

YEAR 3 ANNUAL REPORT

Period Covered by the Report: March 1, 2003 to February 29, 2004

Date of Report: April 1, 2004

EPA Agreement Number: R-82867601

Title: Pacific Estuarine Ecosystem Indicator Research (PEEIR) Consortium:

Biological Responses to Contaminants Component: Biomarkers of Exposure, Effect, and Reproductive Impairment

Principal Investigators: Gary N. Cherr¹, Susan L. Anderson¹

Co-Investigators: Michael S. Denison², Frederick J. Griffin¹, Roger Nisbet³, Mark J. Snyder¹, and Barry Wilson²

Institutions: UC Davis Bodega Marine Laboratory¹, UC Davis², UC Santa Barbara³

Research Category: EaGLE Program

Project Period: March 1, 2001 to February 28, 2005

RESEARCH OBJECTIVES:

The overall aim of the proposed research of this section is to develop a suite of molecular, biochemical, cellular, and tissue level indicators which provide rapid assessment and advanced warning of environmental stress in estuarine/coastal habitats. The particular emphasis of this section is assessment of reproductive parameters since: rapid and accurate techniques are not readily available, biomarkers associated with reproductive impairment can be early warning indicators of stress, and reproductive impairment can be directly linked to effects on populations through modeling efforts. The research proposed here is integral to the overall goals of PEEIR which are to establish indicators that environmental managers can use for: 1) developing an approach for synthesizing indicators into technically-defensible assessments of wetland health and integrity, 2) determining biotic integrity for fish and invertebrate populations within wetland communities, and 3) determining toxicant-induced stress and bioavailability for wetland biota. The **objective** in this proposal is to determine the efficacy of a suite of molecular, biochemical, cellular, and tissue level indicators to collectively predict ecosystem responses to contaminant stress. Biomarkers of reproductive impairment are important early warning indicators of ecosystem impacts, but they need complete characterization and validation in an ecosystem context as proposed in PEEIR.

PROGRESS SUMMARY:

Development of a Rapid Indicator of PAH and/or Phthalate Contamination

Previously we established the sea urchin embryo development bioassay could be used to identify possible PAH contamination under laboratory conditions using individual PAHs as well as complex mixtures (Pillai et al., 2002). We have continued the development of this indicator by showing that there are very few classes of compounds that induce the PAH-like response of exogastrulation. These include phthalates (dibutyl phthalate), some of the PAHs, and the positive control lithium chloride. Selected metals, organochlorine pesticides, pyrethroids and PCBs do not induce this specific developmental abnormality (Table 1).

Table 1. List of contaminants tested for their ability to induce exogastrulation in sea urchin embryos.

Exogastrulae	No exogastrulae
Lithium	Selenate/Selenite
PAHs	Lead
Phthlates	Arsenic
	Dichromate
	Cadmium
	Copper
	Nitrate
	Methoxychlor
	Permethrin
	Cyfluthrin
	Esfenvalerate
	Cypermethrin
	Planar and co-planar PCBs

The usefulness of the assay with field samples was demonstrated by testing elutriates of sediments collected at Carpenteria Salt Marsh (stations A-D), where exogastrulation was observed in 2002. In 2003, sediments from stations A-C again showed exogastrulation induction (Fig. 1). Chemical analyses are being conducted but initial screening in 2002 showed the presence of phthalates.

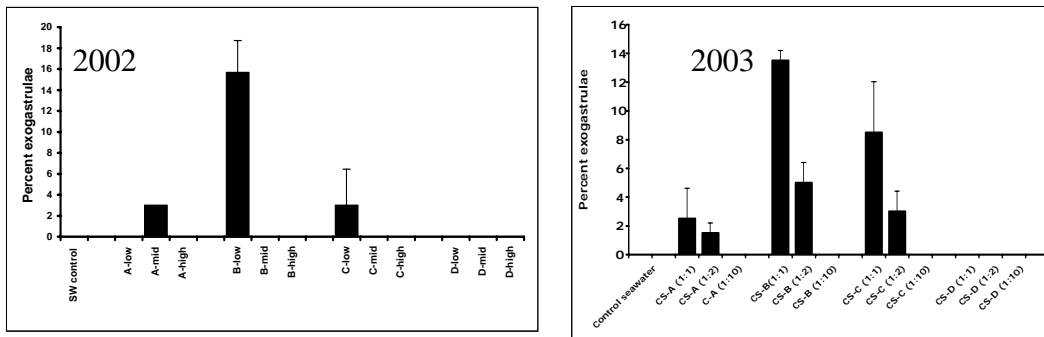


Figure 1. Percentage of embryos showing exogastrulation when exposed to sediment elutriates from Carpenteria Salt Marsh. Both 2002 and 2003 sediments from the same stations showed activity.

