

HTP Special Study 2009-10

Characterization of the Environmental Endocrine Disruption Effects on Male Estrogen Levels and Testicular Estrogen-producing Genes in Hornyhead Turbot from the CLAEMD Outfall Monitoring Program Study Area

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BACKGROUND AND RATIONALE

It is well documented that many California coastal environments are contaminated, such as along industrialized or developed shorelines, in ports and marinas, and in regions around outfalls of publicly-owned treatment works (POTWs), among others. However, it is poorly understood to what extent are existing contaminants –many with continuing inflows into the environment– impacting the biota. In the Southern California Bight (SCB), over one billion gallons of treated wastewater (effluent) are released each day into coastal waters. Such effluents are known to contain contaminants classified as endocrine-disrupting compounds (EDCs). The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) of the US EPA defined an EDC as an “*exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle*” (EDSTAC, 1998). Putative or demonstrated EDCs include (but are not limited to) organochlorine pesticides, polychlorinated biphenyls (PCBs), surfactants, plasticizers, polycyclic aromatic hydrocarbons (PAHs), DDT and metabolites, active steroid hormones and their mimics, and a large variety of pharmaceuticals.

The vertebrate endocrine system is highly conserved, and this holds particularly true for steroidal systems. For example, the primary ovarian steroid in humans, 17 β -estradiol (E2) is the same primary ovarian steroid hormone in fish (Lintelmann et al., 2003). E2, estrone (E1), as well as the pharmaceutical 17 α -ethinylestradiol (EE2), are the most potent forms of estrogens, active in animals at <1 ng/L environmental concentrations (Purdum et al., 1994; Tyler et al., 2005; Khanal et al., 2006). Humans (as well as domestic animals, livestock, etc.) excrete E2, E1, and EE2 (if it is being taken) via urine on a daily basis, and pregnant women can excrete E2 at levels as high as 5 mg/day (Duguet et al., 2004). Studies worldwide have demonstrated that these steroids are present in treated wastewater, surface waters and sediments (both freshwater and seawater environments), ground water, and even drinking water, often at concentrations with the potential to cause reproductive effects, particularly in male vertebrates (Koplin et al., 2002; Matthiessen, 2003; Johnson and Williams, 2004; Campbell et al., 2006; Khanal et al., 2006). Effects in fish exposed to estrogens include the development of intersex (fish expressing sexual characteristics of both sexes, such as ovo-testis), altered gamete production, and reduced reproductive success (e.g., above papers and Rodgers-Gray et al., 2001; Jobling et al., 2006). New data are emerging from studies in the SCB indicating that these steroids are present in our local marine environments. However, it is also important to consider the potential that other (non-steroidal) EDCs can also have important reproductive effects, as will be discussed further below.

The hornyhead turbot (HT), *Pleuronichthys verticalis*, is a flatfish species that serves as a model organism in ocean monitoring efforts conducted by southern California environmental monitoring agencies, including the City of Los Angeles’ Environmental Monitoring Division (see CLAEMD, 2007). This species is believed to have high site fidelity and it maintains a focused diet of benthic tube-dwelling polychaetes and clam siphons (Allen, 1982; Cooper, 1997; Gibb 1995). HT typically reside at depths of <100 m, but are found at depths ranging from 10 – 200 m (Allen, 1982). Since the outfalls of ocean-discharging southern California POTWs are located at average depths of 60 m in soft-bottom habitats, HT are often abundant in these areas. This species, therefore, represents a particularly good opportunity to study and understand exposure and effects of environmental EDCs (endocrine disruption) due to wastewater discharge.

A significant number of known EDCs have lipophilic properties, which means they are most likely to be bound to particles which then settle and accumulate in the benthos. This can then facilitate EDC accumulation in the membranes and tissues of benthic animals. Vertebrate animals at high risk for

EDC accumulation and effects are flatfish, particularly those with diets specializing in sediment-dwelling invertebrates, as for the HT.

