Environmentally-Mediated Endocrine Disruption in Estuarine Crustaceans: A 3-Taxon Multi-Generational Study of Sediment-Associated EDC Effects from the Genetic to Population Levels

Project Scope

Endocrine function in arthropods has been characterized principally in insects and malacostracan crustaceans (e.g., crabs, shrimp). However, the microscopic benthic crustaceans that live interstitially (i.e., as part of the meiofauna in sediment pore water) are the most abundant of the marine invertebrates, with harpacticoid copepods comprising the second most abundant arthropod taxon. Their diminutive biomass has made characterization of endocrine components difficult, so little is known about endocrine control of reproduction, molting, and growth in interstitial crustaceans.

The primary objective of this research was to develop a multi-tiered approach for screening known or suspected endocrine disrupting chemicals (EDCs) using two ecologically important estuarine crustaceans: grass shrimp (*Palaemonetes pugio*), amphipods (*Leptocheirus plumulosus*), and benthic copepods (*Amphiascus tenuiremis*). Tier I and Tier II screening assays that involved *in vitro*, *in vivo*, and life-cycle assessments were developed to evaluate reproductive and endocrine toxicity in estuarine crustaceans.

A coupled abiotic-contaminant transformation / reproductive-impact model was used to characterize toxicological risks associated with EDCs in estuarine crustaceans up to the population level. This predictive model assessed potential mechanisms by which environmental transformation of EDCs by UV photolysis affects their apparent toxicological properties in the environment. It also addressed how biochemical manifestations of toxicants are expressed and ultimately impact crustacean population fitness and maintenance (specifically related to reproductive success) at the genetic, individual, and population levels.

The hypothesis tested whether photoactivation products of certain contaminants, including two organochlorine insecticides (endosulfan and fipronil), an organophosphate insecticide (chlorpyrifos), a herbicide (atrazine), and a

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Key Findings

- Reproduction in the grass shrimp was decreased by exposure to the organochlorine (OC) insecticide endosulfan (ES) in a dose-dependent manner.
- The OC insecticide fipronil (FP) significantly decreased adult grass shrimp survival at 0.2 µg/L; however, it had no significant effects on female reproduction.
- In gravid female grass shrimp, exposure to the organophosphate (OP) insecticide chlorpyrifos resulted in significant increases in ecdysteroid concentrations, but had no significant effects on reproductive parameters.
- In a benthic copepod, trace FP concentrations halted female egg extrusion and altered male spermatogenesis.
- Exposure to the herbicide atrazine increased the incidence of reproductive failure in the copepod and reduced the number of offspring per successful female.
- Elevated yolk deposition occurred in embryos of female copepods cultured throughout their life cycle in sediments contaminated with the PAH chrysene; the biological significance of that finding is unclear.
- Of the five EDCs evaluated in estuarine crustacean models, the OC insecticides and the herbicide adversely affected reproduction; the OP insecticide affected survival and ecdysteroid levels, but not reproduction; and the steroidally-structured PAH, chrysene, increased yolk deposition to embryos without other adverse effects.

Project Period: April 1999 to March 2002

steroidally-structured PAH (chrysene), disrupt normal neuroendocrine/ hormonal pathways, inducing molecular and cellular effects with outcomes at the individual and population levels in crustaceans. Also tested was whether these effects are manifested in a fashion that allows development of robust ecological risk assessment models.

The common crustacean yolk protein vitellin (VTN) and the molting hormone 20-hydroxyecdysone (20HE) were explored as potential biochemical endpoints of endocrine—or at least reproductive—disruption in the crustacean models. Coupled with chronic toxicity tests, these biochemical endpoints represent potential biomarkers for EDC exposure.

Relevance to ORD's Multi-Year Research Plan

This project contributes to two important long-term goals of the ORD's MYP: (1) to support EPA's screening and testing program, and (2) to determine the extent of the impact of endocrine disruptors on humans, wildlife, and the environment.

Researchers developed rapid and ecologically meaningful test systems using Tier I Screening and Tier II Life-cycle assays. Antibody-based screening methods and microscopy techniques were developed to assess EDC effects in crustaceans. Full-life cycle microplate assays were developed to assess reproductive and endocrine toxicity of pesticides and/or PAHs to the grass shrimp *Palaemonetes pugio* and the copepod *Amphiascus tenuiremis*.

