolving environmental problems. In addition, the organizational structure of the

CBR has allowed it to qualify for large integrated, interdisciplinary grants that are beyond the scope of many other universities or research organizations, including individual Tulane and Xavier departments. The work of the CBR is strengthened by this partnership that can provide the faculty, students, and resources that are necessary to conduct the bioenvironmental research projects discussed in this report.

The DOE has a 50-year legacy of environmental problems resulting from the production of nuclear weapons. Among the most serious are the widespread contamination of soils, sediments, and groundwater. The huge cost, long duration, and technical challenges associated with remediating DOE facilities present a significant opportunity for science to contribute cost-effective solutions. Between 1999 and 2003, the Department of Energy plans to invest approximately \$38 million in research to address issues related to health, ecology, and risk. Health, ecology, and risk are cross-cutting problem areas, resulting in this investment having an impact on cleanup work throughout the DOE complex.

Scientific uncertainty exists about the levels of risk to human health and the environment at the end stages of DOE cleanup efforts. Therefore, accurate risk analyses relating to the cleanup efforts require thorough knowledge of contaminant characteristics, basic ecological processes and principles, rates at which contaminants move through ecosystems, and health and ecological effects. Data results from bench and field research will assist DOE in protecting the public, workers, and the environment, thereby having an impact on the decision-making process. In addition to known problem areas, environmental endocrine disruption, a new class of toxic disruption, is increasingly factored into health and risk considerations as the result of research in which the CBR is preeminent.

Subsequent funding for related environmental contaminants and endocrine disruptor research at the CBR has been obtained from the Department of Defense and the Office of Naval Research as part of biohazards research management programs, and most recently from the Office of Environmental Management in the Department of Energy. See Section XV.

VII. PROJECT PRODUCTIVITY

Although the CBR had completed all research requirements by the conclusion of its original funding period, a no-cost one-year extension to September 14, 2000, enabled the CBR faculty investigators to more fully integrate the results of the past three years into new studies to enhance the value of the research for DOE. The research that has been done under this grant has been and will continue to be used to leverage funds for continued research projects. See Section VI.

VIII. PERSONNEL SUPPORTED

The mission of the Center for Bioenvironmental Research (CBR) is to conduct and coordinate research and teaching to enhance global understanding of environmental issues and provide solutions through innovative communication and technology. Founded in 1989, the CBR is an innovative, well-established and effective partnership between a Historically Black College or University (HBCU) and a major research university that encourages scientists from multiple disciplines to work together to investigate and resolve environmental problems. Under the leadership of Dr. John McLachlan, Weatherhead Distinguished Professor of Environmental Sciences at Tulane University and an internationally recognized environmental scientist and

administrator, the CBR has earned a reputation for its scientific research into the environmental problems of Louisiana. In extending its spheres of influence to the national and global problems of the environment, the CBR has brought a unique focus that reflects a community-based perspective in conjunction with scientific rigor.

Tulane and Xavier Universities have developed a close working relationship in the past ten years, aided by a common vision of academic excellence and the development of high quality educational opportunities for minorities and women. The Tulane/Xavier partnership is a well-established, effective joint venture between majority and minority universities. It is an extremely complementary relationship with respect to environmental restoration and waste management, with the Xavier University focus on education and undergraduate work, and Tulane University emphasis and experience in graduate education, research, and technology development and transfer. The preeminent reputation of both institutions enhances the ability of the CBR to solicit resources, recruit staff and researchers, sponsor conferences, and execute successful marketing of its education and research programs.

The professional research personnel on this project were primarily collaborative teams of postdoctoral students and faculty. The primary research investigators of this project were two post-doctoral fellows, Drs. Ann Oliver Cheek and Eva Oberdoerster, who both became full-time faculty during this period. Both conducted research in the CBR Environmental Endocrinology Laboratory under the supervision of Drs. Charles Ide, Tulane Associate Professor of Cellular and Molecular Biology, and John McLachlan, Professor of Pharmacology at Tulane and Xavier Universities. Dr. Cheek is interested in a fish model system, linking individual reproductive fitness to biomarkers of xenobiotic exposure. The research of Dr. Oberdoerster looks at the effects of environmental signals on invertebrates. The third primary research investigator, Dr. Robert Blake, Professor of Basic Pharmaceutical Sciences at Xavier University, focuses his research on the development and validation of new methodologies in kinetic and thermodynamic studies.

Tulane and Xavier investigators had students, often undergraduates, in their laboratories assisting with this project. In this way the CBR assists research faculty to build and train sufficient qualified personnel to complete research and also create capacity in undergraduate students. Students, where applicable, were cited as co-authors of the publications.

IX. PUBLICATIONS

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- Parks, L.G., **A. Oliver Cheek, N.D. Denslow, S.A. Heppell, J.A. McLachlan, G.A. LeBlanc, and C.V. Sullivan. 1999.** Fathead minnow (*Pimephales promelas*) vitellogenin: Purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. *Comparative Biochemistry and Physiology*. 123:113-125.
- Tran, D.Q., **C.F. Ide, J.A. McLachlan**, and S.F. Arnold. **1997.** The anti-estrogenic activity of selected polynuclear aromatic hydrocarbons in yeast expressing human estrogen receptor. *Biochemical and Biophysical Research Communications* 229:102-108.

X. INTERACTIONS

Cheek, A. Oliver. Panelist for the EDSTAC Working Group on Thyroid Disruption. Duke University, Durham, NC, June, **1997.**

Cheek, A. Oliver, C.F. Ide, C. Rider, and J.A. McLachlan. "Effects of environmental chemicals on thyroid hormone induced metamorphosis in leopard frogs (*Rana pipiens*),"

presented at the Society of Environmental Toxicology and Chemistry annual meeting, November, **1997**.

Cheek, A. Oliver, C.V. Rider, M.A. Holstein, and **J.A. McLachlan**. “Morphological and biochemical effects of environmental estrogens on frog (*Xenopus laevis*) metamorphosis,” presented at the Society of Environmental Toxicology and Chemistry annual meeting, November, **1998**.

Cheek, A. Oliver, J. Chen, K. Y. Kow, and **J. A. McLachlan**. “Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid binding globulin,” presented at the annual meeting of the Society of Integrative and Comparative Biology, January, **1999**.

Cheek, A. Oliver, T. Hoexum-Brouwer, S. Gronen, M. Brouwer, and **J. A. McLachlan**. “Bench to Bayou: Progress on Reproductive Disruption in Fishes,” presented at the US-Japan Workshop on Endocrine Disruption. Tulane University, New Orleans, LA, February, **1999**.

Cheek, A. Oliver, T. Hoexum-Brouwer, S. Gronen, M. Brouwer, and **J.A. McLachlan**. “Developmental exposure to anthracene and estradiol alters sex differentiation and reproductive success in medaka (*Oryzias latipes*),” presented at the Society of Environmental Toxicology and Chemistry annual meeting in November, **1999**.

Cheek, A. Oliver, T. Hoexum-Brouwer, S. Gronen, M. Brouwer, and **J.A. McLachlan**. “Developmental exposure to anthracene and estradiol alters reproductive success in medaka (*Oryzias latipes*),” presented at the Society of Integrative and Comparative Biology meeting in January, **2000**.

Oberdörster, Eva. “Decline of Macro- and Meio-faunal Communities in Bayou Trepagnier, LA,” presented at University of New Orleans, Mississippi River Water Quality Conference: Clean Enough? New Orleans, LA. September 21, **1997**.

Oberdörster, Eva. Panelist for Discussion Session on Endocrine Disruptor Detection and Risk Assessment. 37th Annual Society of Toxicology meeting, Seattle, WA. March, **1998**.

Oberdörster, Eva, and **J. A. McLachlan**. “Multi-Tiered Approach to Detecting PAH Interactions with the Arthropod Ecdysone Receptor,” presented at Gordon Research Conference on Environmental Endocrine Disruptors. Plymouth, NH. July 12-15, **1998**.

Oberdörster, Eva. SETAC Expert Workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment (EDIETA), London, England, December 12-15, **1998**.

XI. TRANSITIONS

A portable version of the KinExA is currently under development for use in the field. Under the auspices of the Department of Energy's Natural and Accelerated Bioremediation Research Program (grant number DE-FG02-98ER62704), Dr. Robert Blake is collaborating with another

academic investigator and laboratory (Dr. Diane Blake, School of Medicine, Tulane University) as well as private industry (Sapidyne Instruments, Inc., Boise, ID) on the research and development of a portable, hand-held fluorescence immunosensor for conducting protein-binding studies in the field. The optical unit will be comprised of a solid state laser diode light source (to produce excitation light with a narrow bandwidth at approximately 660 nm), an excitation optical filter (to absorb light from all sources except the narrow-bandpass laser light), a dichroic mirror (to reflect the exciting light from the laser diode to the sample chamber and permit only the longer wavelength light emitted from the sample to pass through the mirror toward the detector), an emission optical filter (to further absorb any exciting light that might make its way through the dichroic mirror), and a detection/amplification system to convert the fluorescence signal to a quantifiable electrical signal.

A pre-packaged disposable cartridge will fit into the side of the hand-held instrument and contain the immunochemicals and ancillary reagents necessary to actually conduct the affinity-based molecular interactions that comprise each immunoassay. In addition to a disposable or rechargeable battery source of power, other power options include vehicle cigarette lighters and normal 110 voltage outlets (just as portable computers can operate on all of these external sources of power). Other features of the portable remote immunosensor to be assembled and tested include the following: incorporation of a palm-top microcomputer to provide instrument control and facilitate on-site data analysis and record keeping; and the addition of the hardware and software necessary to enable on-site analyses to be pinpointed geographically with the Global Positioning System.

Sapidyne Instruments will provide all the hardware and experience necessary to fabricate a bench-scale version of the portable device. Personnel at Xavier and Tulane will conduct developmental activities to address practical operating issues such as the most desirable means of presenting liquid samples to the instrument. Once global operating issues have been resolved and the final design of the disposable cartridge has been completed, the design of the hand-held instrument will be finalized. Fabrication and delivery of the final field-ready prototype was completed in late Fall 2000.

