
Annual and seasonal evaluation of reproductive status in hornyhead turbot at municipal wastewater outfalls in the Southern California Bight

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ABSTRACT

Over a billion gallons of treated wastewater effluent is discharged into the coastal waters of the Southern California Bight (SCB) daily, containing chemicals that target the endocrine system of aquatic organisms. Further, many areas within the SCB are contaminated with historical discharges of DDT and PCBs that can also cause endocrine effects in aquatic organisms. This study investigated changes in indicators of reproduction and environmental estrogen exposure in flatfish near wastewater outfalls. Hornyhead turbot (*Pleuronichthys verticalis*) were collected from four discharge areas, two farfield stations, and a reference location in the SCB during May-June 2006 to examine spatial patterns. Quarterly samples were also collected between May-June 2006 and February 2007 to investigate temporal patterns in reproductive indicators. Fish from the Orange County outfall farfield site were often younger and less sexually mature than fish from other sites. Sex ratio was significantly male or female skewed in some hornyhead turbot samples from the outfall sites, as well as from the Dana Point reference site. However, no consistent pattern in sex ratio was present over

time. Low-level induction of the egg yolk protein vitellogenin (vtg) in males was frequently observed in male hornyhead turbot from all sites, suggesting widespread ongoing exposure to estrogenic compounds. The source of this exposure could not be determined from the data since there was no pattern related to outfall proximity, effluent type, or time of year. Male vtg concentrations did not appear to be impacting reproductive function, as there was no incidence of related gonad abnormalities (ova-testis). Analysis of historical hornyhead turbot trawl catch data indicated that populations are either increasing or stable in the SCB, thus environmental estrogen exposure was not adversely impacting fish abundance. Additional research is needed to determine the cause of the estrogenic response in hornyhead turbot, and whether the source of the estrogenic compounds is a consequence of historical contamination of the SCB, ongoing sources, or representative of an uncommon species-specific natural condition.

INTRODUCTION

Southern California is one of the most populated regions in the United States discharging approximately 1.1 billion gallons of treated

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wastewater effluent per day into the coastal waters of the Southern California Bight (SCB; Schiff *et al.* 2000). Wastewater undergoes various degrees of treatment that contain an array of unregulated chemicals, some of which target endocrine systems of aquatic organisms. Further, sediments offshore of the Palos Verdes Peninsula and within Santa Monica Bay are contaminated with DDT and PCBs discharged through wastewater outfalls between the early 1940's and early 1970's. The resulting Palos Verdes Shelf Superfund site encompasses approximately 15 square miles from depths between 30 and 200 m, but these chemicals can be detected at varying concentrations throughout the entire SCB. These compounds are also known to impact endocrine and reproductive systems of aquatic organisms and represent a potential confounding factor to understanding impacts of endocrine disrupting chemicals in current discharges.

Hornyhead turbot (*Pleuronichthys verticalis*) have been used as a sentinel species in monitoring potential impacts of endocrine disrupting compounds (EDCs) in wastewater discharges on aquatic organisms in the SCB since 2000 (Roy *et al.* 2003, Rempel *et al.* 2006, Deng *et al.* 2007). Hornyhead turbot are found in coastal areas with soft-bottom substrates at a depth of 10 to 90 m. They are typically found buried in sand for concealment and primarily feed on polychaetes and clam siphons (Allen *et al.* 1998, Allen 2006). These life history and feeding preferences of hornyhead turbot maximize exposure to sediment-associated pollutants making this species an ideal bio-indicator of fish reproductive health for monitoring programs.

Estrogenic activities were first observed in male hornyhead turbot collected near the Orange County Sanitation District (OCS) outfall in 2000 (Roy *et al.* 2003, Schlenk *et al.* 2005). Schlenk *et al.* (2005) reported sediment extracts from the OCS and Sanitation Districts of Los Angeles County (LACS) outfalls induced estrogenic responses in hornyhead turbot. Subsequent studies showed that hornyhead turbot collected near the OCS outfall had elevated blood plasma vitellogenin (vtg) concentrations and a skewed gender ratio toward males (Rempel *et al.* 2006). However, a study by Deng *et al.* (2007) found limited reproductive impacts in hornyhead turbot collected from the OCS outfall.

The aim of the current project is to expand previous studies by conducting a spatial study investigating hornyhead turbot at several sites in the

SCB during a single sampling event in May-June 2006, and a temporal study between May 2006 and February 2007. Skewness of gender ratio was examined in addition to plasma vtg levels and gonadal development and histopathology at sites near four of the largest wastewater discharges and at a reference site not associated with a large wastewater discharge. Long-term annual monitoring data from the sites were also examined to assess impacts on hornyhead turbot populations.

METHODS

Study Sites

Hornyhead turbot were collected near the outfalls of the four major wastewater treatment plants within the SCB (Figure 1): Hyperion Wastewater Treatment Plant (LA) operated by the City of Los Angeles, Joint Water Pollution Control Plant operated by the Sanitation Districts of Los Angeles County (PV), Orange County Sanitation District (OC), and Point Loma Wastewater Treatment Plant operated by the City of San Diego (SD). Hornyhead turbot were also collected from two farfield stations down current of the Sanitation Districts of Los Angeles County (PVF) and Orange County Sanitation District (OCF) outfalls. In addition, fish were also sampled from a reference site near Dana Point (DP) that does not

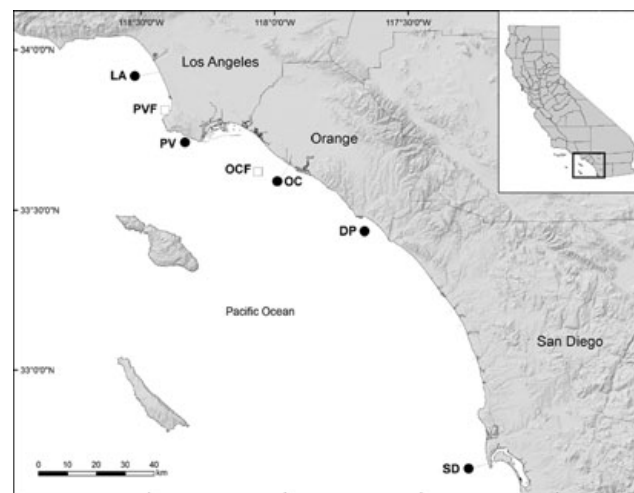


Figure 1. Location of wastewater outfall, farfield, and reference sampling sites in the Southern California Bight. City of Los Angeles outfall (LA), Sanitation Districts of Los Angeles County Farfield (PVF), Sanitation Districts of Los Angeles County Outfall (PV), Orange County Sanitation District Farfield (OCF), Orange County Sanitation District Outfall (OC), Dana Point reference (DP), and Point Loma Wastewater Treatment Plant Outfall (SD).

have a major wastewater effluent discharge in the area and has historically been used as a reference site for the region.