Prior research in collaboration with CLAEMD (HTP Special Study between 2005-2008; Sea Grant Project #CE-17, 2006-2008) determined that **male** hornyhead turbot from Santa Monica Bay exhibit extraordinary plasma concentrations of ('female') E2, with levels as high or higher than that of reproductive females (300-1000 pg/ml). In males of other local fish species tested (including English sole, Pacific sanddab, longfin sanddab, Dover sole), E2 concentrations are all <50 pg/ml, typical of vertebrate males generally. Comparisons of HT males from the HTP outfall area and CLAEMD reference sites in Santa Monica Bay inconsistently point to outfall-related effects, with significantly increased E2 in males from the outfall site observed in some studies, but not in others. These differences may relate to differences in oceanic conditions (especially currents) between studies, as well as to apparent widespread impacts in Santa Monica Bay. All Santa Monica Bay HT males measured to date (from various CLAEMD study sites) have consistently exhibited 2-4 times greater E2 concentrations as compared with HT males from the Orange County coastline, suggesting that Santa Monica Bay is relatively more impacted in terms of this putative endocrine disruption effect in the fish (Reyes, 2006; Hagstrom, 2008; Petschauer, 2008; and unpublished).

In Orange County, the OCSO outfall is located in a more open oceanic environment and handles a relatively smaller human population (200 MGD from ~3 million residents). Santa Monica Bay, in contrast, is a more enclosed oceanic environment, impacted by the larger volume (~340 MGD) HTP outfall and a larger service population (>4 million residents), in addition to possible impacts from JWPCP-LACSD given predominant up-coast currents off of Palos Verdes.

Potential Mechanisms Underlying Effects

Studies are beginning to emerge indicating that EDCs in the environment can alter endogenous gonadal steroid production in fish and wildlife (e.g., Hayes et al., 2002, 2003; Henson and Chedrese, 2004; Arukwe, 2008; Gracia et al., 2008). Thus, in addition to possible accumulation of estrogen from the environment, other EDCs may act directly on the gonad's steroidogenic machinery, potentially activating E2 production. Our preliminary work has pointed to the latter possibility, that the high E2 phenotype in male HT from Santa Monica Bay may be related to altered steroidogenic enzyme gene expression in the testis.

Steroidogenesis is the process by which steroids are synthesized via progressive modifications from the precursor, cholesterol (see Figure 1). The majority of steroid hormones are synthesized in the gonads and adrenal (=interrenal in fishes) tissue. The synthesis of all steroid hormones begins with the conversion of cholesterol into pregnenolone by **P450scc** (side chain cleavage enzyme). The P450scc enzyme is localized to the inner mitochondrial membrane, and as a result, the cholesterol molecule must first be transported across the membrane. Steroidogenesis acute-regulatory protein (**StAR**) plays an essential role in regulating steroidogenesis by allowing for the initial translocation of cholesterol through the inner aqueous mitochondrial membrane (Tsuji-shita and Hurley, 2000). StAR has been referred to as the most important global regulator of steroidogenesis – its activation is a vital rate-limiting step in steroidogenesis (Bauer et al., 2000; Stocco, 1999; 2003). StAR is highly expressed in ovary, testis, and interrenal of fish, with low levels of expression in other non-steroidogenic tissues (Young, 2005). StAR and P450scc are potential targets of environmental EDCs (Arukwe, 2008), as alteration in their expression will have important effects on the overall rate of steroid production in a given steroidogenic tissue, like testis.