XII. PATENTS None during grant period

XIII. FUTURE WORK

This CBR research, conducted for DOE-EMSP, forms the foundation for future experiments into the genetic manipulation of plants to potentially promote greater or more specific symbiotic relationships between plant and *Rhizobium* allowing this biological phenomenon to be used in a greater number of crop types. Future technology developments could include the genetic engineering of crops suitable for *in situ* vadose zone bioremediation (via microbes) and phytoremediation (through the crop itself) in contaminated DOE sites.

XIV. LITERATURE CITED

The focus of one component of this research has been to provide an analysis of the contaminants located in or around various Department of Energy (DOE) sites that are or have the potential to function as endocrine disruptors and to correlate the need for studying endocrine disruptors as a priority in the DOE programmatic agenda.

An extensive literature research has been performed using the following key words and search engines: Department of Energy/Environmental Management (DOE/EM) Complex cleanup needs, plans, and progress; Endocrine disrupting chemicals and effects; U.S. Environmental Protection Agency (EPA) programs affecting DOE/EM; Environmental Risk Analysis and Management; Community/stakeholder issues surrounding cleanup at the DOE complex.

Since a majority of DOE cleanup efforts have focused on sites with heavy, radioactive waste contamination, the focus in the literature search was limited to those DOE sites listed on the EPA National Priorities List (NPL) as a result of hazardous wastes covered by the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA, or Superfund). The Agency for Toxic Substances and Disease Registry (ATSDR), within the U.S. Department of Health and Human Services, was created by the Superfund legislation to help prevent or reduce exposures to toxic chemicals found in the environment and to expand knowledge about the health effects of exposures to hazardous substances. ATSDR is also responsible for conducting public health assessments of all sites that are proposed for listing on the EPA NPL and has thus addressed public health issues in the communities near DOE sites. The NPL and the ATSDR were primary sources for DOE site information.

Environmental Estrogens

The following chemicals have been shown to exhibit estrogenic properties with varying potencies at 18 DOE facilities and 91 waste sites. Some also work as anti-estrogens in different organs in the body.

Organochlorine pesticides: DDT, dicofol, methoxychlor, dieldrin, toxaphene, and endosulfan

Polychlorinated biphenyls (PCBs): (e.g.) Arochlors 1221, 1232, 1242, 1248

Dioxins and furans: 7 dioxins and 10 furans possess hormone-like properties that can profoundly impair the male reproductive anatomy.

Alkylphenol polyethoxylates (APEs): Octylphenol, nonylphenol

Phytoestrogens (plant estrogens): Genistein, daidzen, equol, tetrahydrocannabinol (delta-9 THC), coumestrol, quercetin, luteolin, naringenin

Mycoestrogens (fungal estrogens): Zearalenone, zearanol, taleranol

Industrial Chemicals: Bisphenol-A, butylbenzyl phthalate, di-n-butylphthalate, and butylated hydroxyanisole

Fungicides: vinclozolin, benomyl, hexachlorobenzene, mancozeb, metiram-complex, tributyl tin, zineb, ziram

Herbicides: 2,4-D; 2,4,5-T; alachlor, aldrin, amitrole, atrazine, metribuzin, nitrofen, trifluralin

Insecticides: carbaryl, methomyl, mirex, parathion, petthane, chlordane, oxychlordane, trans-nonachlor, heptachlor, heptachlorepoxyde, hexachlorocyclohexanes (such as lindane), synthetic pyrethroids, DDE (breakdown product of DDT)

Nematocides: aldicarb, DBCP

Metals and Pollutants: cadmium, lead, mercury, polybrominated biphenyls, benzo(a)pyrene, styrenes

Reference: **Environmental Hormones: Chemical Threats to Reproductive Health**

<http://www.rtndf.org/rtndfprograms/hormone.html>

This table of information has been prepared to summarize contaminants identified in groundwater and soils/sediments at 18 DOE facilities and 91 waste sites. Compounds detected in groundwater are those that were disposed of into the ground and subsequently transported

through the soil and vadose zone by water or by nonaqueous liquids such as organic solvents. Compounds from soil and sediment analyses can provide a good assessment of the total chemical composition of wastes and contaminants disposed of to the ground. This table shows the environmental estrogens exhibited in DOE sites listed on the Superfund National Priorities List.

XV. FEEDBACK

Feedback to the DOE has resulted in new funding for the CBR from the DOD, ONR, and DOE for further research and study of endocrine disruptors and environmental hormones related to biohazards research and management. See Section VI. The CBR is currently working with the Office of Environmental Management on an integrated research program in support of the long-term stewardship of the DOE complex. Over the past six years, in partnership with the DOE, the CBR has developed a unique natural laboratory for development of new biosensors and biomarker technologies and a program for risk evaluation and communication. Through a three-year DOE cooperative agreement, initiated in September 2000, the CBR will leverage this capacity into development of an integrated research-based program for monitoring technology development, information management, and risk analyses and communication, effectively positioning the DOE to implement its long-term stewardship strategies in the near future.

XVI. APPENDIX: Unpublished Manuscripts See attached.

Appendix

Unpublished Manuscripts

1. **Developmental Exposure to Anthracene and Estradiol alters Reproductive Success in Medaka (*Oryzias Latipes*).** Ann O. Cheek, et al.
2. **Long-term pyrene exposure of grass shrimp, *Palaemonetes pugio*, affects molting and reproduction of exposed males, and offspring of exposed females.** Eva Oberdörster, et al.

**DEVELOPMENTAL EXPOSURE TO ANTHRACENE AND ESTRADIOL
ALTERS REPRODUCTIVE SUCCESS IN MEDAKA (*ORYZIAS LATIPES*).**

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Running title: endocrine disruption alters reproductive success

**Keywords: endocrine disruption; medaka; sex differentiation; reproductive
success; vitellogenesis; fish**

ABSTRACT

Polyaromatic hydrocarbons (PAHs) impair fish reproduction and appear to antagonize estradiol action *in vitro* and *in vivo*. To test the hypothesis that PAH exposure could alter reproductive success by disrupting the effect of estrogen on sex differentiation and puberty, we exposed medaka (*Oryzias latipes*) for 2 or 8 weeks post-hatch to 0, 12, and 20 ug/L anthracene \pm 0.27 ug/L estradiol-17 β (E2). At the end of the exposure, fish were transferred to clean water, grown to sexual maturity (14 weeks), and placed in mating pairs. Eggs were collected, counted, and scored for % fertilization (mating success). Eight week exposure to E2 resulted in 100% females, while the 2 week exposure resulted in more than 50% females. Exposure to 20 ug/L anthracene for 8 weeks reduced mating success by half, but co-exposure to E2 prevented the effect. Exposure to anthracene for 2 weeks did not alter mating success, but did reduce the proportion of E2-induced sex reversal in co-exposed fish. Anthracene did not prevent the E2 effect in fish co-exposed for 8 weeks. Anthracene also had a transgenerational effect on reproductive success, reducing fertility in the offspring of treated males and females. We conclude that anthracene disrupts estrogen action, since anthracene-induced effects on mating success are prevented by E2, while E2-induced effects on sex ratio are prevented by anthracene.

INTRODUCTION

High concentrations of polyaromatic hydrocarbons (PAHs) in the environment are frequently due to human activity, but most PAHs are natural products – algal aromatic compounds transformed by geologic processes into fossil fuels. The distribution of PAHs in aquatic environments has been characterized (1) and the carcinogenic effects of some PAHs are well known (2), but relatively few studies have examined the reproductive effects of PAHs on wild populations of fish (3-7). Field surveys of benthic fish, including English sole (*Pleuronectes vetulus*), rock sole (*Pleuronectes bilineatus*), and starry flounder (*Platichthys stellatus*) exposed to hydrocarbon-contaminated sediments showed that females had depressed levels of estradiol and reduced fecundity when spawning was induced in the laboratory (4, 8, 9). Laboratory exposure studies of domestic fathead minnows (*Pimephales promelas*) and wild-caught English sole have also shown that PAH-exposed animals have decreased reproductive success (10-12). Effects varied from complete inhibition of spawning in English sole captured at a highly contaminated site (12) to reduced egg production and hatching success in fathead minnows exposed to anthracene and fluoranthene (10, 11).

These data suggest that PAHs may be considered environmental anti-estrogens.

However, the mechanisms by which PAHs decrease sex hormone levels and fecundity are not understood. Many authors have noted the association between PAH-induced expression of cytochrome P450 enzymes and reduced steroid hormone levels (2).

Cytochrome P450s are oxidative enzymes responsible for the synthesis and degradation of steroid hormones and for metabolism of exogenous compounds, including pesticides,

industrial chemicals, fungal and plant-derived compounds and other naturally occurring chemicals, including PAHs. In fact, enhanced expression of cytochrome CYP 1A is a widely accepted marker of exposure to PAHs (2). Although CYP 1A does not metabolize endogenous estrogen, PAH-induced increases in P450 activity may indirectly enhance degradation of endogenous steroids, thereby lowering circulating levels of sex hormones (2). Because estradiol initiates vitellogenesis (egg yolk deposition), decreased levels of estradiol could reduce vitellogenin synthesis, thereby reducing number or size of eggs (7).

Interestingly, in studies of wild-caught English and rock sole, plasma estradiol levels were reduced in fish from highly contaminated sites, but plasma vitellogenin levels were not (7, 8). Still, soles from the most contaminated sites were less likely to spawn after gonadotropin releasing hormone stimulation and had the lowest percentage of fertilized eggs and the lowest numbers of normal larvae after hatch (7). Perhaps maternal transfer of PAHs to the yolk impairs fertilization and development of embryos. In the laboratory, eggs from anthracene exposed female fathead minnows contained significant concentrations of anthracene and the larvae had reduced hatching success and survivorship (11). Parental transfer of lipophilic contaminants into gametes (eggs or sperm) can be a significant route of contaminant elimination in fishes (13, 14).