The sites sampled represent different combinations of effluent treatment and historical contamination. The LA, PV, and PVF locations received 100% secondary treated wastewater, and sediments in these areas contained relatively high legacy contamination from DDT and PCBs. The OC and OCF sites received an effluent which was comprised of 50% primary treated and 50% secondary treated wastewater effluent and contained relatively low legacy sediment contamination. The SD site received advanced primary treated effluent and had little legacy contamination. Finally, the DP area was distant from large wastewater discharges and had little legacy contamination (Vidal-Dorsch *et al.* 2011).

Fish Collection

To document spatial patterns, hornyhead turbot were sampled during a single sampling event between the end of May and beginning of June 2006 at seven locations within the SCB (LA, PVF, PV, OCF, OC, DP, and SD). Fish were also collected quarterly to document temporal and small-scale spatial variations over the reproductive cycle during spring (May/June 2006), summer (August 2006), fall (November 2006), and winter (January/February 2007) at five locations (PVF, PV, OCF, OC, and DP). Samples from the first sampling event (May/June 2006) were the same as those collected for the spatial study.

Fish were collected using a 7.6 meter-wide semiballoon otter trawl. To minimize variation in trawling patterns at different time points during the study, a differential Global Positioning System (dGPS) was used to accurately locate the sampling sites and to control the trawling speed at 50 to 60 m/minute. Upon retrieval of the trawl net, fish were weighed to the nearest gram and standard length was measured to the nearest millimeter. Fish >15 cm were targeted for sampling in order to focus on mature fish. However, smaller fish were included in some collections when a sufficient number of the target size fish were not available. Fish age was determined by otolith examination for a subset (580) of fish. Subsequently, gender-specific second order regressions based on the otolith data were used to calculate the age for all fish.

Blood was collected from the dorsal vein using a heparinized syringe with a 22-gauge needle. Blood plasma was collected after centrifugation for two minutes at 500 rpm with a portable centrifuge and placed on dry ice and then transferred to -80°C until measurement of vtg concentration. Exsanguinated fish were then sacrificed by transecting the spinal cord posterior to the head. One of the paired gonads was removed and weighed to the nearest gram ($\frac{1}{2}$ gonad wet weight). Gonadosomatic index (GSI) was calculated using the following formula: $\frac{1}{2}$ gonad wet weight/total body wet weight X 100; all GSI data presented are reported as $\frac{1}{2}$ GSI since only a single gonad was weighed. The other gonad was preserved for histological analysis.

Plasma Vitellogenin Analysis

Vitellogenin was measured directly from the blood plasma following Rempel *et al.* (2006). Briefly, the wells of a 96-well plate were coated with either 1% milk for non-specific binding wells or 100 μ l of 0.8 μ g/ml California halibut vtg in 50 mM carbonate buffer, and incubated at 37°C for 2 hours. The plate was then washed three times with 10 mM Tris-phosphate buffer saline (TPBS), blocked with 2% milk in TPBS, incubated 37°C for 45 minutes, and washed with TPBS post-incubation. Diluted standard (purified California halibut vtg) or plasma samples and primary antibody (rabbit anti-turbot vtg; Cayman Chemical, Ann Arbor, MI) in TPBS were mixed for an antibody concentration of 1:1000. After primary antibody incubation, a secondary antibody (goat anti-rabbit labeled with alkaline phosphatase; Biorad, Hercules, CA) diluted to 1:2000 in TPBS was added. *p*-Nitrophenylphosphate was used as the detection substrate with absorbance measurements at a wavelength of 405 nm. Non-specific binding samples were included on each plate as negative controls. Plasma from estradiol-treated fish was included with each assay as a positive control. All samples were run in triplicate. An ELISA standard curve with an r^2 value of 0.98 or greater was considered to be a valid measurement. The limit of detection was 0.1 ng/ μ g; if the concentration was below this limit, a value of 0.01 ng/ μ g plasma protein was assigned to facilitate statistical analyses. Values were normalized to total plasma protein (Bradford 1976) using bovine serum albumin as a standard. The normalized vtg protein values were used for annual and seasonal comparisons.

Gonadal Development and Histopathology

A 5 mm-thick piece of gonad tissue was fixed in 10% phosphate-buffered formalin. Fixed gonads were dehydrated in a series of graded ethanol, cleared with xylene, and infiltrated and embedded in paraffin wax. Gonadal tissue was sectioned to a thickness of 5 μm , stained with hematoxylin and eosin, and examined using a light microscope. Gonadal stages and maturity phases were classified based on previous studies (Htun-Han 1978, Johnson *et al.* 1991, Sol *et al.* 1998, Deng *et al.* 2007).

In female fish, ovarian development was characterized into six stages: 1) Regressed stage – ovaries with primary and secondary follicles; 2) Previtellogenic state – follicles with cortical alveoli and zona radiata; 3) Vitellogenic stage – follicles with yolk globules; 4) Hydrated stage – hydrated follicles; 5) Spawning stage – ovary dominated by hydrated follicles and postovulatory follicles; and 6) Postspawning stage – postovulatory follicles and atretic follicles present. Sexual maturation (shown in graphs) was determined by the most developed follicles in ovarian tissue sections and classified into three phases of maturity: 1) Immature phase – the ovary was in a regressed or previtellogenic stage and no vitellogenic follicles present; 2) Maturing phase – the ovary had follicles in the regressed, previtellogenic and vitellogenic stages, but no hydrated follicles were present; and 3) Mature phase – ovarian follicles at all stages were present in the ovary, including previtellogenic, vitellogenic, hyaline and postovulatory follicles.

