Using gene expression to assess the status of fish from anthropogenically influenced estuarine wetlands

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Abstract

The diverse mixture of contaminants frequently present in estuaries complicates their assessment by routine chemical or biological analyses. We investigated the use of gene expression to assess contaminant exposure and the condition of southern California (USA) estuarine fish. Liver gene expression, plasma estradiol concentrations and gonad histopathology were used to study biological condition in longjaw mudsuckers (*Gillichthys mirabilis*). Metals, legacy organochlorine pesticides, polychlorinated biphenyls, and contaminants of emerging concern were detected in sediments and whole fish. Overall gene expression patterns were characteristic to each of four sites investigated in this study. Differentially expressed genes belonged to several functional categories including xenobiotic metabolism, detoxification, disease and stress responses. In general, plasma estradiol concentrations were similar among fish from all areas. Some fish gonads had pathologic changes (e.g., infection, inflammation) that could indicate weakened immune systems and chronic stress. The differential expression of some genes involved in stress responses correlated with the prevalence of histologic gonad lesions. This study indicates that gene expression is a promising tool for assessing the biological condition of fish exposed to environmental contaminants.

Introduction

In recent years, developments in gene expression analysis via microarray techniques have helped us to understand how fish health is impacted by anthropogenic stressors. Gene expression data can provide information regarding contaminant exposure and potential effects at higher biological levels (Garcia-Reyero *et al*. 2008). This type of information can be valuable to investigate the extent to which the presence of contaminants of emerging concern (CECs), such as current use pesticides or legacy contaminants may be impacting aquatic organisms. These data can be especially important in aquatic systems for which the fate and effect of many chemicals are largely unknown. Laboratory studies designed to explore the relationship of fish gene expression and contaminant exposure using single compounds or simple mixtures have been extensively investigated (Yadetie and Male 2002, Yu *et al*. 2010). However, the significance and applicability of gene expression data from wild fish exposed to environmental contaminant mixtures is still unclear.

The study of gene activity in sentinel fish is important because it provides information pertaining to the biological significance of contaminant exposure. Various methods are available to measure gene expression, but microarrays have gained popularity because they gauge changes in the expression of a large suite of genes, allowing

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simultaneous screening of multiple biological processes. As a result, gene microarrays could help to detect biological responses to a wide variety of contaminants. This tool could be particularly useful in aquatic systems where contaminant mixtures often complicate assessments using only chemical methods. Furthermore, effects caused by low chemical levels present in the environment may not be detected using routine toxicity assays examining a limited number of endpoints.

Previous studies in coastal areas of California (USA) found the presence of CECs and legacy contaminants in marine and freshwater systems (Schiff and Allen 2000, Schiff *et al*. 2001, Schlenk *et al*. 2005, Loraine and Pettigrove 2006). The occurrence of CECs such as pharmaceuticals, personal care products, and industrial compounds was recently characterized in southern California coastal marine systems (Bay *et al*. 2011). Some of these compounds were found in marine flatfish tissues (Kwon *et al*. 2009). Additionally, a previous study concluded that marine flatfish exposed to CECs had altered concentrations of plasma hormones and proteins (Rempel *et al*. 2006). However, the impact of anthropogenic contaminants in other southern California coastal systems such as estuarine wetlands has not been comprehensively studied, despite the fact that they receive contamination from diverse inputs (e.g., urban runoff; Hwang *et al*. 2008). Compared to other estuarine areas of the State, some of the highest metal and organic legacy sediment concentrations are found in southern California embayments, which include urban estuaries where monitoring efforts are limited (Schiff *et al*. 2006).

In this study, we used hepatic gene expression to assess biological responses of wild longjaw mudsuckers (*Gillichthys mirabilis*) from anthropogenically influenced wetlands. We investigated the relationships among gene expression responses, chemical exposure and additional biological endpoints. We studied estuarine wetlands with diverse contaminant characteristics (e.g., received different proportions of urban contaminant inputs; Hwang *et al*. 2006, Brown *et al*. 2010). To characterize chemical exposure, we analyzed sediment from areas where the fish were collected and whole fish tissue. We also examined the relationship of gene responses and effects at higher biological levels, such as changes in concentrations of plasma estradiol and gonad pathology.

Methods

Study Sites and Fish Collection

Four sites located in three southern California estuarine wetlands were investigated in this study (Figure 1). Two sites (CR and CM) were located at the Carpinteria Salt Marsh Reserve in Santa Barbara County. These stations were expected to have lower contaminant occurrence and concentrations than other stations in this study; however, contaminant exposure due to urban and agricultural runoff was anticipated. One of the Carpinteria Reserve sites (CR) had been previously dredged and used as a reference area by other investigators (Hwang *et al*. 2008). A third site (SB) was located in the Seal Beach National Wildlife Refuge, in Orange County. It was expected that this site would receive urban and agricultural runoff at a higher level than CR and CM because of its location in a larger watershed and chemical inputs from a nearby marina (GAO 1987). The fourth site (TE) was located in the Tijuana River Estuary in San Diego County. The TE site received urban and agricultural runoff in a watershed area similar to SB; however, TE was expected to have a greater degree of contamination due to periodic inflows of untreated wastewater sewage.

Longjaw mudsuckers were selected for this study because they are commonly found in California estuarine wetlands. These fish create sediment burrows and are exposed to contaminants by multiple routes including water, sediment and food (Gracey *et al*. 2001, Forrester *et al*. 2003, Anderson *et al*. 2006). A previous study found contaminant responses in

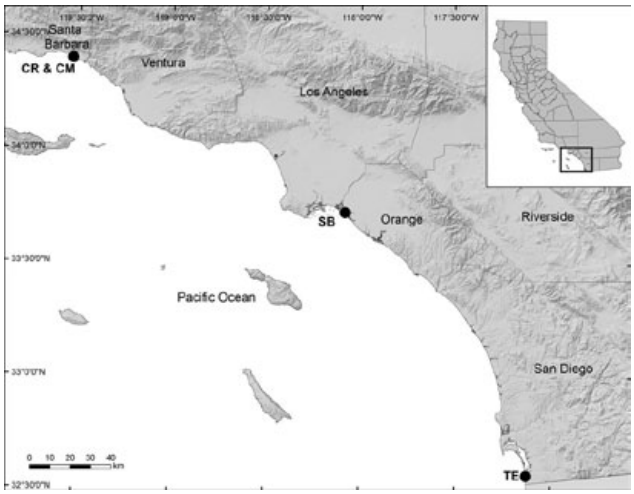

Figure 1. Map of study sites: Carpinteria Salt Marsh (CR and CM), Seal Beach National Wildlife Refuge (SB) and the Tijuana River Estuary (TE).

mudsuckers from contaminated areas (Anderson *et al*. 2006). Our field collections occurred between the last week of May and the first week of July, 2009. Fifteen adult fish were collected in minnow traps at each site, with the exception of TE where only eight fish could be collected. The desired number of fish per site was selected *a priori* to optimize effort and statistical power. Once retrieved from traps, fish were placed into buckets with fresh, aerated, *situ* water, in which they were anesthetized until they exhibited a lethargic behavior; we used 1 L of water and 0.2 g of MS-222 (Sigma-Aldrich, MO, USA) per fish.