Results of the Tier I and Tier II screening assays were used to characterize toxicological risks associated with EDCs in estuarine crustaceans at the genetic, individual, and population levels. The two OC insecticides and the herbicide adversely affected reproductive success via several different modes of action. The OP insecticide affected ecdysteroid levels without impacting measured reproductive parameters. The biological significance of the elevated yolk deposition to embryos of female copepods cultured in the PAH chrysene throughout their lives is unclear.

Project Results and Implications

Screening Assays Developed for Assessing Endocrine Toxicity in Crustaceans

Fluorescence-based enzyme-linked immunosorbent assays (ELISAs) and microscopy-based techniques were successfully developed and validated for VTN and 20HE quantification and *in vivo* yolk localization.

Ecdysteroid ELISA. A highly sensitive, fluorescent ELISA was developed for non-radiometric quantification of 20HE—the biologically active metabolite of the crustacean molting hormone ecdysone. This assay utilizes polyclonal antibodies raised specifically to 20HE, and is capable of detecting 20HE in a single adult copepod (*A. tenuiremis*), individual copepod egg sacks, and amphipod (*L. plumulosus*) embryos. Furthermore, the assay is capable of differentiating 20HE concentrations between early and late embryo developmental stages for both crustacean species. Based on the assay's sensitivity and cross-reactivity, we used 20HE as an endpoint for detecting endocrine-mediated reproductive effects in our model crustaceans.

Vitellin/Vitellogenin ELISA. Vitellogenin (VTG) has been widely used as a biomarker of estrogenic exposure in fish, leading to the development of standardized assays for VTG quantification. However, standardized quantitative assays for invertebrate, particularly crustacean, VTG or VTN are lacking. For VTN ELISA development, polyclonal antibodies were raised against purified amphipod (*L. plumulosus*) VTN and tested for cross-reactivity with copepod and grass shrimp VTN. These polyclonal antibodies are specific for purified *L. plumulosus* VTN and were strongly cross-reactive with partially purified copepod (*A. tenuiremis*) and grass shrimp (*P. pugio*) VTN. Thus, these anti-VTN antibodies were used to develop and validate an indirect, competitive ELISA for the quantification of VTN in all three crustaceans. The ELISA

significantly discriminated positive (gravid female) and negative (male) grass shrimp, amphipod, and copepod samples.

Semi-quantification of Embryo Yolk. Confocal laser-scanning microscopy (CLSM) represents a powerful, but largely unexplored, ecotoxicological tool for rapidly assessing *in vivo* effects of toxicants on marine invertebrate embryo quality and development. A new semi-quantitative CLSM approach was developed for assessing relative yolk quantity in marine invertebrate (harpacticoid copepod) embryos produced by parents reared from hatching to adult in waterborne or sediment-associated contaminants.

Grass Shrimp (*P. pugio*) Exposures

UV-A Irradiation and Endosulfan (ES). In this study, female grass shrimp were exposed to sub-lethal concentrations of ES (0.2 µg/L or 0.4 µg/L) under both white fluorescent (non-UV) light and UV-A (315-400 nm) light conditions for 50 days in laboratory bioassays. Endpoints associated with female endocrine responses (VTG [VTG is taken up by developing embryos as VTN], ecdysteroid [molting hormone, which also plays key role in reproduction and development], and cholesterol levels), reproduction (percent gravid, clutch size), and embryo development and survival (days to hatch, hatching success, and hatching survival) were assessed. UV-exposure alone caused a significant (> four-fold) increase in total *P. pugio* female egg production over the course of 50 days compared to non-UV lighting exposure. Higher VTG concentrations and lower ecdysteroid and cholesterol concentrations, which correlate with increased female egg production, occurred with exposure to ES plus UV light compared with exposure to ES and white fluorescent light (Figure 1). Only cholesterol concentrations exhibited a dose-dependent change when exposed to ES (positive dose-dependent change under UV-A light; negative dose-dependent change under white light). Cholesterol is the backbone molecule for ecdysone synthesis and is important in yolk lipoprotein production in crustaceans. The fitness implications of these ES:cholesterol and UV relationships are uncertain.