In male fish, testicular development was characterized into six stages: 1) Regressed stage – only spermatogonia present; 2) Spermatocytogenesis – mostly primary spermatocytes; 3) Meiotic division – spermatids present, but testis dominated by secondary spermatocytes; 4) Spermiogenesis – maturation of spermatids occurring; 5) Spermiating stage – majority of seminiferous tubules filled with mature sperm; and 6) Postspawning stage – only a few sperm remain within the tubules. Sexual maturation was determined based on the proportion of spermatogonia, spermatocytes (primary and secondary), spermatids, and spermatozoa: 1) Immature phase – the testis was in either a regressed or spermatocytogenesis stage; 2) Maturing phase – spermatids present; and 3) Mature phase – testis in the spermatogenesis or spermiogenesis stage. Testicular tissue was also histopathologically

examined for the presence of ovarian follicles within the tubules of the testis (ova-testis).

All histology slides were read twice to insure precision of diagnosis. Additionally, a subset (20%) of the samples were randomly selected and sent to a commercial histopathology laboratory for diagnosis confirmation.

Hornyhead Turbot Abundance over Time

Long-term (18 to 40 years depending on agency) demersal fish trawl monitoring data collected from the SD, OC/OCF, PV/PVF and LA discharge areas were used to obtain hornyhead turbot catch per trawl data for these areas. Similarly, demersal fish trawl data from the 1998, 2003, and 2008 Bight Regional Monitoring Programs were used to obtain hornyhead turbot catch per trawl data near the DP site. For each area, the catch per trawl data were binned by decade and averaged to identify trends in hornyhead turbot abundance over time.

Statistics

A chi-square test was used to compare sex ratios among stations during the same sampling event ($\alpha = 0.05$). For spatial data comparisons, a one-way analysis of variance (ANOVA) followed by a Tukey post-test was utilized. Temporal and small-scale data analyses from the quarterly samplings were conducted using a two-way ANOVA followed by a Bonferroni post-test to compare replicate means ($\alpha=0.05$). A non-parametric Kruskal-Wallis test was used to compare $\frac{1}{2}$ GSI values and plasma vtg concentrations since these data sets were not normally distributed. The decadal average hornyhead turbot population data for LA, PV, and OC areas were analyzed for significant differences between decades using one-way ANOVA ($\alpha=0.05$) followed by either Holm-Sidak (parametric) or Dunn's (non-parametric) post-hoc all pairwise comparison test ($\alpha=0.05$) as appropriate for the data. For the DP and SD sites, a Student's t (parametric) or Mann-Whitney Rank Sum test ($\alpha=0.05$) as appropriate was used to identify significant differences between hornyhead turbot populations in the two decades being compared. Data analysis was performed using Prism 4 (GraphPad Software, Inc. 2003) or SigmaPlot 11 (SysStat Software, Inc. 2008).

RESULTS

Age, Standard Length, and Body Weight *Spatial*

In spring of 2006, there was a significant difference in standard length ($p < 0.05$) in male hornyhead turbot collected in the SCB. Fish collected from OCF, SD, and DP were smaller (mean 14.6 to 13.3 cm), whereas LA and PV had larger fish (mean 15.1 to 16.0 cm). There also was a significant difference in the standard length ($p < 0.05$) of female hornyhead turbot. Fish collected from OCF were smaller (mean = 13.5 cm), whereas fish from

LA, PVF, PV and DP were larger (mean > 15 cm; Table 1). These variations in fish size reflect the characteristics of the fish retained for analysis and do not represent the size characteristics of the entire population.

Temporal

The standard length of male and female hornyhead turbot was significantly different both seasonally and by location ($p < 0.05$). The largest hornyhead turbot tended to be sampled from PV in spring and summer. There were few differences in size among fish collected in fall and winter, the

Table 1. Average (+ SE) age, length (cm), weight (g) and sample size of hornyhead turbot collected in the Southern California Bight. Letters identify significant differences in length within a season and gender ($p < 0.05$).

Season	Location	Males				Females			
		N	Length	Weight	Age	N	Length	Weight	Age
Spring	LA	23	16.0 ± 0.2 ^{AB}	108.5 ± 5.0	8.0 ± 0.2	27	17.2 ± 0.2 ^{AB}	135.4 ± 6.4	7.6 ± 0.1
	PVF	13	15.8 ± 0.2 ^{ABC}	99.6 ± 4.6	7.4 ± 0.2	17	17.2 ± 0.5 ^{AB}	135.9 ± 13.9	7.7 ± 0.2
	PV	31	16.4 ± 0.2 ^A	107.1 ± 5.0	8.2 ± 0.2	19	18.3 ± 0.6 ^A	169.8 ± 18.2	8.2 ± 0.3
	OCF	5	13.3 ± 0.7 ^C	70.4 ± 9.9	6.4 ± 0.2	9	13.5 ± 0.8 ^C	75.1 ± 14.0	6.1 ± 0.3
	OC	24	15.1 ± 0.3 ^{BCD}	97.5 ± 6.4	7.3 ± 0.2	26	16.3 ± 0.4 ^{ABC}	123.7 ± 10.4	7.1 ± 0.2
	SD	35	13.5 ± 0.3 ^E	59.9 ± 2.8	6.4 ± 0.2	15	15.8 ± 0.5 ^{BC}	107.7 ± 8.8	6.9 ± 0.2
	DP	32	14.6 ± 0.3 ^{DE}	89.1 ± 5.4	7.0 ± 0.2	19	18.0 ± 1.0 ^{AB}	175.8 ± 29.3	8.1 ± 0.5
Summer	PVF	8	15.7 ± 0.2 ^A	95.0 ± 2.7	7.8 ± 0.2	22	16.8 ± 0.3 ^B	129.5 ± 5.9	7.4 ± 0.1
	PV	10	16.2 ± 0.4 ^A	110.0 ± 11.5	8.1 ± 0.5	21	18.8 ± 0.5 ^A	188.8 ± 16.1	8.3 ± 0.3
	OCF	19	13.2 ± 0.3 ^B	66.7 ± 5.0	6.2 ± 0.1	11	14.2 ± 0.7 ^C	86.7 ± 13.0	6.3 ± 0.3
	OC	11	15.5 ± 0.3 ^A	101.5 ± 5.8	7.2 ± 0.2	19	16.9 ± 0.5 ^B	141.7 ± 13.2	7.4 ± 0.3
	DP	20	13.8 ± 0.3 ^B	72.6 ± 5.0	6.4 ± 0.1	10	14.2 ± 0.4 ^C	75.8 ± 7.2	6.3 ± 0.2
Fall	PVF	1	16.5 ^{AB}	120	8	29	17.5 ± 0.2 ^A	142.3 ± 5.7	7.8 ± 0.1
	PV	15	16.2 ± 0.3 ^{AB}	104.0 ± 6.5	8.0 ± 0.4	15	17.4 ± 0.6 ^A	144.0 ± 15.5	7.7 ± 0.3
	OCF	23	16.6 ± 0.2 ^A	122.7 ± 4.0	8.6 ± 0.2	7	17.9 ± 1.1 ^A	153.7 ± 32.1	7.9 ± 0.6
	OC	19	16.2 ± 0.3 ^{AB}	107.9 ± 8.1	8.24 ± 0.4	11	16.8 ± 0.3 ^A	114.7 ± 6.9	7.4 ± 0.2
	DP	23	15.4 ± 0.2 ^B	93.7 ± 4.0	7.4 ± 0.2	7	17.8 ± 0.6 ^A	150.6 ± 16.1	7.7 ± 0.2
Winter	PVF	5	15.8 ± 0.4 ^A	98.0 ± 9.7	7.6 ± 0.4	25	17.1 ± 0.2 ^A	146.4 ± 6.0	7.5 ± 0.1
	PV	13	16.3 ± 0.2 ^A	113.1 ± 5.9	8.2 ± 0.2	17	18.7 ± 0.6 ^A	195.3 ± 16.9	8.4 ± 0.3
	OCF	28	15.4 ± 0.2 ^A	86.2 ± 4.3	7.3 ± 0.2	2	19.5 ± 0.1 ^A	187.5 ± 7.5	9.0 ± 0
	OC	20	16.1 ± 0.3 ^A	102.7 ± 7.9	8.1 ± 0.4	10	17.9 ± 0.6 ^A	148.1 ± 19.0	7.9 ± 0.3
	DP	15	15.8 ± 0.4 ^A	95.5 ± 8.0	7.7 ± 0.3	15	18.5 ± 0.4 ^A	169.9 ± 12.5	8.2 ± 0.2