Biological Indicators

Biological indicators studied in the fish included liver gene expression, plasma estradiol, and gonad histopathology. After the fish were anesthetized, a heparinized syringe was used to obtain blood from the caudal artery. Subsequently, the blood was placed in vials and centrifuged for plasma isolation. Following humane sacrifice via cervical dislocation, the male or female right side gonad was removed, weighed and preserved in Dietrich's solution for histopathologic analysis. The left side gonad was used for chemical analysis. Livers were removed, weighed, and thin slices were cut and preserved in RNAlater (Qiagen, Valencia, CA, USA) for gene expression analysis. The remaining sub-sampled livers and dissected fish were stored individually on dry ice for whole body chemical analysis. Method descriptions for hormone and histopathology analyses can be found in the SI.

Gene Microarray Analysis

Liver samples of individual fish were used to conduct gene expression analysis. Total ribonucleic acid (RNA) was extracted from each liver sample, converted into complementary deoxyribonucleic acid (cDNA), and amplified into complementary ribonucleic acid (cRNA) (Poynton *et al*. 2008a,b). Agilent's Quick Amp, one color labeling kit was used to fluorescently label samples (Agilent, CA, USA). For calculation of differential gene expression we compared site gene expression to a laboratory reference sample which consisted of CR mudsuckers kept under laboratory conditions for one month. The acclimation followed previously used conventions (Alvarado *et al*. 2005, Davoodi and Claireaux 2007). Attempts were made to provide conditions similar to those observed in the field including water temperature, and lighting regime.

All microarray data reported in this study are MIAME compliant (http://www.ncbi.nlm.nih.gov/geo/info/MIAME). Raw and normalized microarray data have been submitted to the GEO database (GSE28695). Additional, gene expression analysis methods including differential expression, normalization and clustering are shown in SI.

Chemical Indicators

Three replicates of the top five centimeters of sediment were collected from each site to characterize chemical exposure. In addition, composites of whole fish tissue were analyzed. Composites for each gender were made at each site. Each sample type was analyzed for a suite of metals and organics, including CECs (analytes and detection limits in Table SI-1).

Sediment and tissue dissolved metals analyses were conducted using EPA method 6020. The samples were digested using a sealed microwave digestion system and strong acids; once digested, the samples were filtered. Metal quantification was performed using internal standard calibration curves, with regression coefficients of at least 0.99. Calibration curve verifications were analyzed every twenty samples and were between 80 and 120% of the expected concentration. Duplicate and matrix spiked samples were also analyzed. Matrix spike samples were available for each target analyte and analyzed in the same manner as unspiked samples to determine whether any matrix interferences were present.

Organic samples were extracted using solvents and an accelerated extraction system (ASE). Deactivated silica gel or florisil column clean up was applied to each ASE extract, fractions were concentrated and quantified using Gas Chromatography-Mass Spectrometry (GC-MS), Gas Chromatography-Electron Capture Detector (GC-ECD) or High Performance Liquid Chromatography-Mass Spectrometry (HPLC/MS). For tissue samples, evaporation of the extract was conducted prior to GC-MS analysis (Table SI-2). Complete details of analytical methods used to analyze CECs are described in Das and Xia (2008), Xia *et al*. (2008), and Kwon *et al*. (2009) . Methods for PCBs, other legacy contaminants and current use pesticides were developed by MSCL.

Quality control measures for organics analyses consisted of procedural blanks, duplicate analyses, and spike analyses. Matrix samples were analyzed using reference sediments and catfish (*Ictalurus punctatus*) tissue samples. At least one laboratory spike and one laboratory blank were analyzed with every set of 10 environmental samples. Standard surrogates were used to determine analyte loss during extraction and processing. Standards were not available for each compound analyzed, but a variety of compound classes were represented with the isotopically labeled standards. Percent recoveries ranged from 80 to 120%.

Statistical Analysis

Clustering, enrichment analysis, analysis of variance and correlation techniques were used to analyze data. We applied hierarchical cluster analysis procedures from *cluster* R packages (www.Bioconductor.org) using Euclidean distance to compare the overall gene expression similarities among stations. Functional enrichment analysis of Gene Ontology terms was performed by Fisher's exact test using JMP Genomics version 5 (SAS, USA). Go terms were considered enriched when Fisher p-vales were <0.05.

Analysis of variance (ANOVA) was used to compare sediment and fish contaminant concentrations, fish size and weight, and plasma estradiol (E2) concentrations among sites. The one-way ANOVA ($p<0.05$) analysis was followed by a comparison among sites using the Tukey test. Spearman correlations were used to compare gene expression to E2 levels, or the prevalence of gonad pathologic changes ($p<0.05$). The ANOVA and correlation analyses were conducted with JMP version 8 (SAS, USA). To compare our sediment data with values from other estuaries, we used southern California estuary area-weighted means for trace metals and legacy organics (Schiff *et al*. 2006). With the sediment area-weighted means we calculated chemical enrichment ratios for sediment concentrations of cadmium, copper, lead, zinc, and DDTs (enrichment ratio= mean site concentration / area-weighted mean).

Results and Discussion

Chemical Analysis

Chemical analysis indicated the presence of diverse compounds in sediment with higher concentrations in SB and TE relative to CR and CM sites. DDTs and CECs such as plasticizers (phthalates), the surfactant nonylphenol (NP), the antimicrobial triclosan, and the ultraviolet filter oxybenzone were detected at all sites (Table 1). Among anthropogenic organics, phthalates had the highest sediment levels, average concentrations ranged from 200 to 517 µg/kg. Current use pesticides, flame retardants (PBDEs), and PCBs were not detected in sediments. Ranking of stations based on sediment concentrations showed that TE and SB were more contaminated than CM and CR, as we hypothesized based on the watershed characteristics for these sites (see Methods section). ANOVA revealed that phthalates and triclosan were present at significantly higher concentrations at TE ($p<0.05$) relative to other sites. The presence of triclosan at higher concentrations in TE may be the result of sewage contamination.