In order to evaluate the apparently anti-estrogenic activity of PAHs, we exposed developing medaka (*Oryzias latipes*) to anthracene alone and in combination with estradiol. As in many other vertebrates, estradiol exposure during sex differentiation

causes feminization of medaka at doses as low as 0.01 $\mu\text{g/L}$ (ppb) (15). Exposure to environmental estrogens also affects sex differentiation in fish. Gray and Metcalfe (16) showed that nonylphenol induced the formation of an intersex gonad, a “testis-ova” in medaka exposed from hatch until the onset of sexual maturity. However, the effective concentrations of nonylphenol (50 – 100 ppb) were four to 100-fold higher than the range of concentrations found in most U.S. and U.K. rivers (0.2 – 12 $\mu\text{g/L}$; (17). Using lower doses (0.5 – 1.9 ppb), Nimrod and Benson (15) treated medaka for one month post-hatch and found no intersex gonads, but did see a decrease in the proportion of females, i.e. masculinization of nonylphenol-treated fry. To our knowledge, no studies have examined the developmental effects of anti-estrogen exposure in fishes.

If anthracene is an anti-estrogen, we would predict that it could inhibit estrogen-induced feminization of fry. Although sex differentiation in fishes is quite labile (18), hormonal effects generally occur within a critical window during development (18-20). To identify the critical window for anthracene and estradiol exposure, we exposed medaka fry during sex differentiation only (for two weeks post-hatch) or throughout sex differentiation and puberty (for eight weeks post-hatch).

Because field studies indicate that PAH exposure reduces reproductive success, we also quantified reproductive success of exposed males and females (F_0) and of their offspring (F_1). Since sexual development in fishes is so sensitive to hormones, we predicted that exposure during the period of sex differentiation would be sufficient to reduce reproductive success once these fish reached maturity. Very recently, studies examining

reproductive success of adult fish exposed to environmental estrogens in the laboratory have shown that fecundity is reduced by alkylphenol (21, 22). Very few studies have examined reproductive success of fish exposed during sexual differentiation and fewer still have examined the transgenerational effects of environmental hormone exposure. We analyzed the effects of maternal (F₀) anthracene transfer by quantifying reproductive success of the offspring (F₁).

We found that anthracene alone does not alter sex differentiation, but does inhibit estrogen-induced feminization. Anthracene does impair fecundity (number of eggs) and fertility (% fertilization) of exposed females. In addition, the effects of anthracene are transgenerational – offspring of anthracene-exposed females produce embryos with reduced hatching success.

MATERIALS & METHODS

Animals

Medaka fry used in this study were hatched from broodstock cultured and maintained at the Institute of Marine Science, University of Southern Mississippi, Ocean Springs, MS. Animal care and experimentation were conducted in accordance with guidelines of the University of Southern Mississippi, Southeastern Louisiana University, and Tulane University guidelines for animal care and use.

Experimental Design

Anthracene exposure concentrations were selected based on laboratory studies with fathead minnows showing that nominal doses of 12 and 20 $\mu\text{g/L}$ (ppb) reduced reproductive success (11). These low dissolved concentrations provide conservative estimates of the potential impact of PAH exposure on field populations, given that sediment concentrations as high as 17 ppt (parts per thousand) have been measured in contaminated waterways (G. Flowers, personal communication). Preliminary exposures of 12 day old medaka fry, five week old juveniles, and 25 week old adults to 20 $\mu\text{g/L}$ anthracene for three weeks showed that anthracene did not cause significant mortality. The estradiol concentration (0.27 $\mu\text{g/L}$ or 1 nM) was chosen to approximate the dissociation constant (K_d) of estradiol binding to fish estrogen receptors (0.7 – 8 nM, depending upon tissue and species) (23-26).

Treatments included a water control (no solvent), a solvent control (triethylene glycol, TEG), 0.27 $\mu\text{g/L}$ estradiol-17 β (E2), 12 $\mu\text{g/L}$ anthracene (ANT), 20 $\mu\text{g/L}$ anthracene, 12 $\mu\text{g/L}$ anthracene + 0.27 $\mu\text{g/L}$ E2, and 20 $\mu\text{g/L}$ anthracene + 0.27 $\mu\text{g/L}$ E2. Fry were exposed for two or eight weeks post-hatch, subsampled for analysis of size at 2, 4, and 8 weeks post-hatch, then transferred to clean water for grow-out until 3 months of age. At 3 months, adults were sorted into mating pairs in order to assess the impact of parental exposure on production and survival of offspring. Three categories of mating pairs were created: both parents treated, only the female parent treated, or only the male parent treated (Table 1).

Chemical Exposures

Anthracene was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). The stock solution was prepared by dissolving 1.9 g anthracene in 4.64 liter triethylene glycol (TEG) for a final stock concentration of 410 mg/L. Estradiol-17 β was purchased from Sigma Chemical Co. (St. Louis, MO). Estradiol stock solutions (7 mg estradiol /L TEG) were prepared periodically throughout the exposure (approximately weekly). Frequent replacement of the stock was performed to compensate for the significant decline (approximately half) in stock concentration observed during preliminary experiments.

Exposures were conducted in a flow-through system. Dilution water was drawn from a 177 m freshwater well. Water was particle and carbon filtered, temperature adjusted, and aerated prior to entering test aquaria. Treated and water control fry were exposed in duplicate 20 L aquaria (45 fry/aquarium) and solvent control fry were exposed in quadruplicate 20 L aquaria (45 fry/aquarium) for two or eight weeks post-hatch. Fry were housed in a 1.5 L cylindrical mesh container within the aquarium to protect against physical damage and to allow more efficient foraging until 3 weeks of age, when they were released into the test aquaria. Fry were fed microworms until day 3, microworms once and brine shrimp nauplii twice daily until day 6, and AquaTox Special dry flakes (Ziegler Bros, Garnder, PA) once and brine shrimp nauplii twice daily from day 7 to day 56. From day 57 to termination fish were fed dry flakes three times and brine shrimp nauplii once daily.

Flow rate was maintained at 100 L/aquarium/day in an intermittent flow-through chamber similar to that described by Walker et al (27). Aquaria were housed in a heated recirculating water bath to maintain constant temperature. A 16L:8D photoperiod was maintained throughout the study. Temperature, dissolved oxygen (DO), and pH were measured twice weekly in each aquarium. The time weighted mean and standard deviation of temperature, DO, and pH were $26.8 \pm 0.5^\circ \text{C}$, $5.3 \pm 0.6 \text{ mg/L}$, and 8.9 ± 0.2 , respectively.

Chemical analysis

Anthracene and estradiol concentrations were measured twice weekly in each treatment. **Anthracene concentrations in water were measured directly by reverse-phase HPLC as described by Oris et al (28). Briefly, 20 ml of sample or standard was injected onto a Waters 3.9 mm x 15 cm mBondpak C18 column at 30° C. Samples were eluted under isocratic conditions (9:1 acetonitrile:water) at a flow rate of 0.8 ml/min. Anthracene was visualized using a xxxx fluorescence detector with excitation at 255 nM and emission at 400 nM. Concentrations were measured using a computerized Beckman System Gold chromatography data station.**

Estradiol concentration was measured using an enzyme-linked immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI) according to manufacturer's instructions. **x ml of water were extracted using a C-18 Bond Elute cartridge prior to assay.** Water samples diluted in parallel with the standard curve and the detection limit of the assay was 17 pg/well.

Analysis of Reproductive Success

F₀ – exposed fish. At the end of the exposure period, fish were transferred to culture aquaria receiving flowing well water at 27° C with a 16L:8D photoperiod. At 3 months of age, adults from replicate treatment aquaria were pooled and indiscriminately assigned to mating pairs (Table 1). Pairs were housed in 1.5 L breeding chambers containing a spawning substrate. Breeding chambers were placed in a flow-through raceway with a flow-rate of 5L/chamber/day maintained at 27.0 ± 1°C. Eggs were collected daily for seven days and the total number of eggs and number of eggs fertilized were scored. A group of 25 fertilized eggs was collected from each mating pair and the chorionic filaments were removed to prevent clumping during the incubation period. Embryos were transferred to 250 ml hatching jars containing embryo rearing solution (0.1% NaCl, 0.003% KCl, 0.004% CaCl₂, and 0.163% MgSO₄ in distilled water) and incubated with aeration at 24°C. A total of 25 embryos were collected for observation. Embryos were assessed daily for abnormal development, survival, and hatch. Newly hatched fry were transferred to 1.5 L chambers to monitor survival for 72 hours post hatch. Fry were fed paramecia, microworms, and brine shrimp nauplii.

F₁ – offspring of exposed fish. To examine the possibility of transgenerational anthracene effects, we reared fry from F₀ fish exposed to vehicle or 20 µg/L ANT for eight weeks post-hatch and fry from F₀ females exposed to vehicle, E2, and 12 and 20 µg/L ANT ± E2. All F₁ fry from an F₀ treatment group were pooled, grown for 13 weeks post-hatch in clean water and randomly selected to form eight mating pairs.

Statistics

Percentage of fry surviving to eight weeks post-hatch was compared between treatments using χ^2 analysis. The effect of treatment on size was analyzed by one-way analysis of covariance (ANCOVA) with time as the covariate. Significant divergence from a 1:1 sex ratio was analyzed by χ^2 tests. The effect of treatment on reproductive success was analyzed by separate one-way ANOVAs for three scenarios: both parents treated, female treated, or male treated. Reproductive success was estimated using the following three parameters: the number of fertilized eggs, the percentage of fertilized eggs, and the percentage of surviving embryos. Dunnett's post hoc test was used to compare treatment means to the control mean if the omnibus F was significant. Alpha was set at 0.05 for all tests.

RESULTS

Chemical dosing

Measured doses of anthracene were 83 – 99% of nominal and measured doses of E2 were 67 – 89% of nominal (Table 2).