average size of samples of fish from OCF and DP collected during these events were larger than those from spring and summer (Table 1). Variations in weight and age (calculated from length) were similar to those in length.

Sex Ratio

Skewed sex ratios in hornyhead turbot collections were found at multiple stations during each sampling event. The skewed ratios often varied by season within a station and did not appear to be related to outfall proximity. For example, the sex ratio in fish from PV was male skewed in spring, female skewed in summer, and not significantly different from 1 in fall and winter (Table 2). The spring collections at the other outfall sites varied in sex ratio, ranging from significantly male skewed at SD to showing no significant difference at LA and OC. Fish collections from the Palos Verdes farfield site (PVF) tended to be consistently female skewed (0.8 to 0.2). Fish collections from the DP reference site also had skewed sex ratios, with male-dominated populations in three of the four collection events.

Gonadosomatic Index

Spatial

There were no significant differences among sites in male $\frac{1}{2}$ GSI ($p = 0.950$) for fish sampled in spring of 2006 (Figure 2). However, female hornyhead turbot $\frac{1}{2}$ GSI was significantly different among sites ($p < 0.01$; Figure 2). Females collected in the spring of 2006 from OCF had significantly lower $\frac{1}{2}$ GSI when compared to DP, OC, PV, SD, and LA sites. Site PVF was statistically similar to both the OCF site and the other remaining sites.

Temporal

Two way ANOVA indicated seasonally collected male hornyhead turbot $\frac{1}{2}$ GSI results were significantly different both seasonally ($p = 0.004$) and by location ($p = < 0.001$; Figure 2). There was also significant interaction between site and season so the data were evaluated by season within each site as well as by site within each season. Differences between sites within a season were only present in summer. Seasonal differences in male $\frac{1}{2}$ GSI were found within the OC and PV sites. At the OC site,

Table 2. Sex ratios of hornyhead turbot collected in the Southern California Bight. * indicates a significant difference from a ratio of 1 for a given sampling event ($p < 0.05$).

Site		Season				Combined
		Spring	Summer	Fall	Winter	
LA	Male:Female	23:27				23:27
	Ratio	0.9				0.9
PVF	Male:Female	13:17	8:22	1:29	5:25	27:93
	Ratio	0.8	0.4*	0.03*	0.2*	0.3
PV	Male:Female	31:19	10:21	15:15	13:17	69:72
	Ratio	1.6*	0.5*	1	0.8	1.0
OCF	Male:Female	5:9	11:19	19:11	20:10	55:49
	Ratio	0.6*	0.6*	1.7*	2*	1.1
OC	Male:Female	24:26	19:11	23:7	28:2	94:46
	Ratio	0.9	1.7*	3.3*	14*	2.0
SD	Male:Female	35:15				35:15
	Ratio	2.3*				2.3
DP	Male:Female	32:19	20:10	23:7	15:15	90:51
	Ratio	1.7*	2*	3.3*	1	1.8