In general, contaminant levels were similar and low at all locations. Our chemical enrichment ratios showed lower sediment DDTs in SB and TE when compared to other southern California locations (CR concentrations were similar). Overall, sediment CEC concentrations were higher than those in samples collected from coastal shelf areas near southern California municipal wastewater discharges (Bay *et al*. 2011). Concentrations of NP were the exception. We found lower NP sediment concentrations when compared to sites near southern California wastewater discharges (Table 1).

We found evidence of tissue contamination at all sites. Ranking of stations demonstrated that SB and TE fish tissue had the highest chemical concentrations, which is consistent with the sediment patterns. Compounds detected in at least one station included PCBs, DDTs, the current use pesticide chlorpyrifos (CP), NP, PBDEs, oxybenzone and triclosan (Table 2). PCBs and PBDEs were only detected in SB and TE fish and were not detected in sediment. DDTs were detected in fish from all stations. TE males had the highest DDT concentrations. Chlorpyrifos was detected in males from all stations, but not in females. The highest average CP concentration was found in SB males. Nonylphenol was found at the highest concentration and averaged 1,080 µg/kg ww in SB tissue.

Tissue DDT and PCB concentrations were similar to mudsuckers from other California estuaries (Hwang *et al*. 2006). These concentrations were lower than those observed in marine flatfish offshore

Table 1. Means and standard deviations (SD) for all compounds detected in sediments. Units for metals reported as mg/kg. Units for industrial compounds (IC), personal care products (PCPs), and legacy pesticides reported as µg/kg. Reporting limits (RL) are also provided. Sediment chemical concentrations from other studies are provided for comparison. ND = Not detected. * = Southern California estuary area weight means used for enrichment calculations. ** = Bay *et al.* 2011

Analyte Type	Analyte	RL	CR		CM		SB		TE		Other Studies
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Metal	Cadmium	0.1	0.4	0.2	0.2	0.0	0.2	0.1	0.3	0.2	0.6 *
Metal	Copper	0.1	21.0	6.0	11.1	3.0	33.7	4.0	33.7	10.0	28.0 *
Metal	Led	0.1	17.7	2.4	8.9	2.0	35.0	2.0	18.1	9.0	19.0 *
Metal	Zinc	0.1	129.7	90.6	43.0	9.5	123.7	3.3	72.3	34.4	94.0 *
Legacy	PCBs	1.0	ND	-	ND	-	ND	-	ND	-	-
Legacy	DDTs	10.0	14.7	7.2	ND	-	11.3	2.3	10.3	0.6	8.8 **
CUPs	Chlorpyrifos	10.0	ND	-	ND	-	ND	-	ND	-	ND **
IC	Phthalates	30.0	200.0	0.0	516.7	333.8	316.7	104.1	500.0	55.7	256 **
IC	Nonylphenol	20.0	80.0	78.1	23.3	15.3	ND	-	80.0	62.5	108 **
IC	PBDEs	1.0	ND	-	ND	-	ND	-	ND	-	43 **
PCPs	Oxybenzone	0.5	0.7	0.7	1.2	0.8	1.8	0.5	0.7	0.7	ND **
PCPs	Triclosan	0.6	ND	-	ND	-	ND	-	1.7	0.4	5 **

of southern California (Table 2). Higher DDT and PCB levels in marine flatfish may be the result of exposure to historic contamination on the southern California shelf.

Most tissue CEC concentrations (except for PBDEs) were similar to or higher than those reported in other fish (Sapozhnikova *et al*. 2004, Kwon *et al*. 2009, Mottaleb *et al*. 2009, Ramirez *et al*. 2009). NP concentrations were highest in SB fish compared to other study sites. Mudsucker NP concentrations in SB fish were also relatively high when compared to offshore southern California marine fish (Table 2)

Table 2. Concentrations of all compounds detected in whole fish tissue composites of female (F) and male (M). Values for metals provided as mg/kg ww. Values for current use pesticides (CUP), industrial compounds (IC), personal care products (PCPs), and legacy organochlorine pesticides provided as µg/kg ww. Reporting limits (RL) are also provided. Tissue concentrations for southern California Marine Flatfish (hornyhead turbot; *Pleuronichthys verticalis*) are also provided for comparison. ND= Not detected. NA= Not analyzed.

Analyte Type	Analytes	RL	CR		CM		SB		TE		Marine Flatfish [8]	
			F	M	F	M	F	M	F	M	F	M
Metal	Copper	0.05	0.6	0.5	0.6	0.6	0.7	0.9	0.7	0.8	NA	NA
Metal	Lead	0.05	0.1	0.07	ND	ND	ND	ND	0.08	0.18	NA	NA
Metal	Zinc	0.05	19.2	17.1	17.8	16.7	19.4	19.1	18.6	21.6	NA	NA
Legacy	PCBs	1	ND	ND	ND	ND	3	3	3	27	766	998
Legacy	DDTs	2	31	37	8	16	14	13	25	54	13036	16225
CUP	Chlorpyrifos	10	ND	13	ND	12	ND	20	ND	16	11	10
IC	Nonylphenol	50	130	130	120	60	1230	930	480	640	56	148
IC	PBDEs	1	ND	ND	ND	ND	ND	ND	27	20	179	401
PCPs	Oxybenzone	0.4	9.8	8.8	9.5	12.2	27.3	12.6	7.7	10.0	ND	ND
PCPs	Triclosan	5.0	9.5	11.4	15.2	7.3	14.7	11.2	9.2	39.2	ND	ND

or fish from other regions (Hu *et al*. 2005, David *et al*. 2009, Mottaleb *et al*. 2009, USEPA 2009). The SB male mudsucker concentrations (1230 µg/kg) were higher than levels reported for other fish, which ranged from below detection to 325 µg/kg in wolffish (David *et al*. 2009). The higher NP concentrations in SB may represent NP contamination from urban inputs, but at this point the sources are unknown. NP has the potential to cause biological effects such as decreased fish size, as well as to cause alterations in reproduction (Ashfield *et al*. 1998). Nonylphenol appears to be a widely distributed contaminant in most coastal locations.

Biological Indicators

Longjaw mudsucker abundance varied among the wetland sites. To characterize these differences we calculated the catch per unit effort at each station (CPUE; calculated as the number of fish/ number of traps set). Fish collected at CR and CM had a higher CPUE (30 and 20 respectively), in contrast to SB (3.5) and TE (0.3). Fish were comparatively scarce at the SB and TE sites.