Survivorship and Size

Neither anthracene nor estrogen exposure significantly altered survival of fry to eight weeks. However, co-exposure to E2 + 20 $\mu\text{g/L}$ ANT for two weeks post-hatch reduced survival by approximately 60% and eight week co-exposure to E2 + 12 or E2 + 20 $\mu\text{g/L}$ ANT reduced survival by approximately 40% (Figure 1). The effects of co-exposure

were both time and dose-dependent, with reduced mortality occurring at a lower ANT dose when exposure was longer.

Size was measured as weight at 2, 4, 8, and 14 weeks. Exposure to solvent, E2, anthracene, and anthracene and E2 in combination for two weeks post hatch had no effect on growth (ANCOVA, $F_{\text{treatment}} = 2.03$, $p = 0.07$; $F_{\text{time}} = 2.26$, $p = 0.13$; time ($F_{\text{treatment} \times \text{time}} = 2.03$, $p = 0.07$). Exposure for eight weeks post hatch did not significantly affect growth either (ANCOVA, $F_{\text{treatment}} = 1.3$, $p = 0.25$; $F_{\text{time}} = 1483.6$, $p < 0.0001$). However, the effects of treatment changed with time ($F_{\text{treatment} \times \text{time}} = 5.17$, $p < 0.001$) and at 14 weeks post hatch, fish exposed to anthracene and E2 were significantly larger than solvent control fish (ANOVA, $F = 6.71$, $p < 0.001$; Games-Howell post hoc test). Since all E2- and anthracene plus E2-treated fish were feminized and mature females are heavier than males, the size analysis at 14 weeks was restricted to female fish. Female size did not differ among treatments (Games-Howell post hoc test).

Sex ratio

Anthracene did not alter sex ratio regardless of dose or exposure duration. E2 exposure feminized fish, producing 83% females and 8% feminized males after two weeks (Table 3) and 100% females after eight weeks. Feminized males had ambiguous secondary sex characteristics but spawned as males. Both doses of anthracene prevented feminization during a two week exposure, but rather than increasing the number of normal males, the higher dose doubled the number of feminized males (Table 3), suggesting that anthracene is not a completely effective anti-estrogen.

Reproductive success

For both exposure periods, reproductive success was quantified as (1) the number of fertilized eggs per treated individual, (2) the percentage of fertilized eggs per treated individual, and (3) the percentage of a treated individual's embryos surviving for 72 hours post hatch.

2 week exposure. Anthracene alone had no effect on male or female reproductive success (data not shown). But ANT + E2 treatment of males significantly reduced reproductive success (Fig. 1). Because some, but not all males in the ANT + E2 mating pairs were feminized, we compared fertilization and hatching success between feminized males and normal males at the same doses of estradiol and anthracene. Feminization reduced the number of fertilized eggs to less than 4% of those fertilized by normal males (Fig. 2, Mann-Whitney U test, $Z = -2.32$, $p = 0.02$ for E2 + 12 ANT; $Z = -2.84$, $p = 0.005$ for E2 + 20 ANT). This suggests that E2, not anthracene reduced sperm production in feminized males. None of the eggs fertilized by feminized males hatched, while 40 – 60% of those fertilized by normal males did, indicating that sperm quality was affected as well as sperm quantity.

Eight week exposure. Females treated with 20 $\mu\text{g/L}$ anthracene had noticeably fewer fertilized eggs than controls (Fig. 3; omnibus $F = 2.9$, $p = 0.03$; Dunnet post-hoc test $p > 0.05$). When both partners were treated, 20 $\mu\text{g/L}$ anthracene significantly decreased the percentage of fertilized eggs (Fig. 4). Females treated with E2 + ANT had the same

number of fertilized eggs as controls, suggesting that E2 prevented the ANT-induced reduction. Males treated with anthracene showed no changes in reproductive success, suggesting that anthracene preferentially affected eggs (Figs. 3 and 4). Neither anthracene nor estradiol affected hatching success of embryos from treated males or females (data not shown).

F₁ generation – 20 µg/L ANT effects. Neither the number nor the percentage of fertilized eggs produced by F₁ fish was affected by ANT exposure of the F₀ fish. However, the percentage of F₂ embryos hatching was significantly reduced by F₀ ANT exposure (Fig. 6A & B), suggesting that anthracene has a genotoxic effect on F₁ germ cells.

F₁ generation – maternal effects. Because females transfer lipophilic substances to embryos via the yolk (13, 29), we investigated the maternal effect of all treatments. The percentage of fertilized F₁ eggs was significantly decreased by F₀ maternal exposure to 12 and 20 µg/L ANT (Fig. 6C & D). E2 prevented the effect of 12 µg/L ANT exposure, but did not prevent the effect of 20 µg/L ANT. Anthracene exposure does appear to have transgenerational effects, particularly on the fertility of F₁ eggs, again suggesting an effect on germ cells. The percentage of embryos hatching was low and similar in all treatments, probably due to inbreeding depression (individuals had a 1:6 chance of mating with a sibling).

DISCUSSION

We predicted that if anthracene were an environmental anti-estrogen, it could inhibit the feminizing effects of estradiol. Results from both the two and eight week developmental exposures show that anthracene alone did not alter the sex ratio of adult fish, indicating that it did not inhibit the action of endogenous estrogen. However, during a two week post-hatch exposure, co-treatment with anthracene did reduce estradiol-induced feminization. As the anthracene dose increased, the total number of males increased, but rather than the number of normal males increasing, the number of feminized males increased. Anthracene could not inhibit estradiol-induced feminization during an eight week co-exposure. Together, these results suggest that the efficacy of anthracene as an anti-estrogen is time- and estrogen dose-dependent.

This dose-dependent anti-estrogenicity is displayed by other CYP 1A inducing PAHs. For example, in immature rainbow trout (*Oncorhynchus mykiss*) injected with physiologically low levels of E2, β -naphthoflavone (β NF) acted as an anti-estrogen, antagonizing estradiol-stimulated vitellogenin (VTG) synthesis. But at physiologically high E2 levels, β NF acted as an estrogen mimic, potentiating VTG synthesis (30). β NF also showed anti-estrogenic activity in coho salmon (*Oncorhynchus kisutch*), inhibiting *in vitro* estradiol production by ovarian follicles (31). A second PAH, 20-methylcholanthrene (20 MC) also inhibited E2 production by coho salmon follicles. Interestingly, both of these compounds also induced CYP 1A production by follicular cells, suggesting that CYP 1A may be directly or indirectly involved in inhibition of steroidogenic enzymes (32).

Our second prediction was that exposure to anthracene during development would reduce reproductive success. When anthracene exposure occurred for only two weeks post-hatch, no changes in reproductive success of males or females resulted. However, when developing fish were exposed for eight weeks, the highest dose of anthracene reduced female fecundity (number of fertilized eggs) by half and significantly reduced fertility (% of fertilized eggs) when both parents were exposed during development. Again, anthracene had no effect on male reproductive success. The apparently female-specific effect of anthracene may be due to transfer of lipophilic contaminants from the female to her eggs. In all species of fish examined, maternal transfer of lipophilic contaminants significantly reduces the female's body burden of contaminants (13, 29). In fact, vitellogenin, the egg yolk protein precursor, may specifically transport contaminants into the developing oocytes (33).

Alternatively, the fecundity reducing effects of anthracene may be due to changes in steroid metabolism. If anthracene exposure enhances degradation of estrogen, fewer eggs with less yolk might be produced. This argument is supported by the observation that co-exposure to estrogen prevents anthracene effects. PAH exposure has been correlated with decreased circulating estrogen levels in other fish, including English sole, rock sole, and starry flounder (4, 7, 8).

Because parental transfer of contaminants to offspring is highly likely, we quantified reproductive success in the offspring (F_1) of exposed fish. Fecundity was not affected,

indicating that development of germ cells and somatic gonadal cells was not altered by any *in ovo* exposure. However, fertility (the % of fertilized eggs) was significantly reduced in offspring of anthracene exposed females. In addition, hatching success (% hatching) of F₂ embryos was abysmal if either the male or female grandparent was exposed to 20 µg/L ANT. These results suggest that anthracene exposure directly alters viability of eggs and sperm, not only in the exposed fish, but in their offspring. We speculate that anthracene is preferentially sequestered in the gonad of exposed fish where it causes some genetic defect in germ cells. If somatic cells were affected, we would expect a decrease in fecundity as well as fertility.

In summary, anthracene has anti-estrogenic activity in the presence of physiological doses of estrogen, inhibiting estrogen induced feminization of developing fish. The efficacy of anthracene is both time and estrogen dose-dependent; anthracene cannot inhibit the feminizing effects of long term estrogen exposure. Anthracene exposure during development also alters adult reproductive success in a time and dose-dependent manner. Reduced female fecundity and fertility occur after longer, higher dose exposure, while male fertility is unaffected. Reduced fertility and hatching success in embryos of offspring show that anthracene has transgenerational effects on reproductive success, although the mechanism is unclear.

ACKNOWLEDGMENTS

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Table 1. Mating Pairs.

Treatment of Male	Treatment of Female	2 week # pairs	8 week # pairs
Male & Female Treated			
water control	water control	6	6
solvent control	solvent control	8	6
E2	E2	6	0 ^a
12 µg/L ANT	12 µg/L ANT	6	6
20 µg/L ANT	20 µg/L ANT	6	6
12 µg/L ANT + E2	12 µg/L ANT + E2	6	0 ^a
20 µg/L ANT + E2	20 µg/L ANT + E2	6	0 ^a
Male Treated			
E2	solvent control	0 ^a	0 ^a
12 µg/L ANT	solvent control	6	6
20 µg/L ANT	solvent control	6	6
12 µg/L ANT + E2	solvent control	6	0 ^a
20 µg/L ANT + E2	solvent control	6	0 ^a
Female Treated			
solvent control	E2	6	6
solvent control	12 µg/L ANT	6	6
solvent control	20 µg/L ANT	6	6
solvent control	12 µg/L ANT + E2	6	6
solvent control	20 µg/L ANT + E2	6	6

^aAll treated individuals were female.