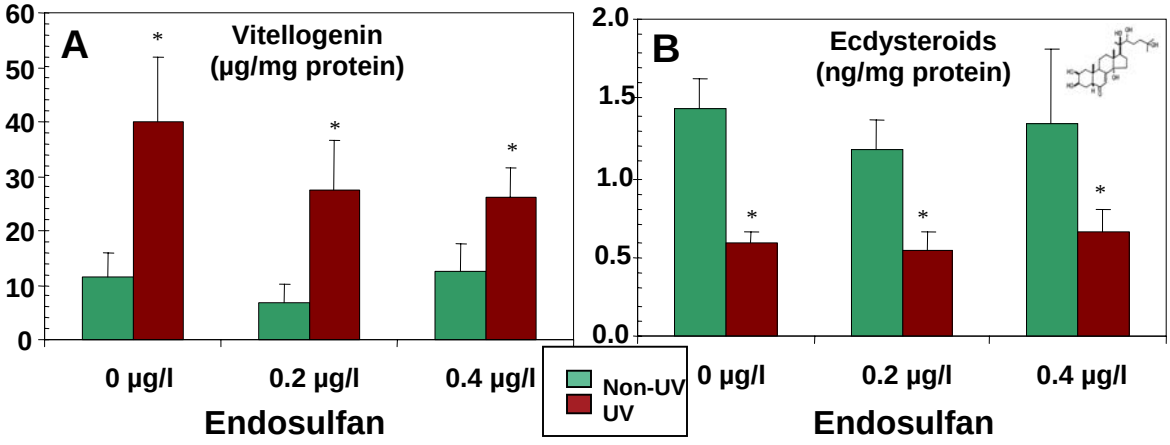

Figure 1. Vitellogenin (A) and ecdysteroid (B) titers in gravid female grass shrimp (*P. pugio*) after exposure to chronic, low doses of endosulfan under ultraviolet (UV) and white fluorescent (Non-UV) light. * Significant difference from non-UV exposure ($p<0.01$).

Reproduction (i.e., the rate at which females became gravid and the numbers of females which became gravid) was decreased by exposure ES in a dose-dependent manner. Numbers of gravid females were reduced 18 percent in non-UV enhanced light and by approximately 40 percent in UV-enhanced exposures. These results indicate that ES countered UV-mediated stimulatory reproductive effects and either: (1) induced shunting of female energy away from reproduction to ES metabolism, (2) interacted with UV-triggered neurotransmitter signaling, or (3) caused poor embryo survival due to ES accumulation in yolk lipids. Clutch size was not affected.

Among treatment groups, embryos from females exposed to UV had significantly lower ecdysteroid concentrations and shorter hatching times, but there were no differences in embryo VTG concentrations, hatching success, or hatching survival.

These results indicate that sub-lethal ES concentrations may have a negative effect on grass shrimp reproductive biology. Specifically, results implicate a population reduction due to a decrease in the overall number of females becoming gravid in a population over time, although not a reduction in clutch size per individual.

Chlorpyrifos and fipronil (FP). In this study, female grass shrimp were exposed to chlorpyrifos (0.1 µg/L or 0.2 µg/L) or FP (0.1 µg/L or 0.2 µg/L) under white fluorescent (non-UV) light conditions for 45 days in laboratory bioassays. To elucidate possible endocrine responses to chronic chlorpyrifos or FP exposure, the study measured the occurrence of gravid females, and body weight, body length, cholesterol, ecdysteroids, and VTG in gravid females. Chlorpyrifos treatments were not significantly toxic, but FP significantly decreased adult survival at 0.2 µg/L (19.6 percent decrease relative to controls). Gravid female body weight and length were not significantly different across all chlorpyrifos and FP treatments. In addition, chlorpyrifos or FP at either dose did not significantly affect total gravid female production based on total survival for more than 45 days. Chlorpyrifos or FP did not significantly increase or decrease gravid female VTG concentrations. Neither chlorpyrifos nor FP affected gravid female cholesterol concentrations. However, there were significant increases in ecdysteroid concentrations after exposure to 0.1 and 0.2 µg/L nominal chlorpyrifos. Enhanced ecdysteroid concentrations were not observed under FP exposure. Ecdysone's role in regulation of molting, growth and reproduction is dynamic over the reproductive cycles of most crustaceans studied to date. Chlorpyrifos-induced disruption of ecdysone balance could lead to changes in reproductive fitness of exposed shrimp depending on timing and strength of disruption. However, in this study, no biologically significant chlorpyrifos or FP effects were observed for female grass shrimp egg production and related reproductive parameters (VTG, cholesterol titers), nor for body weight and length.