Lengths and weights of SB fish were significantly smaller ($p < 0.05$) on average as compared to fish from the other sites (Table SI-3). Sizes were statistically similar among fish collected at the other three sites. Smaller sizes at SB could indicate that these fish belong to a different age class (e.g., juveniles). Another possibility is that contaminants such as NP are affecting the size of SB fish. We cannot determine this from our study results, but we recommend further investigation since our analysis showed that fish size had a negative correlation with NP tissue concentrations (Spearman $r = -0.7$, $p < 0.05$). The size difference could have confounded the results. We did not normalize our data to fish size, since fish size was not correlated with the biological endpoints investigated (except sexual maturity and gonad infections in females).

Gene Expression

Microarray analysis indicated that gene expression patterns in mudsuckers were characteristic to each site (Figure 2). Hierarchical cluster results indicated that CR and CM females had the highest degree of similarity regarding their overall gene expression, and CR and TE males were the most similar (Figure SI-1). We identified 786 genes that were differentially expressed (adjusted $p < 0.05$) in fish from at least one field site relative to laboratory-held fish (used as laboratory reference samples). We selected 120 genes with greater than 2 fold differential expression for further analysis.

Gene expression results suggested that fish were exposed to environmental contaminants and could also reflect adverse responses affecting the fish biological condition. Several gene functional categories were common in differentially expressed genes, including xenobiotic metabolism, detoxification, immune and stress responses (Table 3). Cytochrome P450 subfamily 1A (*CYP1A*; involved in xenobiotic transport and metabolism), heat shock protein 20 (*HSP20*; involved in xenobiotic metabolism and other stress responses), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (*PFKF*; involved in xenobiotic metabolism), and cytochrome oxidase c (*COX8*; which participates in inflammatory responses and in xenobiotic metabolism) were upregulated in fish from at least one site. Interleukin-6 (*IL6*; which regulate immune responses), chemokine receptor (*CXCR*); epoxide hydrolyze 1 (*EPHX1*) and glutathione S-transferase (*GST*) that among other processes, are involved in xenobiotic metabolism, were downregulated in SB or TE fish.

Some compounds detected in this study have been found by other researchers to cause expression changes in genes that were differentially expressed in longjaw mudsuckers. For example, CP has been found to alter the production of interleukins (*IL*), and heat shock proteins mRNA in fish (Eder *et al*. 2009). In longjaw mudsuckers from the present study, we observed downregulation of *IL6* and upregulation of *HSP20* in fish from all stations, but a higher degree of differential expression was observed in SB fish, in which tissue CP levels were greatest. Other researchers found a link between PCB exposure and fish upregulation of *CYP1A* and *HSP* genes (Wiseman 2007). In this study, we observed a higher degree of upregulation of these genes in fish from SB and TE, which were the only fish with detectable levels of tissue PCB. We observed similar linkages for some other chemicals, yet, at this time we cannot attribute specific gene expression changes to individual compounds or contaminant mixtures.

The results of the functional enrichment analysis showed that the fish had gene changes in a variety of gene ontology (GO) terms corresponding to biological processes, molecular functions, and cellular components. The enriched GO terms represented a diversity of metabolic and physiologic

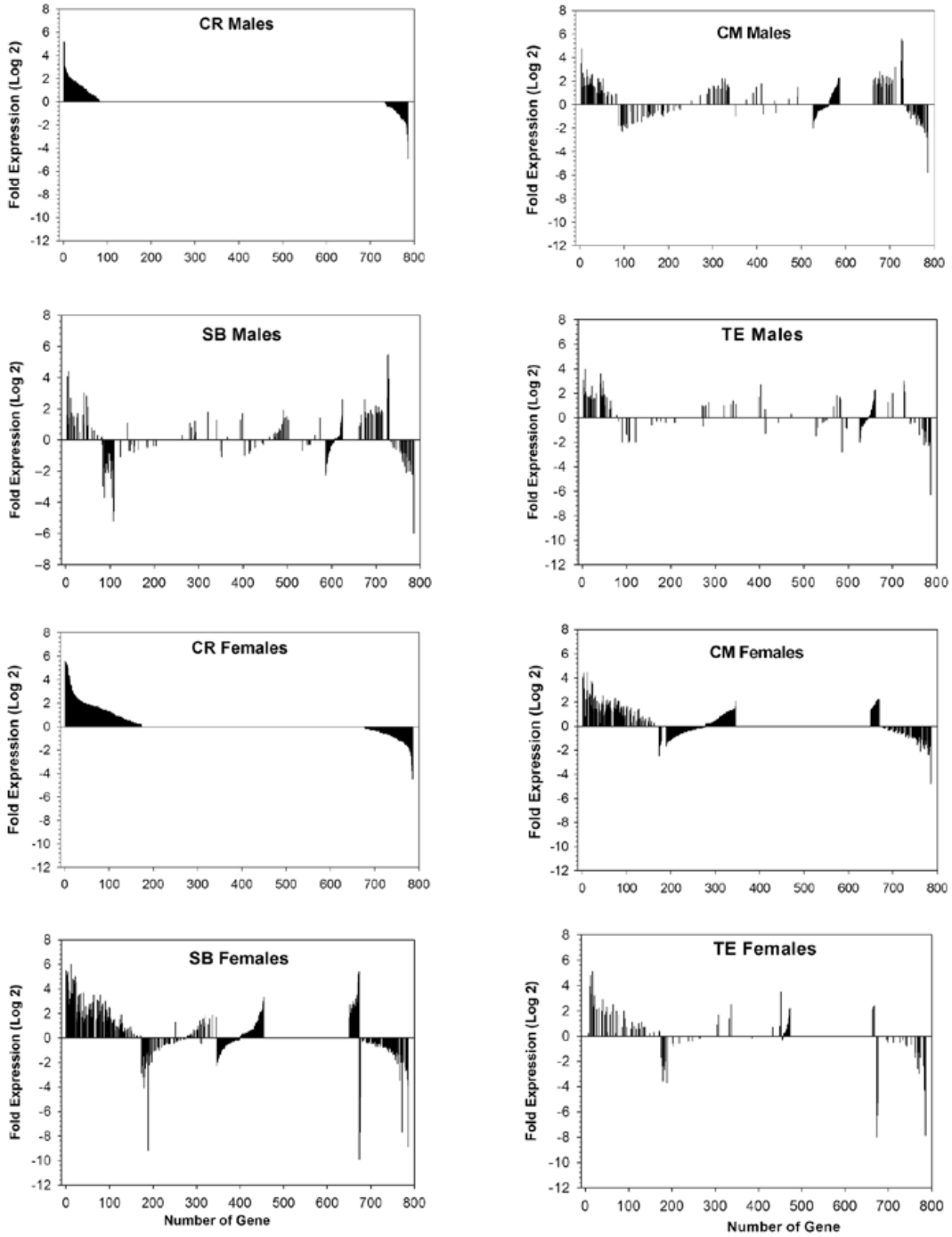

Figure 2. Liver gene expression (expressed as station averages of fold change over expression of laboratory reference samples) for longjaw mudsuckers. All genes were arranged from most upregulated to most downregulated for CR and the same order was kept for all the other stations. Genes with fold expression data that were not significant at p <0.05, were set to 0.

