Title

Development of Molecular and Cellular Tools for the Detection of Environmental Endocrine Disruption in Aquatic Invertebrates

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Publication Date

2003-11-03

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Final Sea Grant Report for Project R/CZ-168.

Environmental endocrine disruptors (EDC's) are environmental contaminants that act by alteration of one or more aspects of the endocrine system of affected individuals. Though well characterized for vertebrates, the extrapolation of EDC effects information to aquatic invertebrates has been inhibited by the large differences in physiological regulatory pathways. There is a significant need for test methodology development and the identification of 'biomarkers' of EDC effects for aquatic invertebrates (Hutchinson and Pickford 2002).

The best studied example to date of aquatic invertebrate endocrine disruption is imposex (sex reversal) in molluscs. Tributyltin, a boat paint additive, causes masculinization of female molluscs by a mechanism(s) thought to involve inhibition of cytochrome P450 aromatase that is responsible of the conversion of testosterone to estrogen (Axiak et al. 2003). The goal of the work described here was to further our knowledge about EDC's and their potential effects on marine invertebrates. A major emphasis was to describe the effects of suspected EDC´s (from vertebrate studies) on invertebrates common to regions with high anthropogenic inputs and to examine these effects at environmentally relevant EDC concentrations.

Our research program has involved a several prong approach. The first direction involved the demonstration of environmental endocrine disruption in a commercially important crustacean. Here, we chose to measure hormone levels and ascertain the effects of a known EDC on biomarkers in this species. In the second approach, we used the same species to examine the effects of vertebrate neurosteroid compounds on a well-defined behavior. We hoped to glean information about potential behavioral pathways that may be directly affected by related compounds or EDC´s that act via the same neuroreceptors. We chose a common west coast marine invertebrate to examine a known EDC´s effect on larval swimming behaviors as potential markers of exposure. Lastly, we used the same species to examine the effects of EDC´s having estrogenic or antiestrogenic actions on vertebrate systems. We studied the effects of these compounds on larval development at three different stages.

(1) How do suspected EDC´s affect an ecologically and economically important marine invertebrate? We used one of the best studied crustaceans, the American lobster *Homarus americanus*. First larval stage lobsters were exposed to the cyclodiene pesticide heptachlor, a known EDC with known affinity for the vertebrate estrogen receptor (Guillette et al. 2002). The purpose of these experiments was to expose larvae for only a single 24 hr period on any of the days of the first larval stage. This was done as an attempt to mimic what might occur as the larva moved in currents past a highly impacted coastal region. We found larvae to be most susceptible to heptachlor during days 4-5 of the normal 8 day first larval stage prior to ecdysis to the second stage (Snyder and Mulder 2001). Delays in the time to ecdysis were related directly to alterations in the ecdysteroid molting hormone levels caused by the heptachlor exposure (Fig. 1). Heptachlor exposure on either the first or second day resulted in an elevation in hormone level, while later exposure either delayed or inhibited the peak production of hormone necessary for successful ecdysis to

stage 2 larvae. This is the first, to our knowledge, demonstration of hormonal cycle alterations by a known EDC.

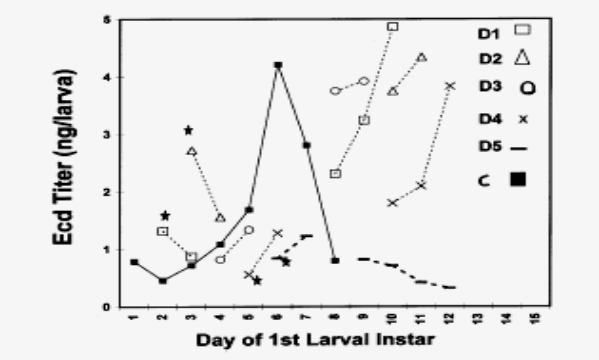

Figure 1. Ecdysteroid levels in whole larvae treated with the EDC heptachlor on the indicated day of the first larval stage. Control larvae are shown by the solid line.