Sediments were obtained through the EPA Narrangansett Lab from the Elizabeth River in Virginia, which is contaminated with creosote. Elutriates from this site also showed exogastrulation and served as a positive control for the Carpinteria sediments (Fig. 2).

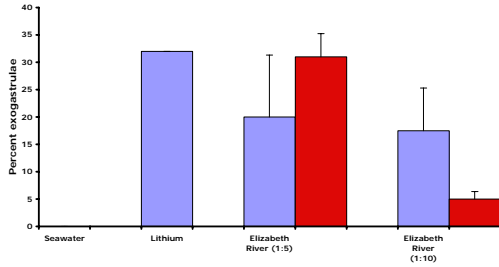


Figure 2. Exogastrulation in elutriates from Elizabeth River sediments. Embryos were exposed before (blue bar) and after (red bar) hatching, and showed as much of a response as the positive control lithium.

Finally, embryos (in semi-permeable enclosures) were outplanted at a PAH-rich site in a local marine to demonstrate the assay can be used *in situ* (Fig. 3).

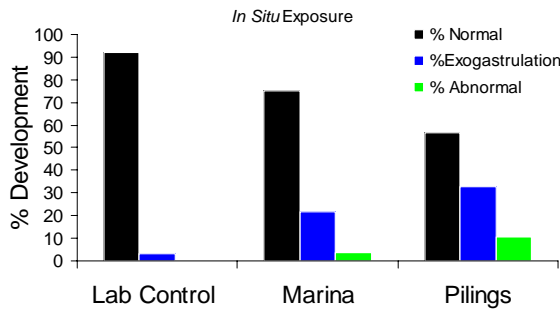


Figure 3. Embryos outplanted at sites high in PAHs (Marina and Pilings) show increased frequency of exogastrulation. Hatched embryos were placed in dialysis bags (semi-permeable membranes) for 48 hrs., retrieved, and analyzed in the laboratory.

Indicators of Exposure to Organic Contaminants: P4501A Enzyme Expression

Biochemical biomarkers of exposure have been suggested as early warning indicators of marsh degradation once they are placed in the proper context and other data sets are available regarding the overall condition of the organisms. Our studies have included the analyses of P450 enzymes in tissues, as these tend to be responsive to exposure to many organic contaminants and have long been used as a biomarker in both laboratory and field studies. We have focused on CYP1A, which is involved in detoxication of many hydrocarbons and related chemicals. We have raised an antibody to a highly conserved peptide domain of CYP1A and have found that it cross-reacts with both vertebrate and invertebrate tissues. Liver samples from mudsuckers collected at all sites have now been analyzed utilizing SDS-PAGE followed by Western blotting with the antibody to P4501A. The assay has been refined since 2002 to be highly specific (Fig. 4). Analyses with crab hepatopancreas is continuing using the same antibody.

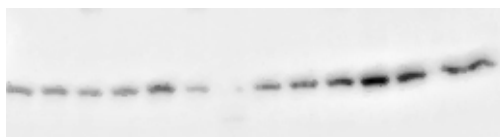
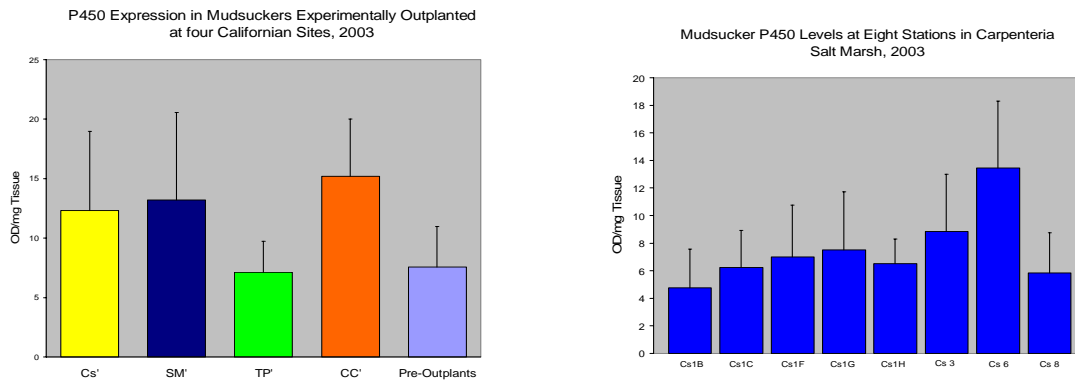


Figure 4. Western blots of individual mudsucker livers from different stations showing specific labeling of the P4501A protein and differing expression levels.