processes (Figure SI-2). The number and types of GO terms significantly enriched varied for each site. None of the enriched GO terms was common to fish from all sites. Both SB and TE fish had enrichment patterns which were very different from the CR and CM fish. A total of 54 GO categories were enriched at SB, 28 at CM, 19 at CR, and 6 at TE. Of these, 4 were unique to CR, 10 to CM, 27 to SB and 0 to TE fish. For CR, CM and SB sites, more than half of enriched categories were upregulated (CR = 84%, CM = 64%, SB = 67%), but only 33% for TE. These results correspond to the individual gene analysis results showing expression patterns that were characteristic to each site.

Table 3. Fold changes (shown as the station mean of the log_2 transformed values) for selected genes involved in different biological processes. For each gene the highest male (M) or female (F) fold change values are highlighted in bold.

Biological Process[1]	Gene Name	Carpinteria Salt Marsh				Seal Beach Refuge		Tijuana Estuary	
		CR F	CR M	CM F	CM M	F	M	F	M
Detoxification	Glutathione S-transferase	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**-2.0**
Immunity	Complement component 1, q	1.9	0.0	0.0	0.0	0.0	0.0	**2.3**	0.0
Immunity	Interleukin 6	-1.6	0.0	-1.9	0.0	**-7.7**	**-5.2**	-3.0	0.0
Immunity	like RAS p21 protein activator 2	**2.5**	**2.8**	0.0	1.5	2.1	1.0	2.1	1.9
Immunity	Purine nucleoside phosphorylase	1.5	2.3	1.6	2.3	**2.2**	**2.7**	0.0	0.0
Immunity & inflammation	like C-type lectin domain family 12	0.0	0.0	0.0	0.0	**2.3**	0.0	0.0	0.0
Immunity & inflammation	like TSC22 domain family	0.0	0.0	0.0	0.0	**2.1**	**1.3**	0.0	0.0
Immunity & inflammation	Tumor necrosis factor	0.0	0.0	0.0	0.0	**2.2**	0.0	0.8	0.0
Infection	like trypsin X3	0.0	0.0	0.0	0.0	**5.4**	0.0	0.0	0.0
Infection & stress	Hepcidin antimicrobial peptide	-1.5	0.0	-1.8	0.0	**-3.5**	**-3.7**	-2.6	0.0
Inflammation	Elastase, neutrophil expressed	0.0	0.0	0.0	0.0	**4.5**	0.0	0.0	0.0
Inflammation	Immunoglobulin heavy constant mu	2.4	0.0	2.5	0.0	**3.5**	**1.9**	0.0	0.0
Inflammation & parasitism	like Carboxypeptidase N, polypeptide 1	1.1	1.1	**1.5**	**2.2**	**1.5**	0.0	0.0	0.0
Infection & parasitism	Chemokine (C-X-C motif) receptor	0.0	0.0	-2.5	-2.5	**-2.9**	**-2.9**	0.0	0.0
Stress	Cancer susceptibility candidate	0.0	0.0	0.0	0.0	**-2.3**	0.0	0.0	**2.7**
Xenobiotic metabolism	Cytochrome P450, family 1, subfamily A	1.7	0.0	2.3	0.0	**3.0**	**1.8**	0.0	0.0
Xenobiotic metabolism	Epoxide hydrolase 1, microsomal (xenobiotic)	0.0	0.0	0.0	0.0	0.0	-2.3	0.0	**-2.8**
Xenobiotic metabolism	Phosphofructokinase, platelet	**2.0**	1.8	1.3	**2.5**	1.3	1.4	0.0	1.6
Xenobiotic metabolism & inflammation	Cytochrome c oxidase subunit 8	0.0	0.0	1.3	0.0	1.9	0.0	**2.5**	0.0
Xenobiotic metabolism & stress	Heat shock protein 20	1.4	0.0	0.0	0.0	**1.8**	0.0	**2.0**	0.0
Xenobiotic metabolism, immunity & inflammation	Complement component 3	2.0	0.0	2.1	**1.3**	**2.6**	0.0	0.0	0.0

[1]The genes included may be involved in additional biological processes not discussed in this manuscript

Female Plasma Estradiol

Lower plasma E2 concentrations were found in SB females. Averaged E2 concentrations ranged from below detection to 1281 pg/ml at TE (Figure 3). Since the females were collected at the same time of the year and during their reproductive cycle (West and Zedler 2000), we expected similar plasma E2 concentrations. Estradiol concentration differences could be related to factors such as size, sexually maturity state and anthropogenic contamination (Giesy *et al*. 2003). We found no statistical correlation between fish size and E2 levels. CR and CM females were at maturing, mature or post spawning stages. While SB and TE females were immature (sexual maturity descriptions provided in SI). The lower E2 concentrations in SB females could be the result of sexual immaturity. However, E2 levels in TE females (also sexually immature) were comparable to those of CR and CM females (which were sexually mature). We do not know what typical E2 concentrations are in immature or mature mudsucker females. These are some of the first measurements of E2 levels in this species and more data are needed before we can determine the cause of these results.

The expression of nine genes of diverse metabolic function was significantly correlated with female plasma E2 concentrations in longjaw mudsuckers. Genes correlated included trypsinogen precursor, zinc-dependent metalloprotease, trypsin-like serine protease, coagulation factors and DNA/RNA non-specific endonucleases. Spearman rank correlations (r) ranged from 0.3 to 0.4, while the p-values ranged from 0.003 to 0.049.

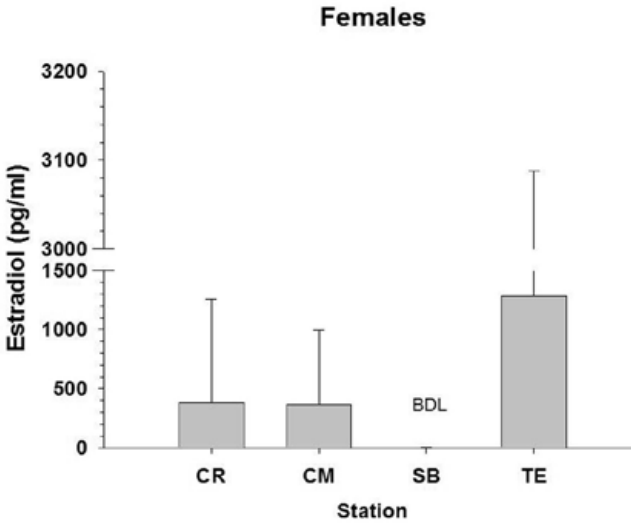

Figure 3. Mean concentrations (+ standard deviation) of estradiol in female longjaw mudsuckers. Estradiol (E2) concentrations in females from SB were below detection limits (BDL).