Amphipod (*L. plumulosus*) Lipovitellin Antibody Production

This project developed the use of *L. plumulosus* as an excellent source of lipovitellin antigen that proved (1) easy to collect in high quantity, (2) easy to extract and purify in high quality, and (3) ideal for producing antibodies that are cross-reactive for vitellin from at least three species of commonly used crustacean bioassay models (*P. pugio*, *A. tenuiremis*, and *L. plumulosus*). The *L. plumulosus* efforts are published in Volz et al. 2002 and 2003. *L. plumulosus* proved to be a less than ideal bioassay model for our endocrine disruption studies because successful lifecycle culture requires sediments, it has a restricted sediment quality range for success, and its full lifecycle (egg to egg) is > 40 days.

Copepod (*A. tenuiremis*) Exposures

Fipronil (FP). In this study, the acute, developmental, and reproductive toxicity of FP to the estuarine harpacticoid copepod *A. tenuiremis* was evaluated. Acute toxicity tests revealed FP to be highly toxic to *A. tenuiremis* (adult 96-hour LC_{50} = 6.8 µg/L) and more toxic to male copepods (96-hour LC_{50} = 3.5 µg/L) than non-gravid female copepods (96 hours LC_{50} = 13.0 µg/L).

Single juvenile (Stage-I copepodite) individuals of *A. tenuiremis* were reared to adulthood, and developmental and reproductive effects (after paired virginal matings) of environmentally relevant aqueous FP concentrations (0.16, 0.22, and 0.42 µg/L) were concurrently assessed. Throughout the entire life cycle, copepod survival in all treatments was greater than 90 percent. However, FP exposures at 0.22 µg/L and higher significantly delayed male and female development from Stage-I copepodite to adult by approximately two days (normally takes about 12 days). More importantly, FP significantly halted female egg extrusion by 71 percent in the 0.22 µg/L FP treatment and nearly eliminated reproduction (94 percent failure) in the 0.42 µg/L FP treatment. A three-generation Leslie matrix-based population growth model of FP reproductive and life-cycle impacts predicted a 62 percent decline in *A. tenuiremis* population size relative to controls at only 0.16 µg/L. Interestingly, acute FP neurotoxicity seems unlikely, because male and female copepods appeared healthy and were reproductively active at all FP concentrations tested. In fact, multiple spermatophore sacs—an observation indicative of active copulation—were noted

in females in all treatments throughout the monitored nine-day mating period. FP-induced reproductive malfunctions, such as alterations in male spermatogenesis, likely account for low reproductive success at treatments greater than 0.2 µg/L.

Sex-specific reproductive and transgenerational effects of FP also were determined for the copepod, *A. tenuiremis*, using a life-cycle bioassay. More than 340 individual Stage-I juvenile copepodites were reared to adults in 12 days in either 200 µL of seawater solution (control) or 0.63 µg FP/L seawater solution. Individual virgin male/female pairs were cross-mated for all possible combinations within and across rearing treatments and were allowed to mate for 12 days in the control or 0.63 µg FP/L solution. Environmentally realistic FP concentrations (0.63 µg/L) showed no significant lethality to any mating combination, but had strong, sex-linked impacts on copepod reproduction. At this concentration, FP evoked 73 and 89 percent inhibition of reproduction when FP-reared males were mated with either control- or FP-reared females in FP solution, respectively. In contrast, when control-reared males were mated with FP-reared females in FP solution, there was no reduction in reproductive success. When FP-reared males were mated with either female group in FP-free solution, these mating pairs displayed a three-day delay in time to brood sac extrusion, but ultimately did reproduce. Sub-lethal FP concentrations had greater reproductive impacts on male *A. tenuiremis* than females.

In this study, trace (environmentally realistic) FP concentrations significantly affected development and reproduction of *A. tenuiremis*. FP is rapidly gaining use in coastal areas and beyond; thus, estuarine crustacean populations exposed to near-detectable or undetectable levels of FP may potentially suffer severe reductions in population growth.