Biochemical markers cytochrome P450 (CYP45, Snyder 1998) and stress protein HSP70 were also monitored in first stage larvae exposed to heptachlor. Both CYP45 and HSP70 were upregulated during the premolt and postmolt periods of the first larval stage. Larval exposure to heptachlor resulted in multi-fold elevations in CYP45 that were transitory and returned to normal levels within two days following the heptachlor exposure (Fig. 2). HSP70 levels were also increased following heptachlor exposure, and at a critical stage Day 5, HSP70 levels did not return to normal levels and none of the larvae exposed on that day survived to Stage 2. The HSP70 data support the contention that prolonged elevations in HSP levels may be detrimental to normal physiology (Krebs and Feder 1997). Taken together, the results of these experiments show that it is possible to measure endocrine disruption and altered biomarker levels as indicators of EDC exposure.

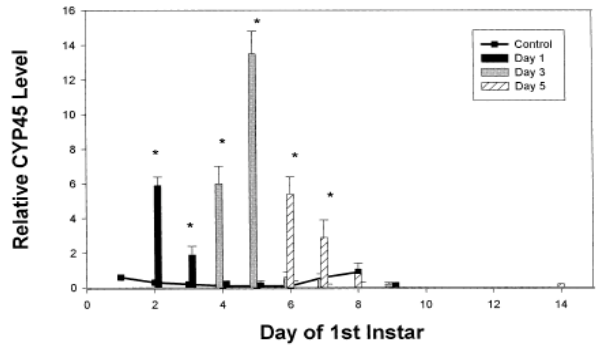

Figure 2. Cytochrome P450, CYP45, levels in lobster larvae treated with the EDC heptachlor on the indicated day. Large transitory increases in CYP45 are seen for 2 days following heptachlor exposure.

(2) The second major emphasis of this work was to develop behavioural alterations indicative of EDC effects on marine invertebrates. We used juvenile lobsters in experiments designed to test whether steroid hormones may regulate aspects of behaviors as found in vertebrates (refs). Lobster locomotor behavior was studies in a dark field circular arena marked in equal intervals (Snyder et al. 2000). We previously developed this test protocol to study gamma aminobutyric acid receptor modulators and how they affect lobster behavior. For the present work, we showed that neurosteroids commonly found in vertebrate brain, and which modulate reproductive behaviors, stress/depression, and others (ex. Noda et al. 2000), either promote or depress lobster locomotor activity in manners

similar to vertebrates (Snyder and Peeke 2001). The potential for EDC´s and other pollutants to alter behaviors directly remains to be elucidated.

An additional marine invertebrate was utilized to determine whether alterations in larval behavior could be ascertained with EDC exposure (Snyder et al. in prep.). *Lytechinus anamesus* larvae are common constituents of the Southern California coastal plankton. Early pluteus stage (approximately 72 hrs from fertilization were exposed to tributyltin, a EDC known to cause sex reversal (imposex) in molluscs (see Axiak et al. 2003 for review). We used environmentally relevant concentrations of tributyltin for 1 hr, then captured swimming behaviors of groups of larvae concurrently with video.

The dilution series (in part per trillion) included 0.1, 0.3, 1.0, 3.0, 30.0, 100, and 300. Six different behavioral measurements were done for the paths in each data set. These six measurements were: speed, angular velocity, linear accelerations, net to gross displacement ratios, and direction of travel. Linear and angular statistics were calculated for speed and angular velocity. These measurements were conducted on a computer integrated video system that digitized the images and followed the movement of each individual larva in real time. The capture duration was for 40 seconds at 5 frames per second. Real world reference points were set to millimeters per time unit (e.g., mm/sec/sec).

Definition of Operators

Direction of Travel—the direction of travel operator estimates the direction in which an object moves as it is displaced from one position in its path to the succeeding location. This angle is measured in degrees with respect to the horizontal. Note that the measurement of angles depends upon the orientation of the frame of reference. The origin (i.e., direction of travel equal to zero or any integer multiple of 360^0) us defined to extend horizontally from left to right. For example, the direction of travel of an object moving along a counter-clockwise circular path (i.e., turning constantly to the left) increases monotonically with time and may successively accumulate a direction of travel exceeding 360^o, 720^o, 1080^o, etc. Turning to the right along a clockwise circular path would, analogously, decrement the direction of travel.

This movement measurement is likely to produce additional separation in behavior characteristics between treatments. Like the angular velocity measurement the use of directional statistics may provide data on the statistical significance between treatments. Preliminary evaluation of the data shows little change at 0.1, 0.3 pptr, less clear at 1.0 pptr., some change trends at 3.0 pptr., 30 pptr., less clear at 100 pptr but larger changes at 300 pptr exposure level.