Initial analyses indicate that as a group, outplanted (12 weeks) Carpinteria Salt Marsh fish show higher P450 levels than the other sites (Fig. 5A). In particular, station C6 shows the highest levels (Fig. 5B) both in collected fish and in outplants. However, a complete analysis by individual fish, including sex, sexual maturity, histology, etc., is underway to tease out this biomarker response. It should be noted that we would expect Stege Marsh fish, which are exposed to the highest level of contamination throughout their life histories, to have decreased P450 expression as part of a compensatory response as they adapt to a high organic contaminant load. This has been observed in other fish in several studies. Complete analysis of data is underway and involves a significant effort to mine these raw data.



Figures 5A,B.

- A. Mudsuckers outplanted at Carpinteria show higher levels of P450 in their livers.
- B. Livers from fish collected in 2003 show elevated P450 at station 6.

Indicators of DNA Damage as Assessed Using the Comet Assay

An indicator of contaminant stress can be DNA strand breaks. These strand breaks can be repaired but may lead to mutations or overall diminished energy budgets; unrepaired strand breaks will usually lead to cell death. We have been assessing DNA strand breaks in blood cells from fish and crabs from the different marshes using the Comet assay that determines the percentage of DNA migrating from nuclei under electrophoretic conditions. Figure 6 shows DNA damage in blood cells from mudsuckers collected in 2003. Animals from Stege Marsh, the most contaminated site, show significantly elevated DNA stand breaks in their hemocytes, and this is seen at station S in Figure 6 below. Large numbers of samples have been prepared and are being analyzed using a new software package. Example data are shown below.

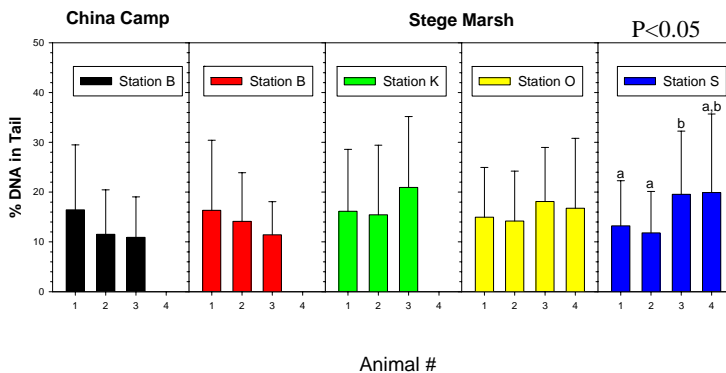


Figure 6. Blood cell Comet data showing statistically significant increases in DNA damage fish from station S at Stege Marsh. Other stations did not show an increase in DNA damage above reference levels (China Camp). Analyses are continuing for all fish and crabs collected at all sites.

Indicators of Endocrine Disruption

For studies of endocrine disruption, we are applying immunologic assays to detect induction (estrogenic activity) of choriogenins (egg shell protein precursors) that are made by the liver in response to estrogenic compounds, including environmental estrogens and estrogen mimics. For this research, we are applying both commercial antibodies and antibodies we have developed as routine tools for detecting endocrine disruption in male and immature fish (Fig. 7). This approach is more broadly applicable than the more commonly used vitellogenin assay in fish because the choriogenins are