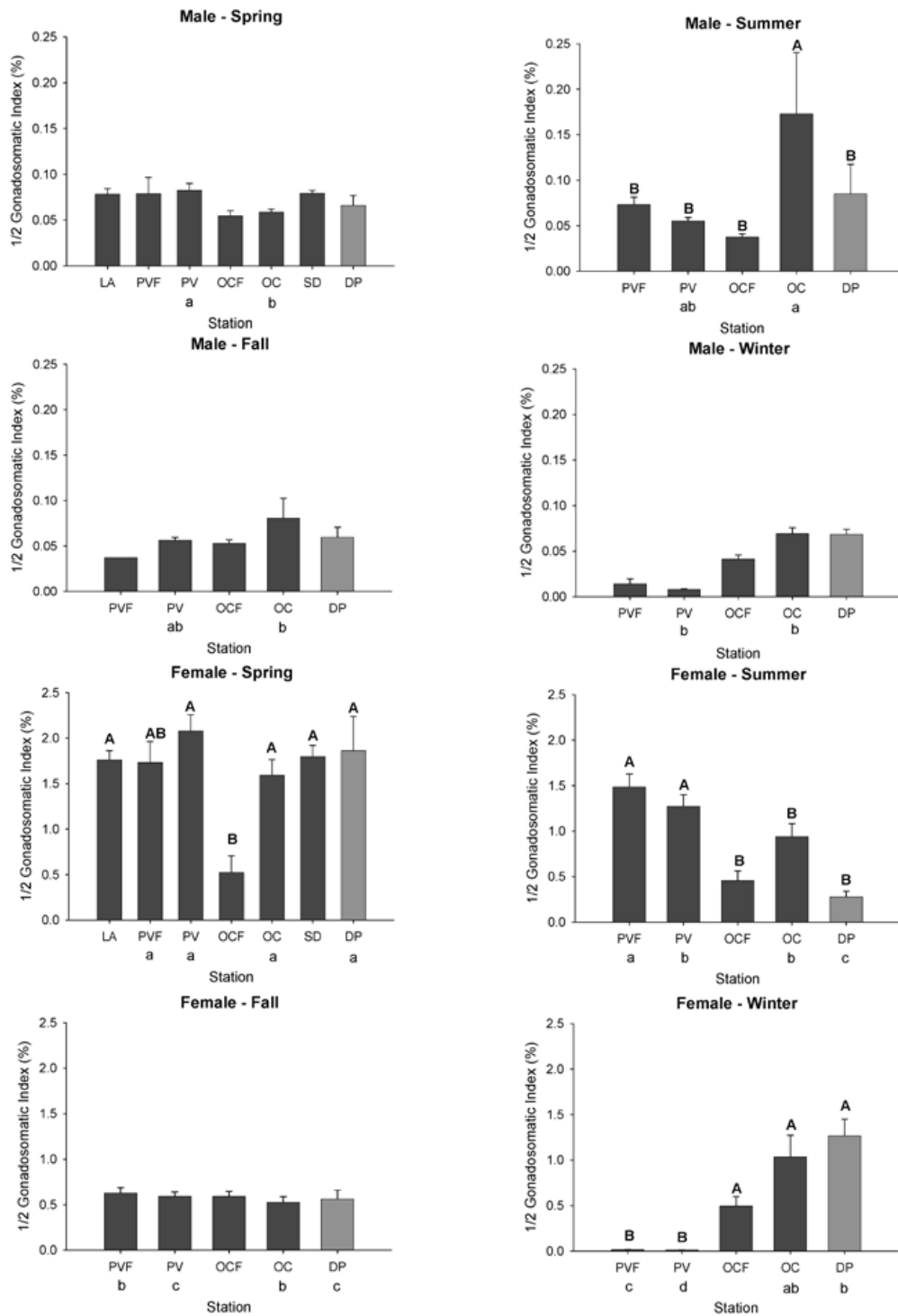


Figure 2. Hornyhead turbot 1/2 gonadosomatic index (GSI). Uppercase letters indicate significant differences between sites during the same season. Lowercase letters indicate differences between seasons for a site (p < 0.05). The LA and SD stations were only sampled in spring.

male 1/2 GSI values were significantly higher in the summer when compared to the other seasons. At the PV site, male 1/2 GSI values were significantly higher in the spring when compared to the winter.

Female hornyhead turbot 1/2 GSI values varied seasonally (p < 0.001) but not by site. However,

there was significant interaction between season and site so the seasonal differences were evaluated within each site. Variable patterns of differences among sites within a season were present in spring, summer, and winter (Figure 2). Seasonal differences were found for each site except for OCF, which

had relatively low 1/2 GSI scores year round. At all other sites, the highest female 1/2 GSI values were observed in the spring, but the patterns and statistical groupings associated with the other seasons were site specific. 1/2 GSI values for DP and OC were lowest in fall, with intermediate values in winter. A different pattern was present at PV and PVF, where 1/2 GSI values were lowest in winter; these winter values were also significantly lower than OCF, OC, and DP.

Plasma Vitellogenin

Spatial

Plasma vtg was detected in most male hornyhead turbot, although at levels approximately 100-fold less than females (Figure 3). Male hornyhead turbot vtg was significantly different among stations in fish sampled in June 2006 ($p < 0.05$). Fish collected at SD had the highest average vtg concentration among