Table 2. Anthracene and estradiol doses ($\mu\text{g/L}$) during exposure (mean \pm 1 s.e.m.).

Treatment	Actual		
	Nominal	Anthracene	E2
water control	0	nd	nd
solvent control	0	nd	nd
E2	0.27	nd	0.20 \pm 0.01
anthracene	12	10.0 \pm 0.66	nd
anthracene	20	19.5 \pm 0.61	nd
anthracene + E2	12 + 0.27	11.3 \pm 0.51	0.18 \pm 0.01
anthracene + E2	29 + 0.27	19.7 \pm 0.73	0.24 \pm 0.02

Table 3. Sex ratio of adults after two week post-hatch exposure to estradiol alone (0.27 $\mu\text{g/L}$) or in combination with anthracene (ANT, 12 or 20 $\mu\text{g/L}$). Feminized males were defined as having ambiguous secondary sex characteristics and the ability to fertilize eggs.

Treatment	# males	# feminized males	# females
E2	2	2	20
E2 + 12 ANT	6	3	15
E2 + 20 ANT	5	7	12

FIGURE LEGENDS

Figure 1.

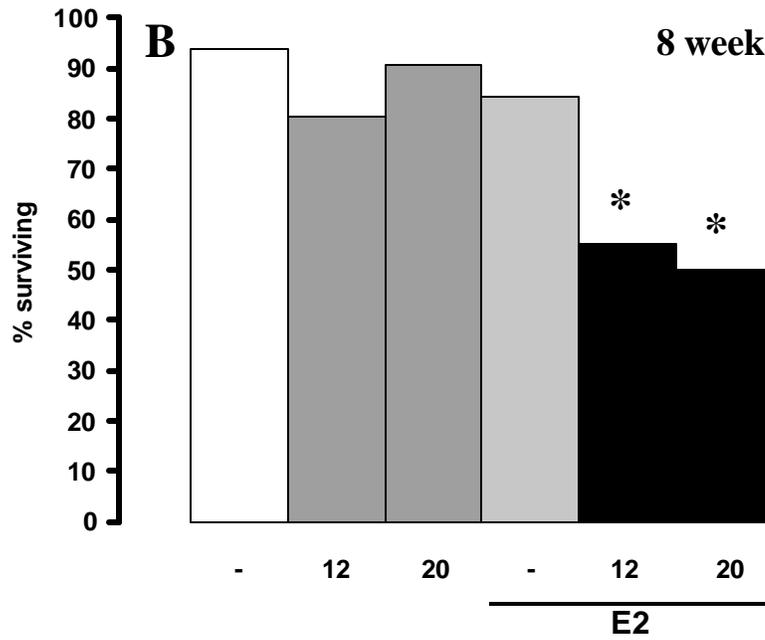
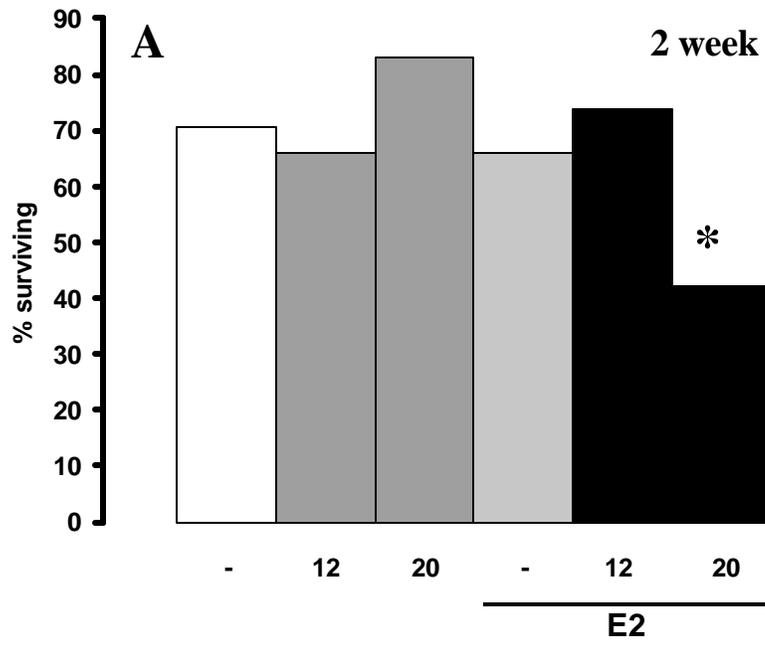
Figure 2. Reproductive success of males exposed to ANT & E2 for two weeks post-hatch. -, vehicle; 12, 12 $\mu\text{g/L}$ ANT; 20, 20 $\mu\text{g/L}$ ANT; E2, 1 nM estradiol. * indicates means that are significantly different from the vehicle ($p \leq 0.05$).

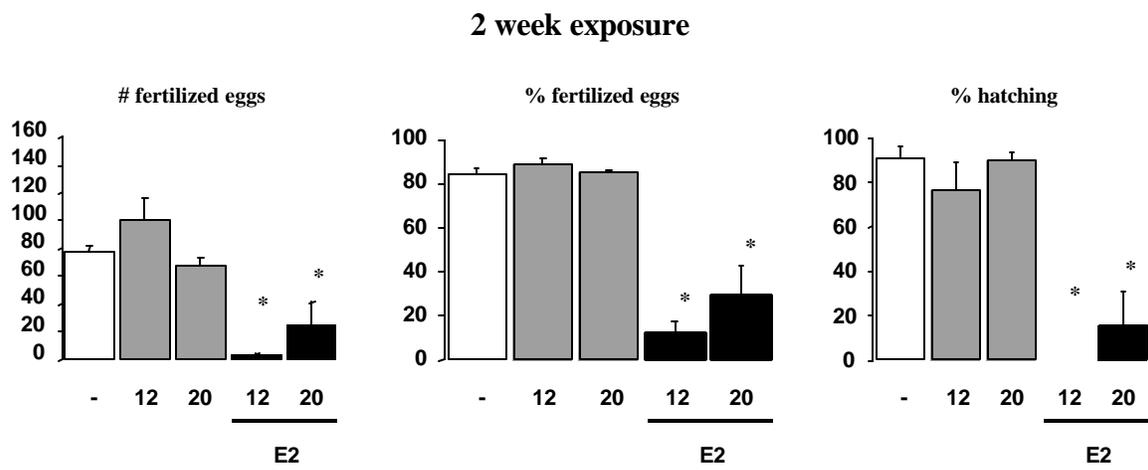
Figure 3. Number of fertilized eggs in normal (M) and feminized males (FM) treated with E2 or ANT + E2 for two weeks post-hatch. Numbers in parentheses are numbers of individuals. C, control (vehicle). * indicates means that are significantly different ($p \leq 0.05$) between males and feminized males within the same treatment (see text for details).

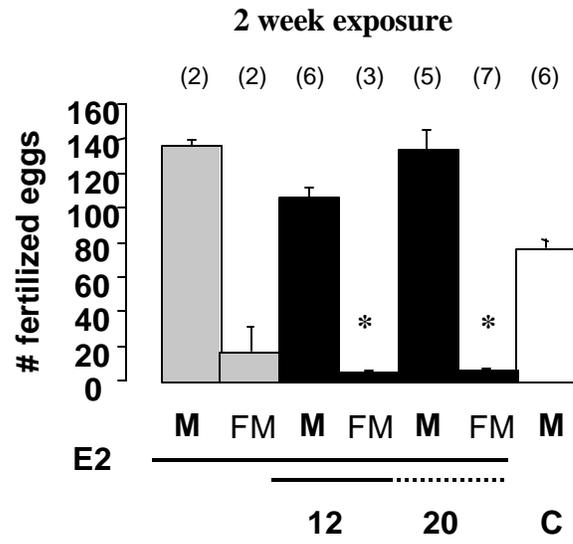
Figure 4. Number of eggs fertilized in pairs with both partners exposed for 8 weeks post-hatch, pairs with only the female exposed, and pairs with only the male exposed. Symbols as described for figure 1. The arrow indicates a tendency for 20 $\mu\text{g/L}$ ANT to reduce the number of fertilized eggs produced by treated females relative to control females.

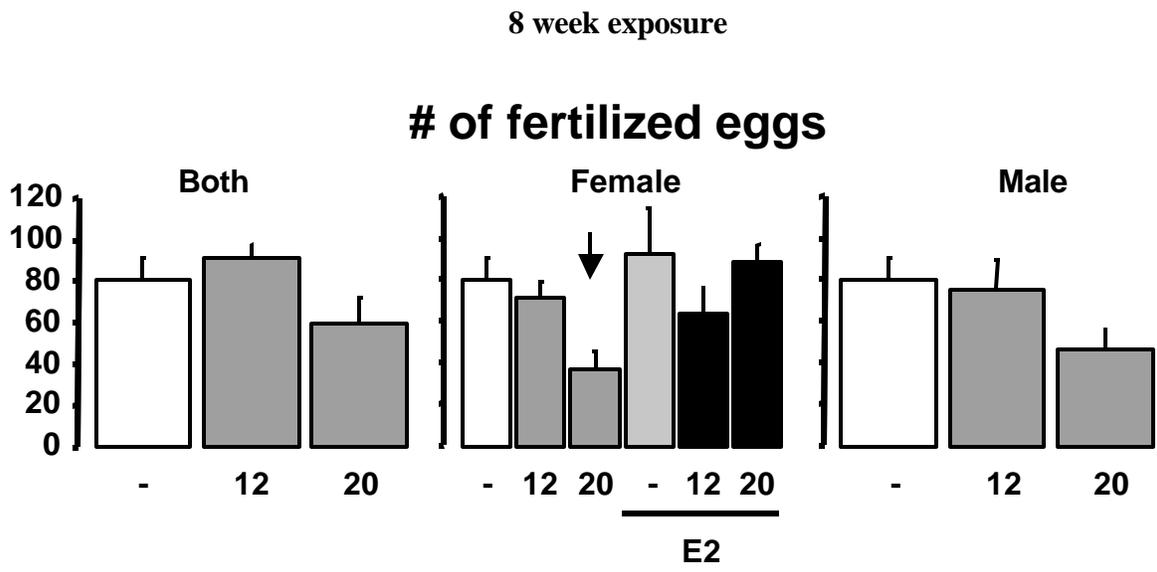
Figure 5. Percentage of eggs fertilized in pairs with partners exposed for 8 weeks post-hatch. Symbols as described for figure 1. * indicates means that are significantly different ($p \leq 0.05$) from the control. Treatment of both partners with 20 $\mu\text{g/L}$ ANT significantly reduced fertility.