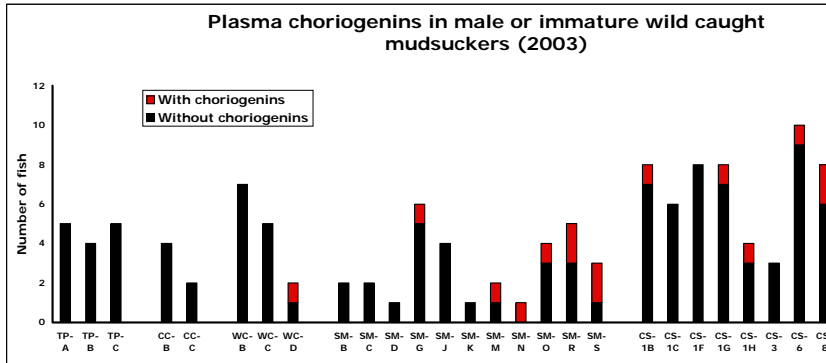


Figure 7. Presence or absence of choriogenins in plasma of male or immature (no gonad) mudsuckers. As expected, Toms Point (TP) and China Camp (CC) show no response. Specific stations at Stege and Carpenteria show an EDC response.

more highly conserved, and the antibodies can be used on a very broad range of fish species. By also utilizing data on the presence of estrogenic and/or androgenic activities from sites determined by Dr. Denison's reporter bioassay, subsequent chemical analyses, and demographic data collected by the EIC, we should be able to determine cause and effect relationships for reproductive impairment. Dr. Denison has completed extractions of all sediments from 2003 collections and is conducting reporter bioassays at present. These data will be correlated with the choriogenin response as well as chemical characterization being conducted.

Indicator of Exposure to Contaminants: Programmed Cell Death (Apoptosis)

Another area of research has been the development of the incidence of apoptosis, or programmed cell death, as an indicator of stress as well as decreased reproductive output in fish. Cell death in liver and gonad can directly impair reproductive output, as well as health of individuals, based on histological condition and biomarker responses such as P450 and Comet. We have utilized a well-developed technique that measures DNA cleavage (the TUNEL assay) on field and laboratory samples, as well as caspase (cysteine aspartate protease) activity as indicators of apoptosis. We have now established a correlation between caspase activity and the TUNEL assay for fish exposed to cadmium; this is the first time both of these assays have been used in an aquatic organism. The TUNEL assay has been applied to livers of fish collected from all sites for 2002 and 2003. An example of the data is shown in Figure 8. All data for each individual fish will be correlated with histological condition and biomarker responses in order to tease out individual variability in all responses and to determine station and site responses. These analyses will be completed later this year.

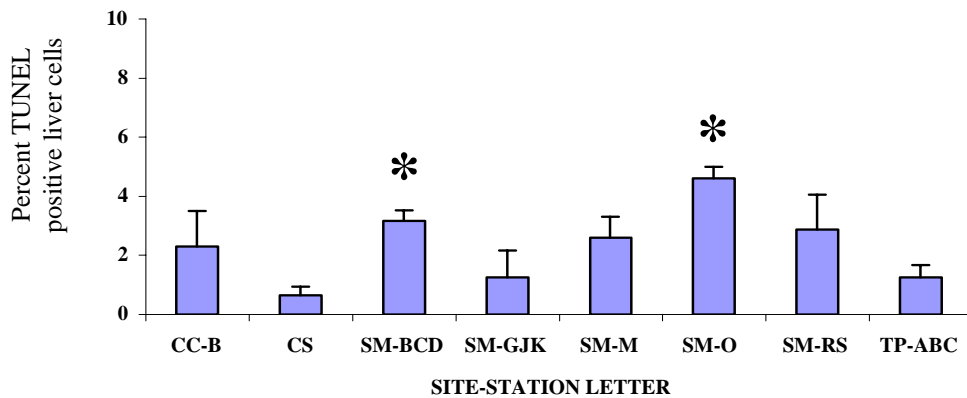


Figure 8. Incidence of DNA damage as assessed by the TUNEL assay in histological sections of mudsucker livers from wetland sites. Stege Marsh (SM) stations B, C, D and R,S showed statistically significant increases in TUNEL-positive cells.

Developing a Larval Fish Model for Stressor Effects on Growth, Respiration, and Biomarker Responses

A series of experiments utilizing larval topsmelt exposed to cadmium were conducted to develop indicators of contaminant-impaired growth and effects on physiology. These data are being used to develop a dynamic energy budget model for larval estuarine fish. Examples of the types of data collected are shown below. These include growth (based on weight and otoliths), respiration, food consumption, and apoptosis in different tissues.

