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Evaluation of relationships between reproductive metrics, gender and vitellogenin expression in demersal flatfish collected near the municipal wastewater outfall of Orange County, California, USA

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Abstract

Estrogenic activity in fish has primarily been evaluated using vitellogenin (vtg) expression in male and juvenile animals. Although the response has been widespread in field and laboratory studies, the relevance of the response to higher level adverse effects, particularly in the field, is less than clear. Previous evaluations of vtg within flatfish species collected near the Orange County Sanitation District (OCSD) outfall and stations as far as 7.7 km down current indicated bioavailable estrogens within demersal flatfish populations. In order to evaluate the persistence of estrogenic activity and relationships to reproduction and development, fish were sampled in the winter and summer of 2003 and 2004 at the outfall and a reference location. Vtg, plasma estradiol (E2) concentrations, gonadosomatic indices (GSI), sperm DNA damage, development, and gender ratios were measured in English Sole (*Pleuronectes vetulus*) and Hornyhead Turbot (*Pleuronichthys verticalis*). Variable levels of vtg were continually observed in the plasma samples of fish collected at both locations. Vtg expression and plasma E2 levels were significantly correlated in females. A positive relationship was demonstrated between plasma E2 levels and sperm DNA damage. Rather than an expected feminization of populations, a trend toward masculinization was observed particularly at the OCSD outfall, as indicated by gender ratios and significantly higher GSI in males versus females. These results are consistent with previous studies showing vtg expression in male flatfish, but no alteration in overall flatfish abundance at the sampled sites.

Keywords: Vitellogenin; Estrogenicity; Endocrine disrupter; Estradiol; Reproduction; DNA damage

1. Introduction

Much of the world's population resides in coastal areas resulting in oceanic and estuarine discharge of domestic and industrial wastewater. In estuarine areas that have low flushing, wastewater can dominate the receiving system and be the primary freshwater contributor (Ferguson et al., 2001). Numerous natural and "xeno"estrogens have been identified in wastewater and include a diverse array of substances including steroid hormones, derivatives of industrial polymers or surfactants, and pharmaceutical agents (Thomas et al., 2001, 2004). Recent fate and transport studies have predicted (Williams et al., 1999, 2003) and measured (Peck et al., 2004) significant concentrations of estrogens as well as estrogenic activity derived from in vitro and in vivo bioassays in sediments from freshwater and marine ecosystems (Legler et al., 2002; Peck et al., 2004; Schlenk et al., 2005). Due to the deposition of these agents into sediments, benthic organisms, such as flatfish, which bury and feed in sediments, would likely experience significant exposure.

Initial studies with European Flounder (*Platichthys flesus*) in the UK indicated significant estrogenic activity determined by measurements of ova–testes and vitellogenin (vtg) in the plasma of male fish exposed to sewage effluent (Lye et al., 1997). Although subsequent studies observed somewhat lower histological deformities of the testes, vtg values in male flounder throughout the UK were elevated (Allen et al., 1999). However,

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gender ratios calculated across size class and gonadosomatic indices were not associated with vtg measurements observed in the male fish. Estrogenic activity in flatfish has also been observed in Japan (Hashimoto et al., 2000), the Netherlands (Vethaak et al., 2002), the northeastern United States (Pereira et al., 1992) and southern California (Roy et al., 2003).

In a southern California study carried out in 2000, significant differences in vtg expression of males collected near the outfall of the Orange County Sanitation District (OCSD) relative to a reference collection site were not observed. However, vtg values in males were similar to that of gravid females collected from each location. OCSD releases a 50% blend of primary and secondary treated effluent at approximately 240 million gallons per day. Subsequent investigations of sediments collected at the outfall revealed the occurrence of 17β-estradiol (sub ng/g), several alkylphenol ethoxylates (∼100 ng/g) and ∼3 μg/g concentrations of alkylphenols (Schlenk et al., 2005). Direct exposure of sexually mature male California Halibut (*Paralichthys californicus*) to sediments and injection of sediment extracts significantly induced vtg. Comparison of vtg expression with flatfish abundance and species diversity metrics in 2000 failed to demonstrate relationships at the OCSD outfall (Roy et al., 2003). Unfortunately, reproductive endpoints and other population metrics, such as gender and developmental measurements, were not examined.