Figure 6. Reproductive success of F₁ fish. Panels A and B: Effects of F₀ exposure on F₁ fertilization and hatching. F₀ fish were exposed to vehicle or 20 µg/L ANT. Panels C and D: Effects of maternal F₀ exposure on F₁ fertilization and hatching. F₀ females were exposed to vehicle, 12 and 20 µg/L ANT and 12 and 20 µg ANT + E2. * indicates means that are significantly different from vehicle (-)(p ≤ 0.05).



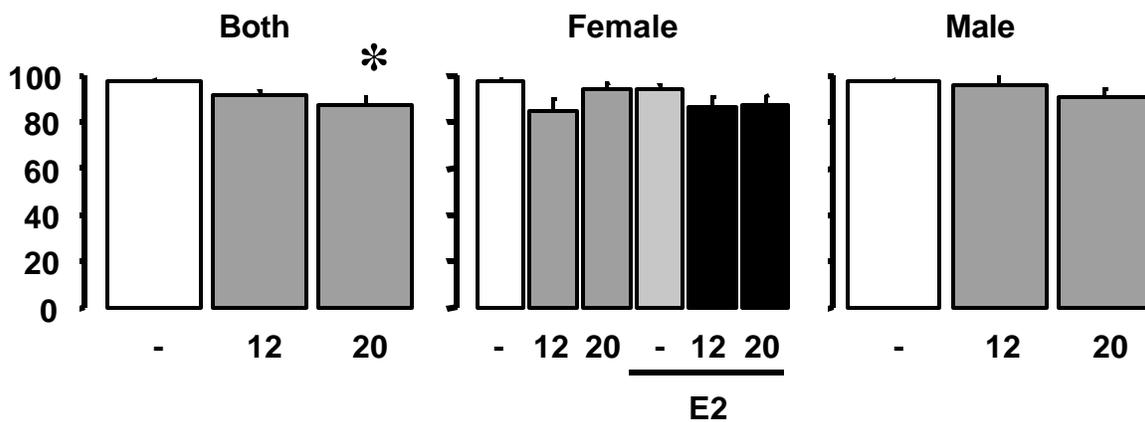


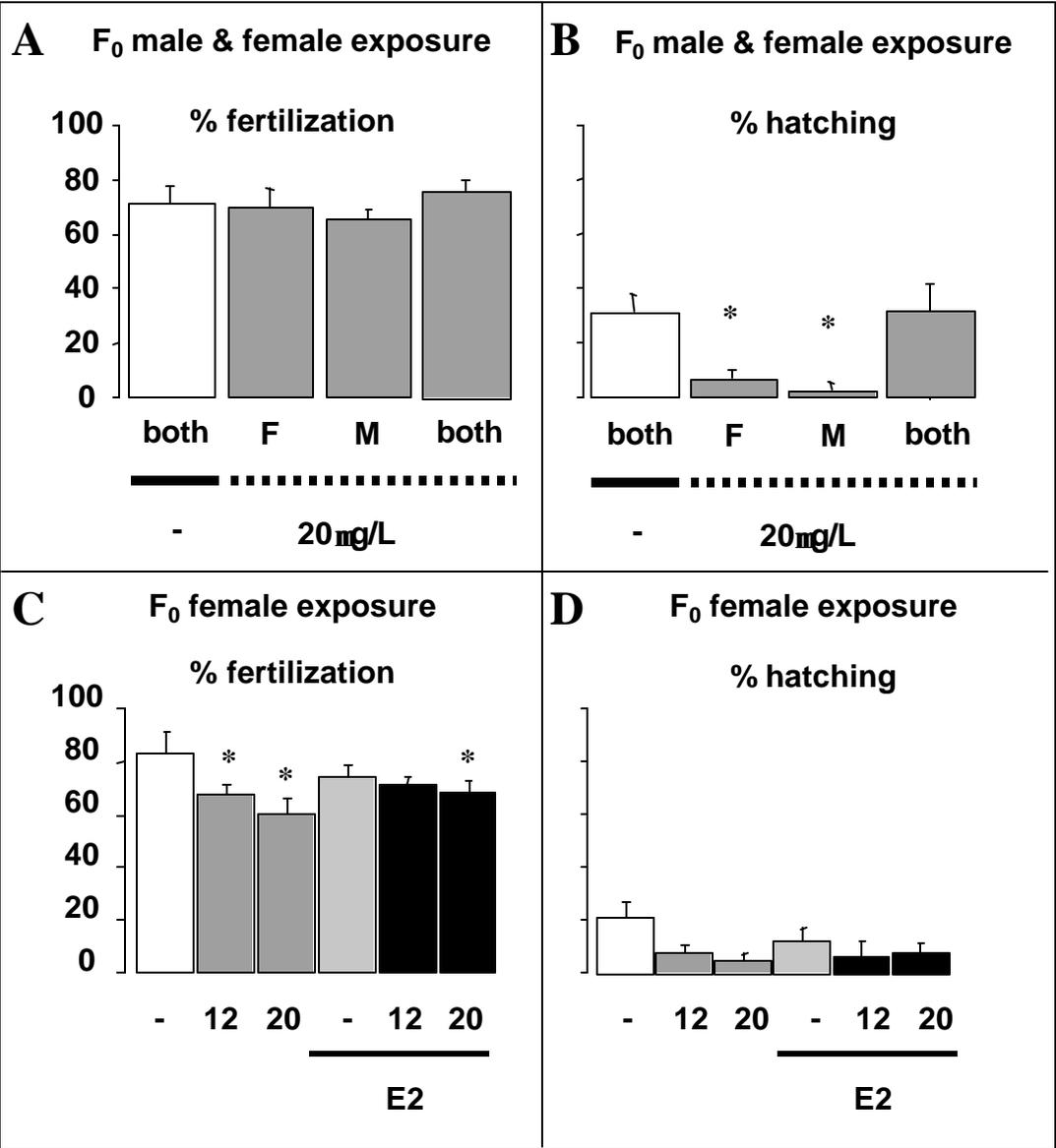




8 week exposure

% fertilization





Long-term pyrene exposure of grass shrimp, *Palaemonetes pugio*, affects molting and reproduction of exposed males, and offspring of exposed females.

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Effects of chronic pyrene exposure to grass shrimp

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lipovitellin
cytochrome P450

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Abstract

The objective of this study was to investigate the impact of long-term pyrene exposure on molting and reproduction in the model estuarine invertebrate, the grass shrimp (*Palaemonetes pugio*). Grass shrimp were exposed to measured concentrations of 5.1, 15.0 and 63.4 ppb pyrene for six weeks, during which time molting and survivorship were determined. At the end of the exposure, some shrimp were immediately sacrificed for biomarker (CYP 1A and lipovitellin) analyses. The remaining shrimp were used to analyze fecundity and larval survivorship during an additional six weeks following termination of pyrene exposure. ECOD activity was measured as a marker of CYP 1A-like activity. Lipovitellin (Vt) levels were measured using dot-blot and an anti-Vt monoclonal antibody. Male shrimp at the highest pyrene dose (63 ppb) experienced a significant delay in molting and in time until reproduction, and showed elevated ECOD activity immediately after the 6 week exposure period. In contrast, 63 ppb pyrene did not affect these parameters in female shrimp. Females produced the same number of eggs per body weight, with high egg viability (98-100%) at all exposure levels, but with decreased survival for the offspring of the 63 ppb pyrene-exposed females. In addition, Vt levels were elevated only in females at 63 ppb pyrene after the 6 week exposure. We hypothesize that the elevated lipovitellin binds pyrene and keeps it biologically unavailable to adult females, resulting in maternal transfer of pyrene to the embryos. This would account for the lack of effect of pyrene exposure on ECOD activity, molting and reproduction in the adult females, and for reduced survival of their offspring.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) have been found as anthropogenic contaminants in the aquatic environment. In some areas, sediment concentrations up to 1.2 mg/g have been found, including Bayou Trepanier, LA near New Orleans, and in the Elizabeth River, VA near Portsmouth (1)[Alden, 1987 #2663].

Biomarker induction, especially cytochrome P450 1A (CYP 1A) induction, has been well documented after exposure of fish to PAHs (2). The induction of CYP1A by PAHs involves binding to the aryl-hydrocarbon receptor (AhR) which is translocated to the nucleus by the accessory protein, ARNT. The complex binds to xenobiotic response element (XRE) in the promoter/enhancer region of the CYP 1A gene resulting in gene transcription and ultimately CYP 1A protein induction (3). The ligand which best fits into the AhR is a 10Å X 3Å planar ring (4), such as those rings found in the PAHs and polychlorinated biphenyls (PCBs).

PAHs are able to induce CYP1A-like protein activity in crustaceans as well (5-12). However, the specificity of this inducibility is slightly different from that in vertebrates. For example, 3-methylcholanthrene (3MC) does not induce EROD activity or benzo[a]pyrene hydroxylase in lobster (6), while in vertebrates (reptiles, birds, fish and mammals) 3MC induces a wide variety of P450s, specifically CYP1A (13-18).