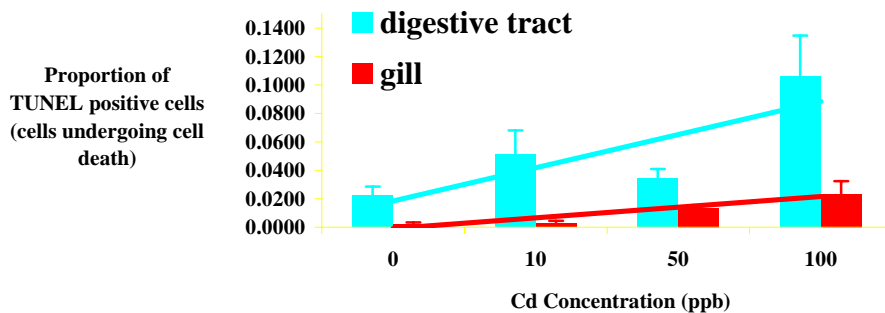


Figure 9a. Select data from cadmium exposures with topsmelt larvae. Cadmium induces apoptosis in digestive tract cells and gill cells at higher concentrations

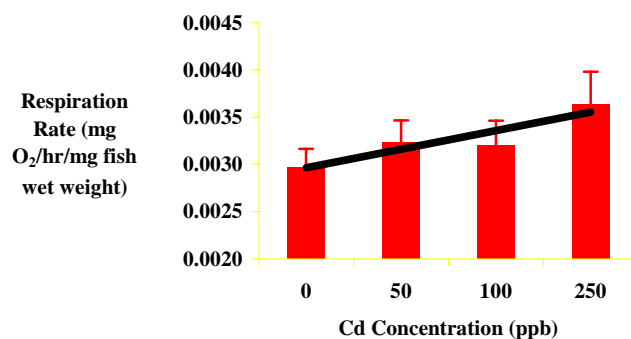


Figure 9b. Cadmium also increases respiration rates, with significant increases at 250ppb. This is an indicator of stress and of metabolic compensation.

FUTURE ACTIVITIES:

Analyses of samples and data will continue from 2002 and 2003 samples and results. New outplant experiments will be conducted this summer at select stations at Carpinteria Salt Marsh and Stege Marsh in order to further define EDC responses. In addition to choriogenin responses, we will analyze plasma using the cell reporter system in order to identify EDC chemicals in mudsucker circulation. The DEB modeling will proceed rapidly now that all of the organismal and physiological responses have been measured. Throughout the winter we will be working with the integration team to assess the most effective suites of stressor indicators using our animal models.

PUBLICATIONS & PRESENTATIONS:

Publications

Pillai, M.C., C.A. Vines, A.H. Wikramanayake, and G.N. Cherr. 2003. Polycyclic aromatic hydrocarbons disrupt axial development in sea urchin embryos through a β -catenin dependent pathway. *Toxicology* 186: 93-108.

Presentations

Cherr, G.N. 2003. The PEEIR Program: Contaminant Responses. Sonoma State University, November, 2003.

Cherr, G.N. 2003. Embryo Defense Mechanisms and the PEEIR Program. University of California Santa Cruz, September, 2003.

12th International Symposium: Pollutant Responses in Marine Organisms (Tampa FL), May 9-13, 2003. "Exogastrulation in Sea Urchins as an Indicator of Exposure to Polycyclic Aromatic Hydrocarbons (PAHS) or Phthalates". Carol A. Vines, Murali C. Pillai, Athula H. Wikramanayake, and Gary N. Cherr. 3rd Prize Best Poster Competition.

Estuarine Research Federation: Seattle, WA, Sept. 14-18,2003; “A Bioindicator Approach to Assessing the Effects of Contaminants in Estuarine Species” C.A. Vines, E. Fairbairn, S. Walsh, R.M. Higashi, S. Anderson, and G.N. Cherr.

Society for Environmental Toxicology and Chemistry (Austin, TX) Nov. 9-13,2003; “A Bioindicator Approach to Evaluating the Environment”, C.A. Vines, R.M. Higashi, S.L. Anderson, and G.N. Cherr.

PEEIR meeting (Bodega Bay, CA), Nov 2003; “Choriogenins as an Indicator of Endocrine Disruption in Fish”, C.A. Vines, G.N. Cherr; “P450 Biomarkers as One of a Suite of Animal Indicators of Estuarine Health”, G.N. Cherr, F.G. Griffin, V. Rashbrook, M. Snyder, C.A. Vines.

SUPPLEMENTAL KEYWORDS:

aquatic, indicators, biomarkers, wetlands, reproduction, cellular, molecular, biochemical, bioavailability, ecosystem, ecological impacts, estuary.