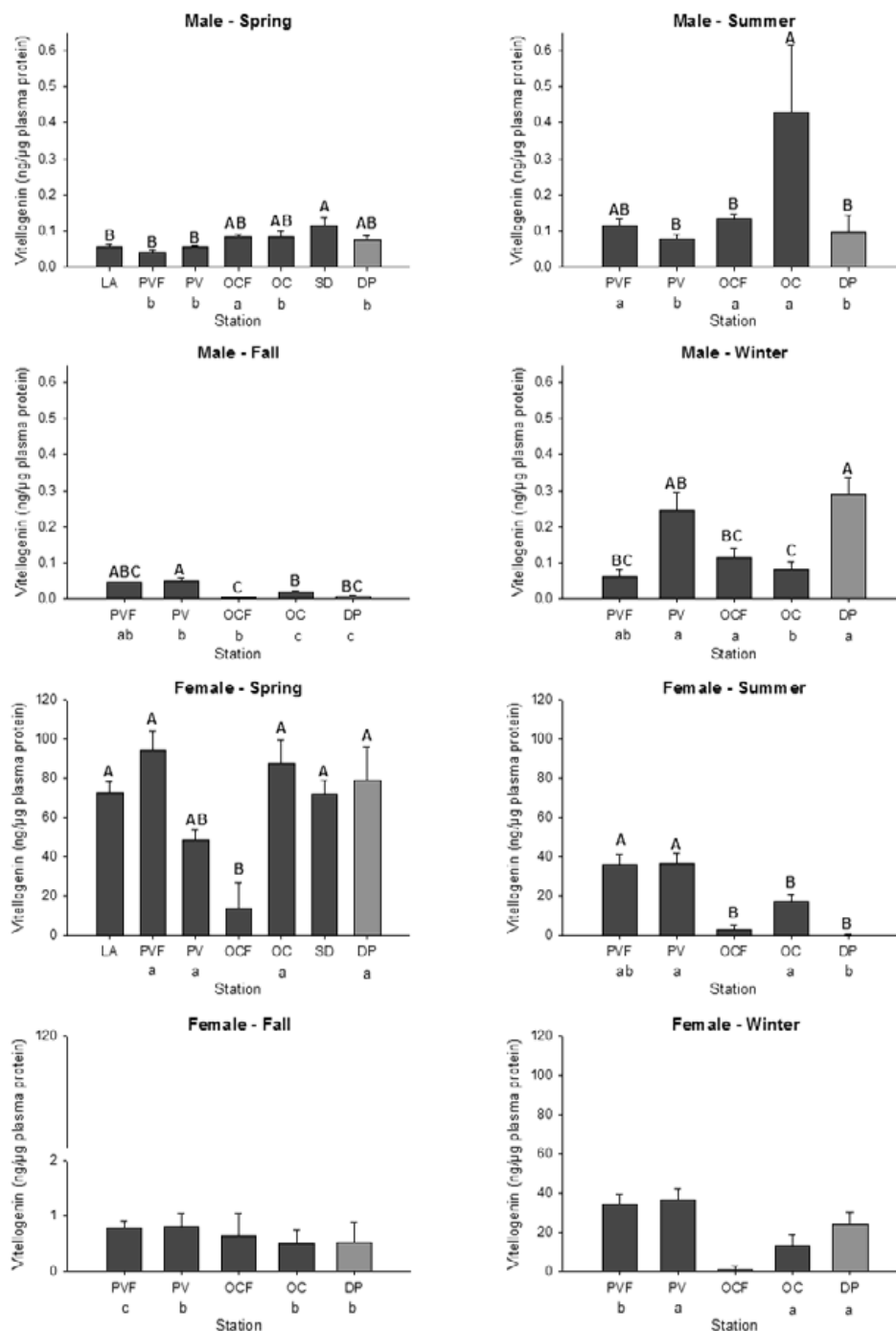


Figure 3. Plasma vitellogenin levels in hornyhead turbot. Uppercase letters indicate significant differences between sites during the same season. Lowercase letters indicate differences between seasons for a site ($p < 0.05$).

males in the spring, which was significantly higher than males from LA, PV, and PVF, but similar to fish from DP, OCF, and OC. Plasma vtg levels in spring female hornyhead turbot were also significantly different, with fish from OCF having lower vtg concentrations than most other stations (Figure 3).

Temporal

Seasonally collected male hornyhead turbot vtg concentrations differed both seasonally and by location, and also showed a significant interaction between site and season ($p < 0.05$; Figure 3). Differences between sites were observed in all seasons. Average male vtg concentrations were in general lowest during the fall. Male vtg levels showed different spatial patterns by season. In summer, OC males had the highest vtg concentration, DP and PV males had the highest vtg concentrations in winter. Seasonal peaks in male vtg also varied by station. Vtg peaked in DP and PV fish in winter, while PVF, OC, and OCF fish showed peak vtg concentrations in summer.

Seasonally collected female hornyhead turbot vtg concentrations varied by site and season ($p < 0.05$) and also showed a significant interaction between season and site. Variable differences among sites were present in spring and summer, but no differences were present in fall and winter. In spring, female OCF vtg concentration was lower than most other stations, while in summer, female vtg at OCF, OC and DP were significantly lower than concentrations at PV and PVF (Figure 3). Vtg concentrations for a given station were generally highest in spring, corresponding to the seasonal pattern in $\frac{1}{2}$ GSI. OCF females did not show any significant variation in vtg concentration by season.

Gonadal Histology

Spatial

The phase of gonad maturity (1 = immature, 2 = maturing, 3 = mature) of both male and female hornyhead turbot was significantly different among sites in fish sampled in spring 2006 ($p < 0.05$). None of the males collected from OCF were classified as maturing or mature, and males from this site were significantly less mature than LA or PV males (Figure 3). There were no significant differences in maturity state of the males from any site relative to the DP reference site, however. Female hornyhead turbot collected from OCF in spring also were less mature and had a significantly lower gonad maturity grade

than all other stations ($p < 0.05$). Gonad maturity in females from all other outfall sites was similar or greater than that of fish from the DP reference site.

Temporal

The quarterly collection data showed that male hornyhead turbot from each site had seasonal changes in gonad maturity and that there were differences among sites within each season (Figure 4). Differences between sites varied depending upon the season, suggesting differences in timing of gonad maturation at different sites. For example, PV and PVF males had greater gonad maturity in winter than males from OC, OCF, and DP, possibly indicating an earlier maturation cycle. Additionally, of the 373 gonads collected from male hornyhead turbot, none had ova-testis.

Female hornyhead turbot from all sites except OCF showed a peak in gonad maturity in spring (Figure 4). Gonad maturity in females from OCF was low in all collections and showed no significant differences between seasons ($p < 0.05$). Gonad maturity in fish from the outfall sites was similar to or greater than DP females in most instances. Fall was a period of low female gonad maturity at all sites. Females from PV and PVF showed evidence of a greater increase in maturity in winter relative to the other sites, similar to that observed in males.

Hornyhead Turbot Abundance over Time

Hornyhead turbot mean catch per trawl increased at all locations (significantly in all cases but DP) in the past decade (2000s) relative to the previous decade (1990s). Further, the mean catch per trawl observed in the last decade was the highest observed at these locations during their respective monitoring periods (Figure 5). Mean hornyhead turbot catch per trawl significantly ($p < 0.05$) increased from 1990 to 2000 at SD, as well as near OC after three decades of relatively lower catches in this area. Hornyhead turbot catches near PV significantly increased between the 1970s and the two subsequent decades (1980s and 1990s) with another significant increase from these catches observed in the 2000s. Catches of hornyhead turbot from LA declined ($p < 0.05$) from 1980 to 1990, but have since increased to levels statistically similar to and slightly higher than found in the 1980s.