Net to Gross Displacement—for a given point in a path, the net displacement is the distance along a straight line from the first point in the path to the given point. In contrast, the gross displacement is the distance along the actual path from the first point in the path to the given point. The ratio between these two quantities is called the net to gross displacement ratio. The ngdr is a dimensionless number between 1 and 1 inclusive. It is an index of path twisted ness or contortion: simple linear (straight line) movement results in ndgr = 1;

repeated circular motion results in a declining ngdr which approaches zero with the progress of time.

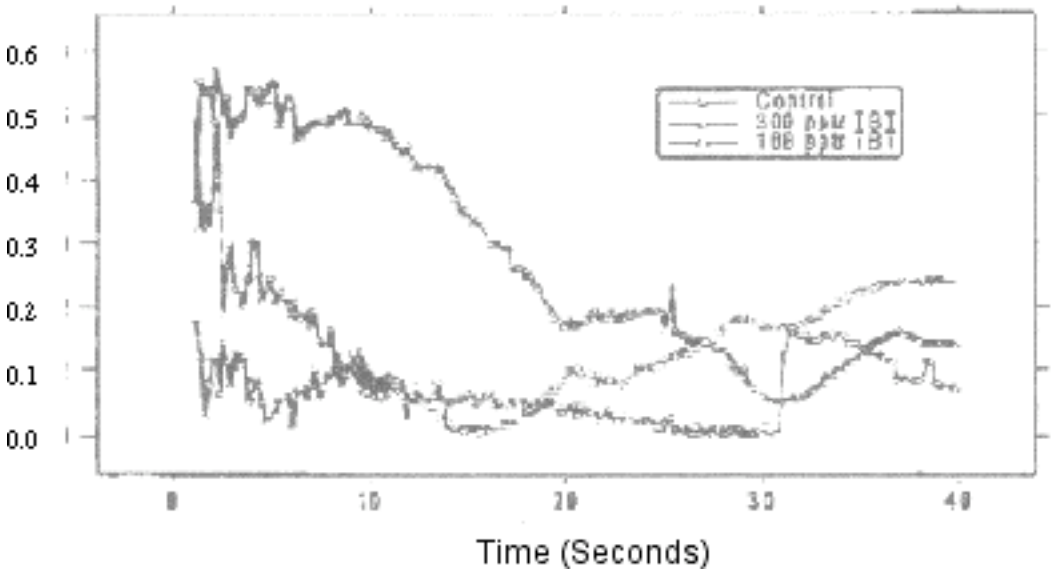

Figure 3. Net to Gross Displacement Ratio. This function is an index of path twistedness or contortion: simple linear movement results in a ngdr = to 1; repeated circular motion results in a declining ngdr. Echinoderm larvae exposed to two levels of TBT compared to a control.

This function measures the twistedness or contortion of the paths. The larvae show a tumbling movement that changes with exposure to TBT. An example of the increased rate of tumbling is shown in Figure 3. This change can be seen at 100 and 300 pptr exposure levels but is less clear at 0.1, 0.3, 1.0, 3.0, and 30pptr exposure ranges.

Linear Acceleration—is the rate of change in speed with which an object traverses its trajectory and may be positive or negative. Linear acceleration is equal to zero when an object is moving at constant speed. Speeding up is indicated by a positive value, slowing down is indicated by a negative value. This is a more difficult operator to analyze, however, when the mean of each path is calculated for each exposure and then compared to the control it can be seen that there is no significant difference between treatments. However, the frequency of change may be used to separate the response at exposures. Some change at 1.0 ppt., and 30 ppt. At 100 ppt. Change is more evident with less certainty at 300 ppt.

The altered swimming behaviors resulted in another discovery, that food capture was significantly reduced by 1 hr TBT exposure. In these experiments, larvae were exposed to TBT for 1 hr, and then allowed to feed for a 5 min period on algal cells (*Isochrysis*) at 50,000 cells per ml density. The number of algal cells was visualized under fluorescence with the aid of a confocal microscope. By focusing up and down through the

plain of each larva, the number of algal cells in the gut could be counted. TBT at 0.1-10 pptr. reduced feeding by approximately 50% within 1 hr of exposure.