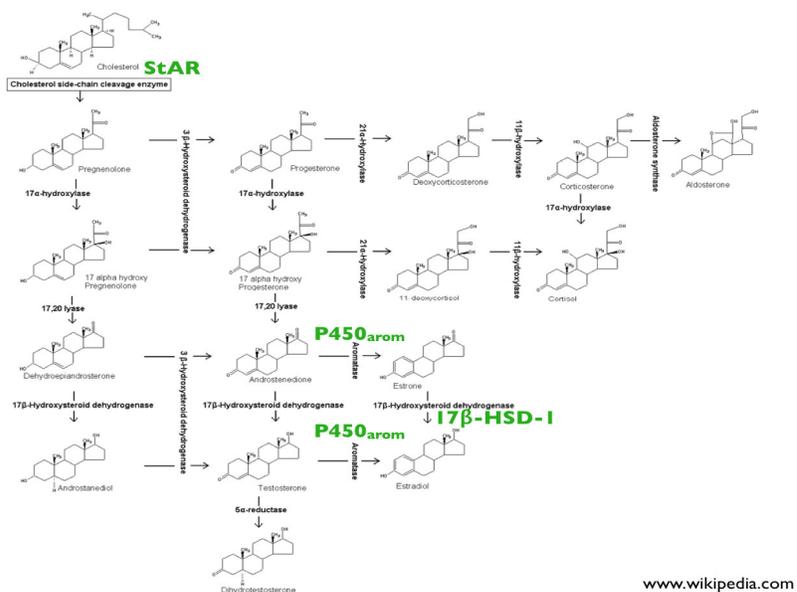


Figure 1. Vertebrate steroidogenic pathways. Enzymes investigated in the present study are shown in green. Further descriptions in text.

The conversion of androgens (e.g. androstenedione, testosterone) into estrogens (e.g. E1 & E2) is carried out by P450aromatase-A (**P450arom**). P450arom is essential to reproductive and developmental processes in vertebrates, since it is the terminal enzyme in the steroidogenic pathway responsible for producing E2 in both females and males (Blasquez & Piferrer, 2004; Gorski, 1998). A limited number of studies have shown that EDCs may alter P450arom expression or activity. For example, very low (ppb) concentrations of the pesticide, Atrazine, are reported to stimulate P450arom activity in frog testis, leading to increased E2 production and feminization in males (Hayes et al., 2001, 2002, 2003). In other examples, some EDCs (e.g., the PAH, benzopyrene) can reduce gonadal aromatase expression in killifish (Patel et al., 2006), while a study by Halm et al. (2002) demonstrated that exposure of male fathead minnows to E2 induces testicular P450arom gene expression in a dose-dependent manner.

The steroidogenic enzyme, 17 β -hydroxysteroid dehydrogenase-type 1 (**17 β -HSD-1**), can convert E1 into E2 (Kazeto et al., 2000; Baker, 2004a,b) and it has been identified as a target of some EDCs (e.g., Whitehead & Rice, 2006). E1 is typically poorly removed from wastewater by secondary WWTPs (Khanal et al., 2006), and it is often present in receiving environments. E1 has been consistently detected in SCB inshore environments, and therefore a possibility exists that aberrant E2 production in HT males could result from EDC-activated testicular 17 β -HSD-1 expression, leading to conversion of environment-derived E1 into E2.

In this Special Study, the steroidogenic genes, StAR, P450sc, P450arom and 17 β -HSD-1, will be investigated in male HT for potential environment-associated disruption in testicular mRNA expression, and will be correlated with the degree to which plasma E2 concentrations are elevated. Preliminary data (outlined in next paragraph) suggest that at least three of the genes may exhibit altered mRNA expression in HT associated with differing plasma E2 concentrations. In addition, this Special Study will characterize the concentrations of testosterone (T) and E1 in the fish, since enhanced P450arom (and plasma E2) would be expected to be correlated with reduced T levels (T is the precursor to E2), whereas altered 17 β -HSD-1 would be expected to alter E1 levels (E1 is a precursor to E2).

Our most recent findings indicate that HT males exhibiting elevated plasma E2 concentrations show expression of steroidogenic enzymes involved in E2 synthesis within their testis, including StAR, P450arom and 17 β -HSD-1 (P450scc has not yet been tested). Importantly, preliminary experiments indicate that the expression of each specific mRNA in testis was positively correlated with plasma E2 concentrations. These findings point to the possibility that elevated concentrations of E2 in male HT could be due to environmental endocrine disruption causing aberrant testicular E2 production.