Gonad Pathologies

Fish from all stations had gonad pathologies. A higher proportion of fish from SB and TE presented such lesions. Pathologies included microbiological infections, inflammatory responses to infection, non-specific inflammation, and fibrovascular proliferation associated with inflammation (Table 4). Morphologic changes specific for endocrine disruption were not observed. Microsporidian infections were found in males and females, but a higher proportion of females were affected (Figure 4). Microsporidia formed large, lobose xenomas in female ovaries, but appeared to be primarily contained within macrophages in male testes. Microsporidian spores were ovoid and ranged from approximately 2.5 to 4 microns in diameter; such spores, and the pattern of xenoma formation, were morphologically consistent with *Ichthyosporidium giganteum*. In most infected females, the microsporidian infection was graded as severe, as it replaced nearly all of the gonad parenchyma in the histologic sections. Infections had negative correlations with fish length and sexual maturity (Spearman r = -0.3 and -0.5 respectively, $p < 0.05$).

A higher number of fish from the SB and TE stations had microsporidia in their gonads (percentages of fish infected at these stations ranged from 18 to 36%). Parasites such as nematodes and trematodes were also found in the gonads of some fish from CM, SB and TE. Granulomatous responses, perhaps caused by such infections, were found in females from TE and in males from CR, CM and TE. Fibrovascular proliferation associated with inflammation was found in males from CM and SB. Fish with gonad infections had a greater magnitude of additional types of pathologies (Spearman $r=0.8$, $p<0.05$).

We found significant Spearman correlations between gonad pathologies and 75 genes. Spearman correlation r values ranged from -0.5 to 0.5, while p values ranged from 0.002 to 0.044. Several of those correlated genes corresponded to genes known to respond to infection and inflammation, as well as immune and stress responses (Table 5). None of the genes that correlated with the gonad pathologies also correlated with plasma E2. Of particular

Table 4. Percentage of male and female longjaw mudsuckers with gonad abnormalities at each station.

Abnormality	Males				Females			
	CR N = 6	CM N = 4	SB N = 4	TE N = 5	CR N = 9	CM N = 11	SB N = 11	TE N = 3
Microsporidiosis	1	0	0	20	0	18	36	0
Endoparasitism	1	0	25	20	0	18	36	33
Fibrovascular Proliferation	0	0	0	0	0	9	18	0
Granulomatous Inflammation	0	0	0	20	11	9	0	33
Other Inflammation	0	0	0	20	11	18	0	33

interest was the unexpected downregulation of *IL-6*, a Chemokine receptor and hepcidin anti-microbial peptide, that would be anticipated to be upregulated in response to infection (Hu *et al*. 2007 , Alvarez-Pellitero 2008, Fischer *et al*. 2008, Rodriguez-Tovar *et al*. 2011).

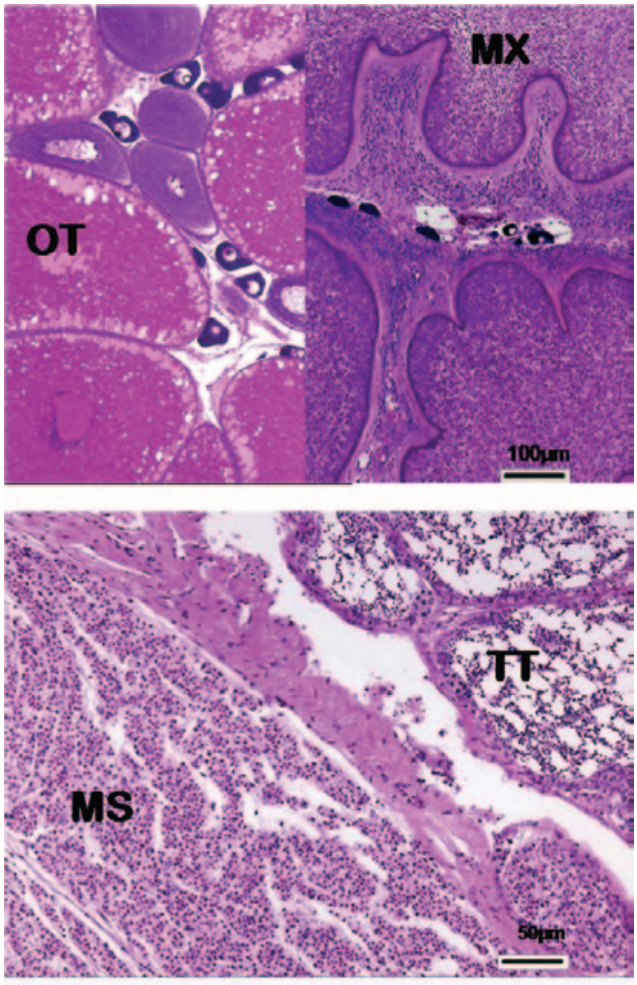

Figure 4. Normal ovarian tissue (OT) of a CR female and ovarian tissue of a SB female with microsporidian xenomas (MX; Top). Uninfected testicular tissue (TT) located adjacent to testicular tissue inhabited by phagocytized microsporidian spores (MS) in a TE male (Bottom).

The observation of infectious and inflammatory gonad conditions in some of the fish suggests that these animals may have had weakened immune systems. We hypothesize that inappropriate downregulation of immune related genes may play a role in this susceptibility. Of the histopathologic findings, the microsporidian infections stand out because of the severe degree of gonad tissue compromise associated with some infections. The presence of massive xenomas and/or xenoma-induced necrosis in the gonads of two thirds of affected fish suggests that this type of infection had the potential to negatively affect fish reproduction (Williams 2009). Infected ovaries tended to have relatively lower maturation scores (data not shown) than uninfected ovaries, which was likely due to the concomitant microsporidian infections. Combined gonad infection and immunologic responses can affect reproductive ability (Williams 2009).

There was no relationship between gonad infection and E2 levels in affected fish. Other researchers have found that microsporidia and other parasites can alter female fish E2 production (Jobling and Tyler 2003, Trubirohaa *et al*. 2010). Nevertheless, spearman correlation results of longjaw mudsucker gonad infections and E2 concentrations were not significant.