The altered sea urchin larval swimming behavior patterns and corresponding feeding rates can be explained by TBT´s inhibition of larval external cilia beating. TBT alters larva calcium dynamics (data not shown), which is a major regulatory mechanism of beat rate control. We conclude that it is highly probable that current environmental levels of TBT near areas of high shipping activities will negatively impact local sea urchin larval survival. TBT has been banned for use as a boat paint additive in developed countries (to prevent settlement of organisms on the boat surface which increase surface drag and cause increased fuel consumption). Correspondingly, TBT levels may be dropping in harbors and embayments in those countries in which the ban is in effect (Diez et al. 2002).

(3) A final area of emphasis for this funded work on EDC effects was performed by Troy Roepke as part of his Sea Grant Traineeship supported Ph.D. thesis. For this project, a wide range of EDC´s with estrogenic or antiestrogenic actions (EEDC´S) on vertebrate systems were chosen. Three different *L. anamesus* larval stages were exposed to environmentally relevant concentrations of each of the compounds (early cleavage/blastula, gastrulation, and post-gastrulation stages).

Sea urchin development was affected by the range of different EEDC´s in the same order (Fig. 4) in which vertebrates respond to the same compounds (estrogen > ethynylestradiol > estrone > progesterone > estriol). The environmental levels of such compounds fall within 10 ng – 1 □g per liter, levels that are physiologically active for vertebrate systems.

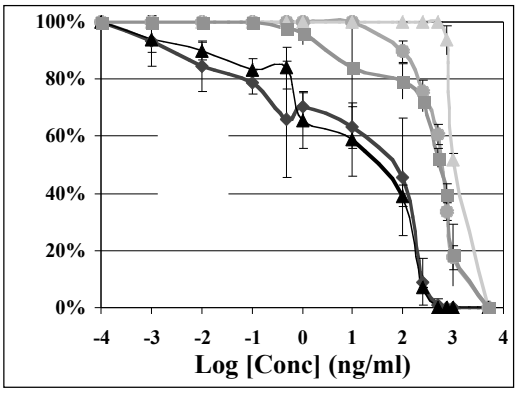

Figure 4. Percentage of normal sea urchin embryo development after 96 hr exposure to natural and synthetic reproductive hormones.

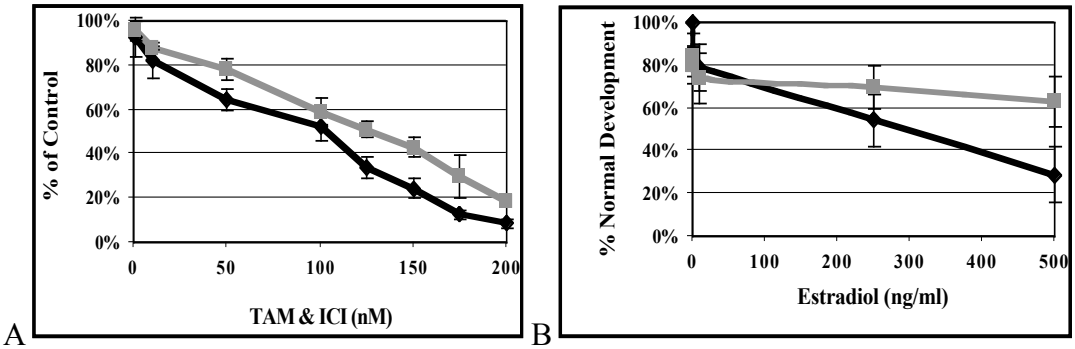

Figure 5. Percent of normal sea urchin development following a 96 h exposure to estrogen with and without selective estrogen receptor modulators. A: Percent normal development after exposure to Tamoxifen (grey) or ICI 182,780 (black). B: Percent normal development after exposure to estrogen with (grey) or without (black) 50 nM Tamoxifen.

The effects of environmental estrogens was examined further with the use of a known estrogen receptor agonist Tamoxifen and a known antagonist ICI 182,780. Both Tamoxifen or ICI 182,780 alone inhibited development in the low nanomolar range. The addition of Tamoxifen and estrogen partially cancelled the development inhibitory effects of estrogen. These results argue for an estrogenic hormonal pathway in sea urchin development. Recently, a similar result was found in another study involving a comparison between fish and molluscs. It is also likely that environmentally relevant concentrations of estrogenic EDC's will negatively impact several critical periods in sea urchin larval development.

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