Other than the very limited studies cited above, little is known to date about the potential environmental disruption of steroidogenic genes in animals, both aquatic and terrestrial (Arukwe, 2008; Zhou et al., 2008). No work to date has been reported in California, let alone from the SCB. Studies are strongly warranted, given the known presence of multiple contaminants in the SCB, and the observed testicular expression of estrogen-producing enzymes and elevated E2 levels in male HT, particularly individuals from Santa Monica Bay.

This Special Study is being proposed in order to address an essential issue requiring resolution: can the extraordinary E2 phenotype in male HT be unequivocally connected to effect of an impaired environment in Santa Monica Bay? Thus, if HT are well isolated from exposures to putative acting environmental factors (EDCs), to what extent do they exhibit a typical male phenotype? Further, do depurated fish exhibit an amelioration of the endocrine-disrupted condition?

STUDY AIMS AND METHODOLOGY

This Special Study will test whether elevated concentrations of E2 in male HT from the HTP study area are positively correlated with testicular expression of steroidogenic enzyme genes (P450arom, P450scc, 17 β -HSD-1, StAR), and whether separation of the fish from the environment (either experimentally or by sampling at remote field sites) will be correlated with reduced testis steroidogenic enzyme expression and E2 concentrations.

The **specific aims** are as follows:

1. To sample HT males from HTP 5-Mile outfall and Santa Monica Bay reference sites (yielding fish with varying E2 concentrations) and measure plasma concentrations of E2, E1 and T, and testicular expression of P450arom, P450scc, 17 β -HSD-1 and StAR. Correlation analyses between hormonal concentrations and testicular mRNA expression will be carried out to characterize relationships between changes in gonadal enzyme expression and steroid levels among individuals. Comparisons between field sites will test for outfall-associated effects.
2. To collect HT from the HTP 5-Mile outfall site (to obtain fish with elevated E2 levels) and rear them in synthetic seawater (lacking contaminants) in a laboratory setting for 4 or 8 weeks, followed by hormone and enzyme expression measurements as above. Potential differences will be evaluated between field-sampled animals (from Specific Aim 1) and the laboratory reared animals before and after “depuration” (isolation).

Studies under Aim #1. HT will be collected by otter trawl at the HTP 5-Mile outfall (site Z2) and at CLAEMD reference site C1 off-shore of Malibu. Trawls will target 30 fish per site, with roughly half

of the caught fish expected to be males. All fish will be measured for body length (mm) and weight (g), bled using a syringe needle at the caudal vein, and dissected to ascertain sex. Gonad (testicular) and hepatic (only for potential use) tissues will be removed and weighed to the nearest 0.1 g, in order to calculate gonadosomatic index (GSI) and hepatosomatic index (HSI) (indicative of reproductive status and fuel storage/metabolic status, respectively; Evans and Claiborne, 2006]. Tissues will then be placed into RNA-Later™ buffer (Invitrogen, Inc., Carlsbad, CA) to protect them from RNase degradation, until qPCR analyses are carried out, as described below (see Specific Methods). Blood plasma will be stored at -80°C until measurement of concentrations of E2, E1 and T, as described below (see Specific Methods). Although the hypothesis of the present study centers on potential aberrant testicular steroidogenic enzyme mRNA expression, samples from the females (ovary, liver, plasma) will be saved alongside male samples for potential subsequent measurements.

Studies under Aim #2. During the same trawls as above, 40 additional fish will be caught from site Z2, blood sampled (time-0), placed into on-board live wells. The fish will then be transported to the laboratory and placed into aquaria with cooled (16 °C) re-circulated synthetic seawater (Instant Ocean™) for 6 weeks, with 50% water replacements occurring every 3rd day during the experimental period, similar to published “deuration” protocols (e.g., Hano et al., 2007). Twenty fish will be sampled at week-4, and then 20 again at week-8, with roughly half of the individuals expected to be male. Fish will be blood sampled and their gonad (testicular) and hepatic tissues will be removed and weighed (as above, calculation of GSI and HSI) and then placed into RNA-Later™ buffer until qPCR analyses are carried out (Specific Methods). Blood plasma will be stored at -80°C until measurement of E2, E1 and T concentrations (Specific Methods).