When all four sites were compared across decades, fish from SD had the lowest catch per

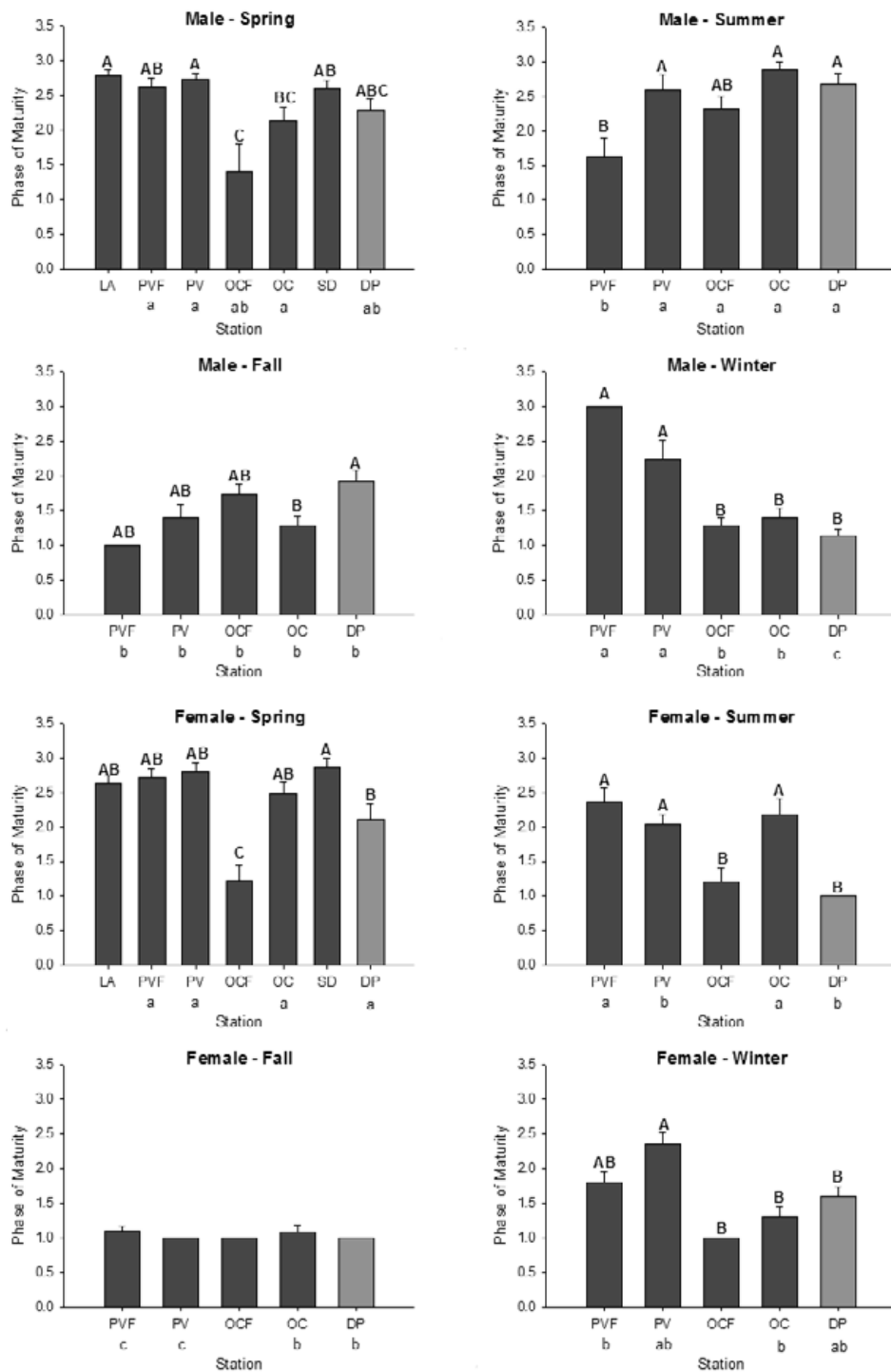


Figure 4. Hornyhead turbot phases of gonadal maturity (1 = immature, 2 = maturing, 3 = mature). Uppercase letters indicate significant differences between sites during the same season. Lowercase letters indicate differences between seasons for a site ($p < 0.05$).

trawl, with abundances generally 10 to 20% of those present at the other sites (Figure 5). This reduced abundance may be due to sampling at the deeper discharge depth of the SD outfall (100 m, relative to 60 m for the others) which is deeper than the preferred depth range for this species.

DISCUSSION

This study is the first to investigate hornyhead turbot reproductive condition and impacts throughout the Southern California Bight, and expands upon previous studies that were primarily focused on