In addition to interacting with AhR, PAHs can interact with the vertebrate steroid hormone systems by acting as anti-estrogens in reporter gene assays and MCF-7 breast cancer cell proliferation assays, and as anti-androgens in whole animals (19-21). PAHs are also able to interact with the invertebrate ecdysteroid hormone system (22). Benzo[a]pyrene, pyrene, chrysene and benzo[b]fluoranthene enhance ecdysone-dependent reporter gene expression and cell differentiation, most likely by interaction with cell signaling pathways, such as the MAP kinases.

Several studies have been done on whole animals to investigate physiological responses to PAH exposure in crustaceans (23; 24)[Flowers, 1997 #1733]. These studies have shown that molting patterns are altered in crabs exposed to crude oil (24). Tanner crabs exposed to 0.56 mL crude oil/L seawater autotomized several limbs, which also stimulates ecdysis (molt). At 0.32 mL/L, molting success was increased, and crabs closest to molt were resistant to acute toxicity. Crabs exposed to PAHs during molt were less able to metabolize PAHs, presumably because PAHs are competitive substrates for some of the P450s which also metabolize 20-OH ecdysone to its inactive form (23; 25; 26). It could be argued that PAHs in molting crabs would compete with and interfere with normal molting due to interaction with the metabolism of ecdysone. In a study using Elizabeth River sediments, a mixture of PAHs was used, including pyrene levels up to 2 µg/g sediment and over 160 µg/L [Alden, 1987 #2663]. At these levels, there was a slight increase in mortality from 5% in controls to 12% in exposed shrimp after 96 hours.

In blue crab, PAHs are taken up primarily into the hepatopancreas where they are metabolized and eliminated (9; 27). During PAH metabolism, reactive intermediates are often produced, which lead to toxicity. Organic contaminants (dinitrochlorobenzene, hexachlorobiphenyl, and organotins) are not only distributed to the hepatopancreas for metabolism, but are also bound by lipovitellin (the protein that serves the nutritional needs of the embryo before feeding begins), and are found in the ovaries and oocytes (28). In fish, Vt can bind both dioxin and benzo[a]pyrene (29). This may be one mechanism by which organic contaminants can become biologically unavailable for metabolism to reactive intermediates, thereby mitigating toxicity to the adult animal, but promoting toxicity in their offspring.

The objective of this study was to investigate the impact of long-term exposure of pyrene on molting and reproduction in the model estuarine invertebrate, the grass shrimp (*Palaemonetes pugio*). Since several PAHs, including pyrene, are able to interact with ecdysone-dependent gene transcription and cell proliferation *in vitro* (22), we hypothesized that pyrene will interfere with normal growth, reproduction and lipovitellin production of grass shrimp *in vivo*.

Materials and Methods

Grass shrimp, *Palaemonetes pugio*, were collected near Ocean Springs, MS and held at the IMS aquatic facility for 6 months prior to use in the exposures. Shrimp for all treatments were maintained in flow-through condition at 27°C at 15 ppt well-aerated seawater and fed *ad libitum* with brine shrimp once daily and commercial flake food once daily. Seawater was filtered (10 µm) natural seawater transported from the USEPA Environmental Research Laboratory in Gulf Breeze, FL, and adjusted to 15 ppt salinity with non-chlorinated well water. Female shrimp were separated when identified (gravid), and held separately until use.

The six week exposure of shrimp to pyrene was done using the methods of Walker et al. (30). Twelve test aquaria with duplicates of three exposure levels of pyrene (10, 25 and 100 ppb) plus seawater control and solvent control (triethylene glycol, TEG) were housed within a central water bath to maintain tank temperature at $27 \pm 1^\circ\text{C}$. TEG concentrations in solvent controls and all treatments were kept below 0.1 mL/L seawater. Prior to the start of the exposures, the flow through system was allowed to operate at the selected pyrene concentrations for approximately one week, and measured daily by HPLC (Beckman Gold System with 4.6 mm X 25 cm column eluted with 90% acetonitrile at 0.8 mL/min) with fluorometric detector (Jasco FP-920 fluorescence detector with excitation at 235 nm and emission at 390 nm) to assess pyrene concentrations in the aquaria. Measured pyrene concentrations (mean \pm SEM) were 5.1 ± 0.5 , 15.0 ± 0.7 , and 63.4 ± 2.5 ppb. Fifty shrimp, 25 in each of two replicate aquaria per treatment, were exposed per exposure level, each shrimp housed in individual mesh netting cages within the aquaria. This compartmentalization of test organisms precluded cannibalism, isolated males from females to avoid premature mating, and enabled us to enumerate individual molt data. A

16 h light:8 h dark photoperiod was used. Shrimp were checked daily for molting. Water quality (pH, salinity, temperature, dissolved oxygen) and pyrene levels were checked twice weekly.

After the 42 day exposure, a reproductive phase was initiated for an additional 6 weeks (42 days) by setting up controlled matings of treatment and control shrimp. This phase of the study was carried out in clean seawater using the following scenario: Exposed males X exposed females; TEG males X exposed females; exposed males X TEG females; and seawater control males X seawater control females. Because of mortalities in the 63 ppb exposure group, between 4 and 10 pairs were used in the mating studies (Table 1). Pairs were checked daily for egg production, and sacrificed after the female was gravid for a minimum of 2 days. The thorax, including hepatopancreas and gonads, was frozen at -80°C in 200 μL buffer (100 mM K_2HPO_4 , pH 7.4, 1 mM DTT, 1 mM EDTA, 20% glycerol and aprotinin at 0.67 TIU/mL). Eggs were collected, counted, and viability determined. A subset of 20 viable eggs/female were incubated individually in 4 mL sterile seawater/plate in 24-well polystyrene culture plates at $27 \pm 1^{\circ}\text{C}$ at 60 rpm in a shaker incubator. Percent embryo survival was determined by successful hatch by day 10 post-isolation. Animals not used in the mating studies were sacrificed immediately after the six week exposure period, and hepatopancreas and abdomens were collected as described above.

As a measure of CYP1A-like protein induction, ethoxycoumarin o-deethylase (ECOD) activity was measured in crude hepatopancreas extract. Tissues from the three collection times were used: immediately after the exposure phase, as soon as eggs were produced, and if no eggs were produced, immediately at the end of the reproductive phase. Hepatopancreas/thorax was homogenized with a Teflon homogenizer in storage buffer, and centrifuged at 14,000 rpm at 4°C in a microcentrifuge for 15 minutes. The supernatant was used in the ECOD assay. Protein was measured via the Bradford method (31). A 96-well plate assay was developed for the ECOD assay using the Spectramax Gemini plate reader set at 380 nm excitation and 448 nm emission. Preliminary studies showed that metabolism was linear for at least 30 minutes at 29°C . A standard curve of hydroxycoumarin from 0 to 500 pM was used in incubation buffer (50 mM Tris, pH 7.6, 2 mM 7-ethoxycoumarin). One hundred and forty μL of incubation buffer and ten μL of supernatant proteins were incubated for 5 minutes at 29°C . Three μL of NADPH (5 mM) was added to each well and fluorescence measured immediately for 30 minutes at 45 s intervals. V_{max} values were calculated and relative fluorescence units were converted to pM coumarin. Final numbers are reported as pM coumarin/min/mg protein. Each sample was measured in triplicate.

Dot-blot for lipovitellin were done using the crude hepatopancreas/thorax homogenates which also contained gonads. The PVDF (Millipore) membrane was wetted in methanol, rinsed in water for 5 minutes, and used in the BioRad dot-blot apparatus. Two rinses of 100 μL PBS were done under mild vacuum, and samples were applied to each well afterwards. A standard curve using partially purified lipovitellin from *P. pugio* eggs covered a range from 10 μg to 160 μg of protein. Ten μL of homogenates in triplicate

were applied to each well of the BioRad dot-blot after heating in 2.5 μ L sample buffer (0.8 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptoethanol and 0.05% w/v bromophenol blue) at 95 °C for 5 minutes. Fifty additional μ L of PBS was added to each well, and samples were allowed to gravity filter through the unit for 2 hours at room temperature. The remaining methods were done as described elsewhere (32). Briefly, the membrane was blocked overnight in PBS plus 3% BSA at 4 °C, rinsed 4 times with PBST (PBS plus 0.05% Tween 20), incubated for two hours at room temperature with a 1:3 dilution of S-15-2 monoclonal anti-lipovitellin antibody (32), rinsed 4 more times with PBST, and incubated one hour at room temperature with 1:50,000 dilution of goat-anti-mouse IgG coupled to HRP secondary antibody. Dots were visualized using the Enhanced Chemiluminescence kit (ECL) from Amersham, and multiple exposures of X-ray film were taken. The films developed using a Mini-Med film processor, and were analyzed using the BioRad Gel Documentation system. Western blot analysis was used to ascertain that increased density of the dot blots was indeed due to elevated levels of Vt. To this end, a subset of homogenates (5 μ g each) was subjected to separation on a 6% SDS-PAGE gel and transferred to PVDF membrane for 6 hours at 60V as described elsewhere (32), and blotted as described above.

Statistical analysis was done using SYSTAT version 8.0 for IBM. Since some ECOD and larval mortality data was non-normal, data was square-root transformed (ECOD) or arcsine square root transformed (% larval mortality) using the equation $x^1 = \sqrt{x+0.5}$ or $p^1 = \arcsin(\sqrt{p})$ (33). If outliers still remained (Durbin-Watson t-statistic), they were removed from the data set. ANOVA was run on data from all assays, and if $p < 0.05$, post-hoc Tukey test was used to determine which groups were different. If data had to be transformed to meet normality requirements, ANOVA was done on transformed data. Linear regression analyses of larval mortality was done on percentages, not transformed data.

Results

Molting was affected only in males at the highest exposure level (Table 2). Males exposed to 63 ppb pyrene had fewer molts during the 6 week exposure period than males at other exposure levels. There was no effect of pyrene on molting in females. At 63 ppb pyrene, higher mortality occurred in both males and females. (Table 2). An interesting observation was that deaths of the 63 ppb exposure occurred at the time of, or in close proximity to, molt.