Specific Methods

Radioimmunoassay (RIA). The concentration(s) of E1, E2 and T in HT plasma will be determined using RIAs validated and routinely used in the laboratory for this species (Reyes, 2006; Petschauer, 2008; Kelley et al., 2001). Standards and specific antiserum are obtained from Diagnostic Systems Laboratory, Inc (Beckman-Coulter, Inc., Webster, TX). Intra-assay variabilities for these assays range from CV= 6.9-8.9%, and inter-assay variabilities range from CV = 7.5-11.2%.

Real-time quantitative PCR (qPCR) with SYBR green. Total RNA will be extracted from the testicular tissue samples using TriZol reagent (Invitrogen), quantified by spectrophotometry, and treated with DNase I to avoid genomic DNA contamination. First-strand cDNA will be reverse-transcribed from 1 µg of total RNA using Superscript II RNase H⁻ reverse transcriptase (Invitrogen). Gene-specific primers for StAR, P450arom and 17β-HSD-1 have previously been designed and validated for real-time qPCR assays (Hagstrom, 2008; Hamilton, 2008). QPCR assays will be performed on a MX3000P qPCR system (Stratagene, La Jolla, CA) using a commercially available SYBR green mix (AB Gene, Rockford, IL). A cDNA pool containing 5 µL of cDNA from each sample from the far-field-sampled fish will be used as the ‘calibrator’ cDNA, for calculations of relative mRNA expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Mastermixes for P450arom, P450scc, 17β-HSD-1, StAR, and GAPDH (=normalizer) containing SYBR green, forward primer, and reverse primer, will be used, and each gene, normalizer (GAPDH) and calibrator PCR amplification will be run in triplicate, in addition to no-template controls and no-RT controls.

Data Analysis. Mean ± SEM values of each measured parameter, including for testicular enzyme mRNA and plasma hormones, will be compared among the various reference groups, the HTP outfall group, and experimentally-depurated groups, by ANOVA analyses using SigmaStat v.3.3 software

(SSPS Inc., Chicago IL). For the gene expression data, mean fold differences (relative to the calibrator) will be calculated for each group (Livak and Schmittgen, 2001). The reference site within Santa Monica Bay, or some other appropriate reference site, will be used to produce the calibrator cDNA pool. To assess the potential relationships between the gonadal mRNA expression for each enzyme and the plasma concentrations of each hormone (E1, E2, and T), correlation analyses will also be carried out (SigmaStat v.3.3 software).

DELIVERABLES

- 1) Electronic versions of the data generated by these studies
- 2) Summary tables and/or graphs
- 3) Report providing a summary of the findings and methods used
- 4) Professional conference presentations of the data, with CLAEMD recognition, authorship
- 5) Submittal of scientific articles for peer-reviewed publication, with CLAEMD authorship

ESTIMATED BUDGET

Task	Explanation	Est. #	Cost
Plasma hormone concentration analysis	Anticipated sample measurements Estimated #: 3 hormone measurements*/fish x 95 male samples (90 in Aim-1, 60 in Aim-2, 135 in Aim-3) =285 samples. [*E2, E1, T] \$35/sample (includes reagents, supplies, labor)	285	\$9,975
Gonadal gene expression analysis	Testicular steroidogenic gene expression analysis [qPCR for StAR, 17β-HSD-1, P450_{arom}] 95 samples, \$95/sample	95	\$9,025
Other Project Support Expenses	Additional required supplies (field & sample collection, prep., laboratory), data analysis, report preparation; student assistant salary		\$3,300
Total			\$22,300

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