Anthropogenic stressors such as contamination may have contributed to the results of this study. However, no direct causal associations can be made from the results of this study. We observed a relationship between biological responses (e.g., differential gene expression and gonad pathologies) and greater chemical concentrations in SB and TE

Table 5. Spearman correlations between gonad pathologies and selected genes involved in xenobiotic metabolism, immunity, stress response, inflammation and infection responses. A total of 120 genes were used for the correlation analysis.

Gonad Pathology	Gene Name	Spearman r	Probability
Microsporidia	Complement component 3	0.4	0.015
Microsporidia	Chemokine (C-X-C motif) receptor	-0.4	0.019
Microsporidia	Immunoglobulin heavy constant mu	0.4	0.025
Microsporidia	Interleukin 6	-0.4	0.037
Microsporidia	like C-type lectin domain family 12	0.5	0.002
Microsporidia	like RAS p21 protein activator 2	0.3	0.044
Microsporidia	Tumor necrosis factor	0.4	0.007
Fibrovascular Proliferation	Chemokine (C-X-C motif) receptor	-0.3	0.040
Fibrovascular Proliferation	Hepcidin antimicrobial peptide	-0.4	0.020
Fibrovascular Proliferation	like C-type lectin domain family 12	0.3	0.033
Fibrovascular Proliferation	Tumor necrosis factor	0.3	0.029
Inflammation	Purine nucleoside phosphorylase	-0.5	0.004

fish as compared to CR and CM fish. The results may also have been influenced by other variables not investigated during this study, such as changes in temperature, eutrophication and habitat quality. Additional studies are needed to determine the specific causes of the biological responses observed in this study.

Gene expression data can provide an insight into the health of organisms at a site and provide information to determine if the fish have been exposed to contaminants. This work has provided information regarding the relationship between contaminant presence, gene expression, and biological response. However, limitations in the chemicals analyzed and genomic data restricted our ability to interpret the results. Chemical analyses were done on tissue composites and included a partial suite of analytes, which hindered our ability to relate the chemistry data to biological endpoints. In addition, gene functional analysis was limited by incomplete function information to explore relations between stressors and gene responses, laboratory dose-response studies are needed to obtain such information. It is also difficult to differentiate gene expression that represents "adverse" responses connected to impacts at the organism and population levels. Linking gene expression changes to adverse effects on the survival or reproduction of fish would be an important step towards more predictive and complete assessments.

Literature Cited

Alvarado, N.E., A. Buxens, L.I. Mazon, I. Marigomez and M. Soto. 2005. Cellular biomarkers of exposure and biological effect in hepatocytes of turbot (Scophthalmus maximus) exposed to Cd, Cu and Zn and after depuration. *Aquatic Toxicology* 74:110-125.

Alvarez-Pellitero, P. 2008. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary Immunology and Immunopathology* 126:171-198.

Anderson, S.L., G.N. Cherr, S.G. Morgan, C.A. Vines, R.M. Higashi, W.A. Bennett, W.L. Rose, A.J. Brooks and R.M. Nisbet. 2006. Integrating contaminant responses in indicator saltmarsh species. *Marine Environmental Research* 62:S317-S321.

Ashfield, L.A., T.G. Pottinger and J.P. Sumpter. 1998. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environmental Toxicology and Chemistry* 17:679-686.

Bay, S.M., D.E. Vidal-Dorsch, D. Schlenk, K. Kelley, M. Baker, K. Maruya and J. Gully. 2011. Sources and Effects of Endocrine Disruptors and other Contaminants of Emerging Concern in the Southern California Bight Coastal Ecosystem. Technical

Report 650. Southern California Coastal Water Research. Costa Mesa, CA.

Brown, J.S., M. Sutula, C. Stransky, J. Rudolph and E. Byron. 2010. Sediment contaminant chemistry and toxicity of freshwater urban wetlands in southern California. *Journal of the American Water Resources Association* 46:367-384.

Das, K. and K. Xia. 2008. Transformation of 4-nonylphenol isomers during biosolids composting. *Chemosphere* 70:761-768.

David, A., H. Fenet and E. Gomez. 2009. Alkylphenols in marine environments: distribution monitoring strategies and detection considerations. *Marine Pollution Bulletin* 58:953-960.

Davoodi, F. and G. Claireaux. 2007. Effects of exposure to petroleum hydrocarbons upon the metabolism of the common sole Solea solea. *Marine Pollution Bulletin* 54 928-934.

Eder, K., C. Leutenegger, H. Kohler and I. Werner. 2009. Effects of neurotoxic insecticides on heat-shock proteins and cytokine transcription in Chinook salmon (*Oncorhynchus tshawytscha*). *Ecotoxicology and Environmental Safety* 72:182-190.

Fischer, J., C. Suire and H. Hale-Donze. 2008. Toll-Like Receptor 2 Recognition of the Microsporidia Encephalitozoon spp. Induces Nuclear Translocation of NF-B and Subsequent Inflammatory Responses. *Infection and Immunity* 76:4737-4744.

Forrester, G.E., B.I. Fredericks, D. Gerdeman, B. Evans, M.A. Steele, K. Zayed, L.E. Schweitzer, I.H. Suffet, R.R. Vance and R.F. Ambrose. 2003. Growth of estuarine fish is associated with the combined concentration of sediment contaminants and shows no adaptation or acclimation to past conditions. *Marine Environmental Research* 56:423-442.

General Accounting Office (GAO), United States. 1987. Wildlife Management National Refuge Contamination Is Difficult to Confirm and Clean Up. United States General Accounting Office. Washington DC.

Garcia-Reyero, N., I. Adelman, L. Liu and N. Denslow. 2008. Gene expression profiles of fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota. *Marine Environmental Research* 66:134-136.

Giesy, J.P., E.M. Snyder, K.M. Nichols, S.A. Snyder, S.A. Villalobos, P.D. Jones and S.D. Fitzgerald. 2003. Examination of reproductive endpoints in goldfish (*carassius auratus*) exposed in situ to municipal sewage treatment plant Effluent discharges in Michigan, USA. *Environmental Toxicology and Chemistry* 22:2416-2431.

Gracey, A.Y., J.V. Troll and G.N. Somero. 2001. Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. *Proceedings of the National Academy of Sciences of the United States of America* 98:1993-1998.

Hu, J., F. Jin, Y. Wan, M. Yang, L. An, W. An and S. Tao. 2005. Trophodynamic behavior of 4-nonylphenol and nonlyphenol polyethoxylate in a marine aquatic food web from Bohai Bay, North China: comparison to DDTs. *Environmental Science and Technology* 39:4801-4807.