Reproduction was delayed only in males, but not in females (Figure 1). In both groups where 63 ppb pyrene exposed males were mated with either control females or exposed females, reproduction was delayed until 21 days or 12 days, respectively. Control males were able to reproduce after 4-7 days. Females were able to reproduce at normal levels even after exposure to the highest dose (63 ppb) pyrene (Figure 1). At all exposure levels in all groups, when females produced eggs, the number of eggs/female body weight did not differ between any exposure groups, and percent viable eggs was not different, ranging from 98-100% viability (Table 3). However, there was a significant increase in

larval mortality in pyrene exposed females at 63 ppb, both when mated with control males and exposed males (Figure 2). In addition, linear regression analysis showed a significant increase in mortality with increasing pyrene concentrations for both mating groups ($p < 0.01$ for exposed males X exposed females, and $p < 0.002$ for TEG males X exposed females).

ECOD activity was highly variable within exposure groups (Table 4). At the end of the exposure phase, there was significant elevation of ECOD activity for males in the 63 ppb exposure when compared to all other exposures. There was no significant induction in females after the exposure phase, and there was no significant induction of ECOD at any time during the reproductive phase of the study in either males or females (Table 4).

Percent of protein as lipovitellin identified by dot blots was significantly elevated in females after exposure to 63 ppb pyrene for 6 weeks (Figure 3). Western blot analysis of a subset of 4 females confirmed these results. Lipovitellin levels were not different from control or solvent control levels at any time in males, and in all females that had just reproduced or that had not reproduced after 6 weeks. There was a high variability in Vt levels in both males and females. To further examine the somewhat unexpected presence of Vt in males, a subset of six males which showed Vt via dot blots were analyzed via Western blotting. In three of these we found a single band that corresponded to the molecular weight of the Vt standard, whereas the other three did not contain Vt. There appears to be material in homogenates from some of the males that cross-react with the anti-Vt antibodies. The nature of this cross-reactivity is not clear, but is under further investigation. It is clear however that consistently low levels of apparent Vt in males do not change with pyrene exposure.

Discussion and Conclusions

Grass shrimp, *P. pugio*, are a key link in the estuarine detritus food chain. However, many of our estuaries are impacted by anthropogenic contaminants which can adversely impact this ecologically important resource. Grass shrimp life history is well studied (34), and shrimp can be easily maintained in the laboratory, making them an ideal and important model estuarine invertebrates. Female grass shrimp molt, and must be mated within 7 hours of molting (34). Eggs are fertilized and extruded, and held on the pleopods of the female's abdomen until larvae are released 12-15 days later. The female molts again within a few days after spawning, and produces an additional brood. The breeding season varies with climate, but can be several months long in the southeastern US.

Djomo et al. measured pyrene water concentrations in an uptake/deposition study where 1.2 $\mu\text{g/g}$ pyrene in sediment led to measured water concentrations of 17.8 $\mu\text{g/L}$ [Djomo, 1996 #1394]. In the PAH contaminated Bayou Trepagnier, LA, sediment pyrene concentrations are up to 18 $\mu\text{g/g}$, which would lead to water pyrene concentrations in the range used in this study. In our study, grass shrimp were exposed to 5, 15 and 63 ppb. Mortality was high (56% male and 60% female) at the 63 ppb dose, with shrimp dying

just prior to or during the molt. In a study with blue crabs, animals were unable to metabolize and eliminate PAHs the closer they were to molt, resulting in a higher body-burden (23). Mothershead *et al.* (23) suggested that this was due to competition for substrates by P450s necessary to metabolize ecdysone for the molt. Extrapolating to this current study with a chronic exposure to shrimp, this increased PAH uptake could lead to increasingly higher body burdens of pyrene with each successive molt, eventually leading to increased mortality.

Exposure of pyrene to grass shrimp resulted in significant effects on male shrimp. At the highest pyrene dose (63 ppb), males had significantly decreased number of molts, elevated ECOD activity, and delay in reproduction. Male shrimp sacrificed immediately after they reproduced showed that ECOD activity had returned to control levels. It appears that once males were able to depurate enough pyrene, they were able to reproduce.

In contrast to males, molting, ECOD activity, and reproduction in female shrimp were not affected by any of these doses. However, there is a significant induction of lipovitellin levels at 63 ppb pyrene exposure in surviving females. It is possible that Vt binds up pyrene, resulting in reduced production of toxic reactive intermediates of PAH metabolism. This may explain the lack of adverse effects of pyrene on adult females. Our hypothesis further predicts that Vt may serve as a vehicle for transport of pyrene from the mother to the oocytes of embryos, which is supported by our observed pyrene-dependent decrease in larval survival, and by studies in blue crab and fish, which showed that lipovitellin is able to bind several classes of organic contaminants and transfer them to the oocytes (28; 29). There appears to be Vt cross-reactivity in homogenates of some of the males.

Pyrene is able to increase ecdysone-dependent gene expression *in vitro* (22). Since the active ecdysteroid, 20-hydroxyecdysone (20HE) regulates protein expression via ecdysteroid-dependent gene expression and controls both molting and vitellogenesis (35), this may be the mechanism by which Vt levels are elevated in pyrene-treated shrimp.

Conclusions: This study shows that there may be a link between molting, reproduction, lipovitellin levels, and P450 activity in shrimp. Males are significantly delayed in molting and reproduction after exposure to environmentally relevant levels of pyrene. In the field, where exposure is continuous, this would lead to impairment of reproductive function of male shrimp. In addition, our study suggests that embryo mortality would be higher in contaminated areas than in clean areas, due to Vt-mediated transport of PAHs from the adult to the embryos. Finally, our study shows that high pyrene concentrations can be lethal to adult shrimp. Taken together, these effects of PAHs may result in reduction of grass shrimp population, and hence decreased food availability for animals dependent on grass shrimp for prey, ultimately resulting in effects at the community and ecosystem level.

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Table 1: Numbers of pairs used in the controlled mating study.

treatments male ⇒ ↓ female	seawater	TEG	5 ppb	15 ppb	63 ppb
seawater	8				
TEG		6	8	9	5
5 ppb		10	6		
15 ppb		8		8	
63 ppb		5			4

Table 2: Number of molts and % survivorship of *P. pugio* during 42 days of pyrene exposure.

Treatment		n	% survivorship	molts per shrimp ^a
Control	male	106	96	4.29 ± 0.25
TEG	male	186	100	3.77 ± 0.21
5 ppb	male	86	100	3.70 ± 0.24
15 ppb	male	90	92	3.60 ± 0.22
63 ppb	male	66	56 *	2.86 ± 0.46 *
Control	female	104	100	4.16 ± 0.24
TEG	female	212	100	4.24 ± 0.20
5 ppb	female	99	100	3.96 ± 0.25
15 ppb	female	89	96	3.58 ± 0.21
63 ppb	female	93	60 *	4.00 ± 0.29

^aMolts per shrimp are calculated using only those shrimp surviving to day 43

* p<0.05

Table 3: Number of eggs/mg female weight and % viability.

treatments male ⇒ ↓ female	seawater	TEG	5 ppb	15 ppb	63 ppb
seawater	0.53± 0.12 99.1%				
TEG		0.70 ± 0.07 100%	0.66 ± 0.06 100%	0.59 ± 0.08 99.5%	0.63 ± 0.07 98.3%
5 ppb		0.62 ± 0.05 100%	0.63 ± 0.06 99.6%		
15 ppb		0.68 ± 0.07 99.9%		0.71 ± 0.11 99.5%	
63 ppb		0.71 ± 0.09 100%			0.74 ± 0.14 100%

Table 4: Ethoxycoumarin o-deethylase (ECOD) activity (pmol/min-mg protein \pm SEM) in shrimp hepatopancrei.

	n	males	n	females
Post-Exposure				
Control	5	3.39 \pm 1.86	5	27.51 \pm 12.66
TEG-Control	5	5.46 \pm 2.57	5	3.56 \pm 1.45
5 ppb	5	13.38 \pm 4.81	5	10.08 \pm 5.42
15 ppb	5	4.35 \pm 1.78	4	7.22 \pm 3.98
63 ppb	4	42.25 \pm 8.91 ^{***a}	5	13.16 \pm 4.27
Reproductive				
Control	6	8.00 \pm 3.13	6	13.63 \pm 3.58
TEG-Control	22	7.93 \pm 2.24	21	11.87 \pm 2.67
5 ppb	11	12.64 \pm 3.50	10	17.46 \pm 3.14
15 ppb	10	2.33 \pm 1.28	12	11.58 \pm 3.26
63 ppb	7	9.73 \pm 3.46	7	13.12 \pm 2.90
Non-Reproductive^b				
Control	4	3.84 \pm 2.89	4	3.43 \pm 0.61
TEG-Control	14	8.02 \pm 2.40	11	5.45 \pm 1.71
5 ppb	5	7.86 \pm 5.52	8	5.04 \pm 1.54
15 ppb	9	3.84 \pm 0.72	7	6.81 \pm 3.22
63 ppb	3	12.99 \pm 6.98	2	13.72 \pm 5.32

*** p<0.001 from Control, TEG and 15 ppb; ^ap<0.03 from 5 ppb

^banimals which did not reproduce during the reproductive phase

Figure 1: Number of days until production of the first brood. A) Exposed males were mated with solvent-control (TEG) females. B) Exposed females were mated with solvent control (TEG) males. C) Exposed males were mated with exposed females. Only 63 ppb pyrene exposed males had a significant delay in reproduction (A and C).

* $p < 0.05$; ** $p < 0.01$

Figure 2: Larval mortality from either exposed males mated with control females, control males mated with exposed females, or both males and females exposed. There was a significant increase in larval mortality only in offspring from exposed females at 63 ppb. * $p < 0.05$; *** $p < 0.001$

Figure 3: Vt levels in male and female shrimp immediately after the 6 week exposure period, three days after mating in clean seawater, or after 6 weeks in clean seawater if mating did not occur. Only females at 63 ppb pyrene immediately after the exposure had a significant increase in Vt levels. ** $p < 0.01$