Consequently, the current study was designed to evaluate plasma vtg concentrations from males of the same two species of flatfish collected in the same area near the OCSD outfall and reference sites during 2003 and 2004. In order to assess reproductive function, gonadosomatic indices, sperm DNA damage, and plasma estradiol (E2) concentrations were evaluated in the fish collected. To assess population effects, developmental status and gender ratios (dating to 1988) were documented.

2. Materials and methods

2.1. Sampling locations

The OCSD outfall is located 7 km offshore on the San Pedro Shelf within the southern California Bight at a nominal depth of 60 m (latitude 33°34.641′; longitude 118°00.567′). The reference sampling location (latitude 33°36.055′; longitude 118°05.199′) was 7.7 km north of the outfall at a nominal depth of 56 m. The reference location was based on 25 years of monitoring data and confirmed by USEPA Region IX. The prevailing surface current is upcoast (north). Trawling paths were determined using differential Global Positioning System (dGPS) navigation to accurately locate the area sampled and to control the speed of the trawl (2–2.5 knots).

2.2. Fish sampling

English Sole (*Pleuronectes vetulus*) and Hornyhead Turbot (*Pleuronichthys verticalis*) were collected in January 2003, July 2003, January 2004, and August 2004 utilizing a 7.6 m wide semiballon otter trawl. Three replicate samples for population analysis were collected. All specimens were taxonomically identified, counted, and individually weighed and measured. Gender ratio data were collected by OCSD biologists from 1988 through 2004 for Hornyhead Turbot, and 2001 through 2004 for English Sole. Sex was determined by gross morphology of the gonads. Blood (<1 ml) was collected via heparinized 22 g syringe from the dorsal aorta and immediately spun with a portable centrifuge unit for 2 min at 5000 rpm. The plasma was removed and stored on dry ice until transport to a −80 °C freezer where it was stored until analysis. Only one-half of the gonad was removed for GSI determination, as the other half was collected for use in other studies. Therefore, all GSI data presented are reported as ½ GSI. Fish were size-classed and aged using age/length regression equations reported by the National Marine Fisheries Service (Myers et al., 1993). The regressions were developed for each target species by geographic area. Regression equations from the geographic area either encompassing or nearest to the OCSD study site were used to determine the age of the study fish. The equation used for English Sole was $y = 3.126 - 0.021x + (8.9136\text{E}{-5})x^2$, where y = age and x = length in mm, developed for the Monterey–Moss Landing geographic area. The equation used for Hornyhead Turbot was $y = 1.526 - 0.015x + (2.0965\text{E}{-4})x^2$, developed for the California geographic area.

2.3. Measurement of vitellogenin in plasma

2.3.1. Hornyhead Turbot vitellogenin assay

Wells were coated with 100 μl of 0.8 μg/ml California Halibut vtg (provided by Amanda Palumbo of UC Davis) in 50 mM carbonate buffer. Non-specific binding wells were coated with 1% non-fat milk in 50 mM carbonate buffer. The plates were then incubated at 37 °C for 2 h. Wells were washed three times with 10 mM Tris–phosphate buffer saline (TPBS) then blocked with 200 μl of 2% non-fat milk in TPBS for 45 min at 37 °C. The wells were then washed again three times with TPBS. Standards (purified Halibut vtg) and samples were diluted in TPBS. Primary antibody (rabbit anti-Turbot vtg purchased from Cayman Chemical, Ann Arbor, MI) diluted in TPBS was added to standards and samples at a ratio of 1:1, for a final concentration of antibody of 1:1000. These solutions were then incubated for 2 h at 37 °C. One hundred microliters of each solution was then added in triplicate to the wells and incubated again for 2 h at 37 °C. The wells were then washed three times with TPBS. The secondary antibody (goat anti-rabbit labeled with alkaline phosphatase purchased from Biorad in Hercules, CA) was diluted to 1:2000 in TPBS then added to the wells and incubated for 45 min at 37 °C. The wells were washed twice with TPBS and once with PBS. The substrate *p*-nitrophenylphosphate diluted in diethanolamine buffer was added to each well at volume of 100 μl. The plate was then incubated for about 1 h in dark. The absorbance was measured with a microplate reader at a wavelength of 405 nm.

2.3.2. English Sole vitellogenin assay

Methods were as outlined above with the following exceptions. Wells were coated with 100 μl of 0.8 μg/ml English Sole vtg (provided by Dan Lomax of NMFS) in 50 mM carbonate