Atrazine (ATZ). The objectives of this study were to assess developmental and reproductive effects of ATZ over multiple generation exposures of the copepod *A. tenuiremis*. Copepods were exposed to sub-lethal ATZ concentrations (2.5, 25, and 250 µg/L) using a life-cycle bioassay. Individual Stage-I copepodites (n = 60/treatment) were reared through two generations (F_0 and F_1) to sexual maturity and were individually mated in 200 µL ATZ solution. Copepod survival across all treatments and generations was greater than 95 percent. ATZ did not significantly affect development times to reproductive maturity, time to female egg extrusion, or time to egg hatch. However, reproductive failures [defined as those mating pairs unable to produce viable offspring after seven days of mating plus females unable to extrude more than one brood over a 10-day mating period] increased across generations with increasing ATZ concentrations. Reproductive failures in the 0, 2.5, 25, and 250 µg/L ATZ treatments were 11, 11, 20, and 24 percent for the F_0, respectively, and 4, 9, 26, and 38 percent for the F_1 generation, respectively. Compared to controls, total larval production per female was reduced by approximately 22 percent in F_0 females exposed to 250 µg/L ATZ, and by approximately 23, 27, and 32 percent in F_1 females exposed to 2.5, 25, and 250 µg/L ATZ treatments, respectively. The combined effect of reproductive failure and reduced offspring production significantly reduced total population growth in the F_1 generation, even at ATZ concentrations lower than the draft ATZ ambient aquatic life water quality criterion for animals in saltwater of 17 µg/L.

Interestingly, a small proportion of the F_1 copepods exposed to ATZ (2, 2, and 6 percent for the 2.5, 25, and 250 µg/L treatments, respectively) showed distinct malformations of the abdomen or post-abdomen, specifically the urosome and/or caudal rami. A three percent incidence of these malformations was observed in the ATZ-reared F_2 generation in both the 2.5 and 25 µg/L treatments. None of these malformations have been observed in control copepods in similar bioassays in our laboratory. Individuals showing malformations were unable to swim, and the majority did not survive to sexual maturity; those that survived to adulthood were unable to produce viable offspring. These copepod malformations may lack biological/ecological significance at the population level since they were present at fairly low incidence in exposed individuals.

Chrysene. Chrysene has a chemical structure that is steroidal in nature, particularly when photo-oxidized by UV light to 6-hydroxychrysene. This photo-oxidation product exhibits anti-androgenic properties *in vitro* in mammalian models, and may have endocrine-active properties in marine invertebrates. In this study, we hypothesized that vitellogenesis may be affected in female copepods (*A. tenuiremis*), and that these effects could be detected in day-old embryos.

A fluorescent yolk-labeling method (CLSM) was developed to detect statistically significant differences in concentrations of yolk in copepod eggs/embryos from females exposed to UV and chrysene-

contaminated sediments. Yolk quantity in embryos from females cultured throughout their life cycles in clean sediments were statistically identical with or without UV exposure. UV exposure had no discernible effect on embryo yolk content. In contrast, yolk quantity in embryos of females cultured throughout their life cycle in chrysene-contaminated sediments were significantly higher in the non-UV exposed 2,500 ng-chrysene/g-sediment (65.7 percent higher) and UV-exposed 500 ng-chrysene/g-sediment (76.6 percent higher) treatments. Elevated yolk deposition may be indicative of maternal off-loading of chrysene body burdens which are strongly associated with lipids.

Chrysene exposure during maturation to female reproductive maturity significantly enhanced yolk deposition to eggs/embryos and was strongly enhanced by UV irradiation. Although the direct mechanism of chrysene-induced yolk deposition is unknown, chrysene may exhibit hormonal properties that mimic endogenous crustacean hormones such as ecdysteroids.

Summary. The research assessed the reproductive toxicity and EDC potential of five chemicals – endosulfan and fipronil (organochlorine [OC] insecticides), chlorpyrifos (organophosphate [OP] insecticide), chrysene (steroidally structured PAH), and atrazine (herbicide) – using one or more of three crustacean models developed in this project: *P. pugio* (grass shrimp), *L. plumulosus* (amphipod), and *A. tenuiremis* (benthic copepod). The crustacean models were used to evaluate each chemical for a suite of EDC-specific biomarkers including VTG and ecdysteroid levels, along with survival and reproductive parameters. The two OC insecticides and the herbicide adversely affected several genetic and individual-level parameters influencing individual- and likely population-level reproductive success. The OP insecticide affected ecdysteroid levels without impacting measured reproductive parameters. The biological significance of the elevated yolk deposition to embryos of female copepods cultured in the PAH throughout their lives is unclear. Thus, EDC chemicals can adversely affect populations of several groups of crustaceans, with the mode of action and individual- and likely population-level impacts depending on the type of chemical, exposure levels, and receptor. These studies demonstrated how biochemical manifestations of toxicants may ultimately impact crustaceans at the individual and population levels.

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For More Information

South Carolina Arnold School of Public Health Environmental Health Sciences Website:
http://www.sph.sc.edu/facultystaffpages/facstaffdetails.php?ID=65

NCER Project Abstract and Reports:
http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/447/report/0