Hu, X., A.C. Camus, S. Aono, E.E. Morrison, J. Dennis, K.E. Nusbaum, R.L. Judd and J. Shi. 2007 Channel catfish hepcidin expression in infection and anemia. *Comparative Immunology, Microbiology and Infectious Diseases* 30:55-69.

Hwang, H.M., P.G. Green and T.M. Young. 2006. Tidal salt marsh sediment in California, USA. Part 1: Occurrence and sources of organic contaminants. *Chemosphere* 64:1383-1392.

Hwang, H.M., P.G. Green and T.M. Young. 2008. Tidal salt marsh sediment in California, USA: Part 3. Current and historic toxicity potential of contaminants and their bioaccumulation. *Chemosphere* 71:2139-2149.

Jobling, S. and C.R. Tyler. 2003. Endocrine disruption, parasites and pollutants in wild freshwater fish. *Parasitology* 126 S103-108.

Kwon, J.-W., K.L. Armbrust, Vidal-Dorsch, S.M. Bay and K. Xia. 2009. Determination of 17 alpha-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone in fish livers. *Journal of AOAC International* 92:399-409.

Loraine, G.A. and M.E. Pettigrove. 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. *Environmental Science and Technology* 40.

Mottaleb, M.A., S. Usenko, J.G. O'Donnell, A.J. Ramirez, B.W. Brooks and C.K. Chambliss. 2009. Gas chromatography-mass spectrometry screening methods for select UV filters, synthetic musks, alkylphenols, an antimicrobial agent and an insect repellent in fish. *Journal of Chromatography A* 1216:815-823.

Poynton, H.C., A.V. Loguinov, J.R. Varshavsky, S. Chan, E.J. Perkins and C.D. Vulpe. 2008a. Gene expression profiling in Daphnia magna Part I: Concentration-dependent profiles provide support for the no observed transcriptional effect level. *Environmental Science and Technology* 42:6250-6256.

Poynton, H.C., R. Zuzow, A.V. Loguinov, E.J. Perkins and C.D. Vulpe. 2008b. Gene expression profiling in Daphnia magna Part II: Validation of a copper specific gene expression signature with effluent from two copper mines in California. *Environmental Science and Technology* 42:6257-6263.

Ramirez, A.J., R.A. Brain, S. Usenko, M.A. Mottaleb, J.G. O'Donnell, L.L. Stahl, J.b. Wathen, B.D. Snyder, J.L. Pitt, P. Perez-Hurtado, L.L. Dobbins, B.W. Brooks and C.K. Chambliss. 2009. Occurrence of pharmaceuticals and personal care products in fish: Results of a national pilot study in the United States. *Environmental Toxicology and Chemistry* 28:2587-2597.

Rempel, M.A., J. Reyes, S. Steinert, W. Hwang, J. Armstrong, K. Sakamoto, K. Kelley and D. Schlenk. 2006. Evaluation of relationships between reproductive metrics, gender and vitellogenin expression in demersal flatfish collected near the municipal wastewater outfall of Orange County, California, USA. *Aquatic Toxicology* 77:241-249.

Rodriguez-Tovar, L.E., D.J. Speare and R.J.F. Markham. 2011 Fish microsporidia: Immune response, immunomodulation and vaccination. *Fish Shellfish Immunology* 30:999-1006.

Sapozhnikova, Y., O. Bawardi and D. Schlenk. 2004. Pesticides and PCBs in sediments and fish from the Salton Sea, California, USA. *Chemosphere* 55:797-809.

Schiff, K. and M.J. Allen. 2000. Chlorinated hydrocarbons in flatfishes from the Southern California, USA, Bight. *Environmental Toxicology and Chemistry* 19:1559-1565.

Schiff, K., K.A. Maruya and K. Christensen. 2006. Southern California Bight 2003 Regional Monitoring Program: II. Sediment Chemistry. Southern California Coastal Water Research Project. Westminster, CA.

Schiff, K.C., S.M. Bay and C. Stransky. 2001. Characterization of stormwater toxicants from an urban watershed to freshwater and marine organisms. pp. 71-84 *in*: S.B. Weisberg and D. Elmore (eds.), Southern California Coastal Water Research Project. Annual Report 1999-2000. Southern California Coastal Water Research Project. Westminster, CA.

Schlenk, D., Y. Sapozhnikova, M.A. Irwin, L. Xie, W. Hwang, S. Reddy, B.J. Brownawell, J. Armstrong, M. Kelly, D.E. Montagne, E.P. Kolodziej, D. Sedlak and S. Snyder. 2005. *In vivo* bioassay-guided fractionation of marine sediment extracts from the Southern California Bight, USA, for estrogenic activity. *Environmental Contamination and Toxicology* 24:2820-2826.

Trubirohaa, A., H. Kroupovaa, S. Wuertzc, S.N. Franka, B. Suresd and W. Kloas. 2010. Naturally-induced endocrine disruption by the parasite Ligula intestinalis (*Cestoda*) in roach (*Rutilus rutilus*). *General and Comparative Endocrinology* 166:234-240.

United States Environmental Protection Agency (USEPA). 2009. The National Study of Chemical Residues in Lake Fish Tissue. USEPA, Office of Water. Washington, DC.

West, J.M. and J.B. Zedler. 2000. Marsh-creek connectivity: Fish use of a tidal salt marsh in southern California. *Estuaries* 23:699-710.

Williams, B.A.P. 2009. Unique physiology of host–parasite interactions in microsporidia infections *Cellular Microbiology* 11:1462-5822

Wiseman, S. 2007. Characterization of AhR signaling and the impact of polychlorinated biphenyls on the adaptive responses to stress in fish. University of Waterloo. Ontario, Canada.

Xia, K., M.B. Luo, C. Lusk, K. Armbrust, L. Skinner and R. Sloan. 2008. Polyhrominated diphenyl ethers (PBDEs) in biota representing different trophic levels

of the Hudson River, New York: From 1999 to 2005. *Environmental Science & Technology* 42:4331-4337.

Yadetie, F. and R. Male. 2002. Effects of 4-nonylphenol on gene expression of pituitary hormones in juvenile Atlantic salmon (*Salmo salar*). *Aquatic Toxicology* 58:113-129.

Yu, L., J. Deng, X. Shi, C. Liu, K. Yu and B. Zhou. 2010. Exposure to DE-71 alters thyroid hormone levels and gene transcription in the hypothalamic-pituitary-thyroid axis of zebrafish larvae. *Aquatic Toxicology* 97:226-233.

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Supplemental Information

Supplemental information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2011AnnualReport/ar11_SupplementalInfo_GeneExpressionWetlands.pdf