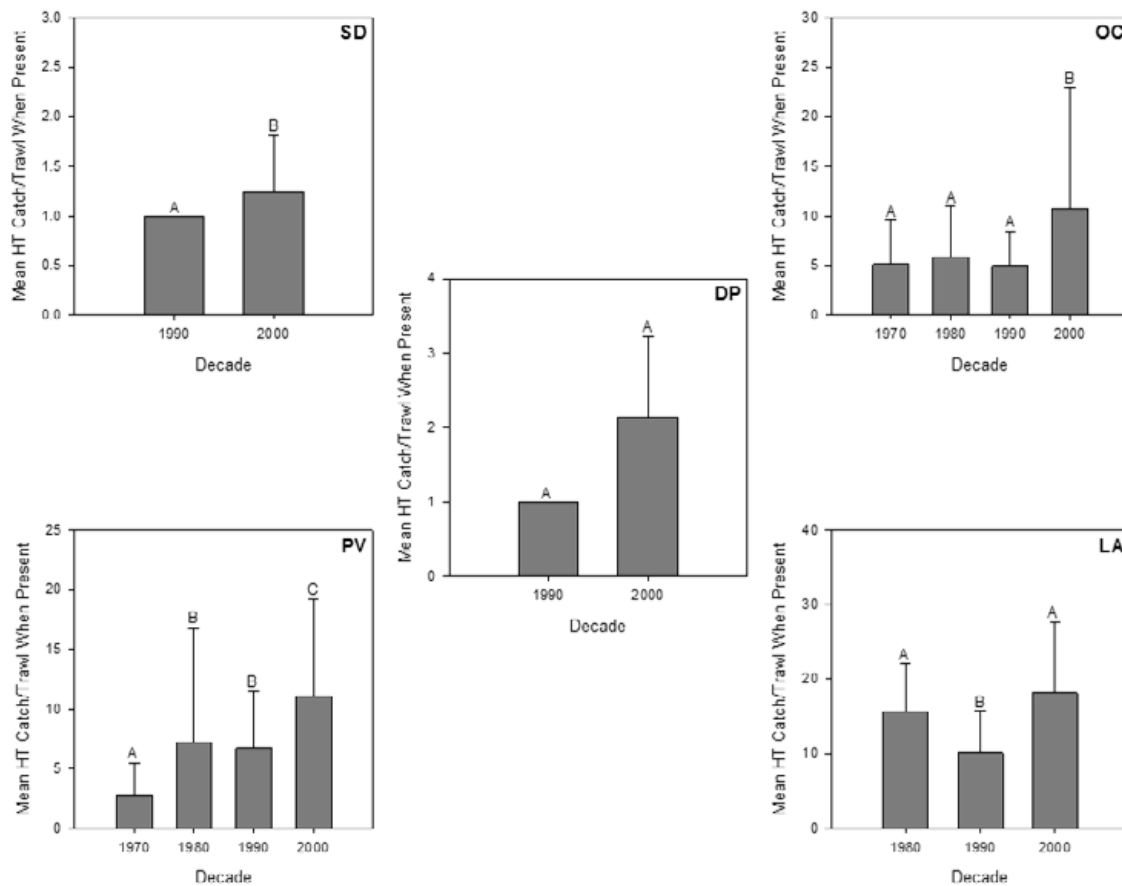


Figure 5. Hornyhead turbot (HT) historical abundance data from five sites in the Southern California Bight. Dana Point reference (DP), Point Loma Wastewater Treatment Plant Outfall (SD), Orange County Sanitation District Outfall (OC), Sanitation Districts of Los Angeles County Outfall (PV), and City of Los Angeles outfall (LA).

fish from the Orange County Sanitation District outfall area (OC and OCF). Significant spatial and temporal variation was observed among hornyhead turbot from some sites for parameters reflecting fish condition and reproductive status, including sex ratio, gonad size, plasma vtg, and maturity. Variations in fish size were also observed, but such differences may reflect differences in trawling efficiency or spatial variation in fish distribution unrelated to discharge characteristics.

Male-dominated sex ratios were observed at three locations in the SCB during the spring sampling event (PV, DP and SD), and at OCF and OC during other seasons. However, there was no consistent difference in sex ratio throughout the year at any site. Rempel *et al.* (2006) and Deng *et al.* (2007) reported that the gender ratios at OC were significantly male dominated in catches from 1988 to 2004, suggesting masculinization relative to the farfield site (OCF). A similar pattern was not observed in the current study, as hornyhead turbot catches at OCF were male-dominated in fall and winter and catches at OC

were not male skewed during the spring of 2006. Given the seasonal variation in sex ratio observed at multiple sites, it is likely that the differences relative to previous studies at OC reflect temporal and spatial differences in fish aggregation behavior related to spawning.

Hornyhead turbot collected from OCF (farfield site) were smaller and younger in spring and summer, relative to other sites. Hornyhead turbot mature at approximately 15 cm in length (Cooper 1996), and therefore it is likely that fish from OCF were sexually immature during spring and summer. This finding is supported by the lower female $\frac{1}{2}$ GSI and maturity state also observed at OCF. The current and previously published data (Rempel *et al.* 2006, Deng *et al.* 2007) showing that hornyhead turbot reproductive condition is lower at OCF, relative to other sites, may be a reflection of differences in turbot population age structure between sites. Thus, OCF may not be an adequate reference site for investigating the impacts of OC wastewater effluent

on age-dependent processes in hornyhead turbot, such as reproduction.

Whether the reproduction differences at OCF are due to environmental factors, fish behavior, or recruitment is uncertain. Three petroleum-drilling platforms are located in relative close proximity to OCF and sediment PAH concentrations are typically higher than the outfall site (OC) and range from 9 to 992 µg/kg PAH (OCSO 2010). Oil-contaminated sediments have been shown to impair steroid biosynthesis in hornyhead turbot (Roy *et al.* 2003) and PAHs interfere with reproductive processes in other fish (Logan 2007). Whether contaminant exposure is a cause for the diminished gonadal development at OCF is only speculation, but is a plausible hypothesis for further study.

Low level induction of vtg was widely observed in male hornyhead turbot from all sites, both outfall and farfield. In teleosts, production of the hepatically-derived egg yolk protein, vtg, is stimulated by estrogen (Nagahama 1994). As such, it has become a widely accepted biomarker of exposure to environmental estrogens in fish (Sumpter and Jobling 1995, Hansen *et al.* 1998, Jones *et al.* 2000, Hutchinson *et al.* 2006). No direct association of vtg induction and wastewater effluent exposure or degree of wastewater treatment was evident from our study. Although mean vtg concentration was highest in fish from one of the outfall stations in spring (SD), summer (OC), and fall (PV), vtg concentrations were generally similar to the reference site (DP) throughout the study. In addition, ova-testis was not observed in any hornyhead turbot indicating that male populations may not be adversely affected by legacy contamination or current discharges of estrogenic compounds into the SCB.

The high incidence of male vtg detection in hornyhead turbot may be indicative of exposure to estrogenic compounds in the aquatic environment. This conclusion was supported in a laboratory study where animals collected from locations where vtg was observed during field sampling were allowed to depurate for four days, and vtg was not detected (Rempel *et al.* 2008). Other studies of demersal flatfish have indicated seasonal variation in vtg in males from pristine locations, suggesting unique life history components that involve a “natural” estrogenic response (Scott *et al.* 2006). Given the removal of the response in depurated animals, the response observed in the SCB does appear to be environmental. The cause is still uncertain as the

response does not correspond to contamination patterns in the SCB or to concentrations of known estrogenic substances.

The site and seasonal variations in reproductive indicators found in this study do not appear to have resulted in adverse impacts on hornyhead turbot populations. Analysis of historical population data from 1970 to 2007 indicated that populations in the SCB are increasing at all outfall locations relative to the 1990s. It should be noted that although hornyhead turbot populations are stable and do not appear to be impaired by estrogenic compounds, other species or different trophic guilds may respond differently. However, examination of long-term monitoring data on fish community structure does not show signs of impact due to anthropogenic sources of contaminants (Bay *et al.*, this report)

In conclusion, evidence of widespread exposure to environmental estrogens was observed in hornyhead turbot. The impacts of such exposure appear to be small, however. The source of this potential estrogenic exposure is uncertain, and may include historically contaminated sediment or food, wastewater, or other sources. Additional studies are needed to determine whether these responses represent a legacy of past activities or a response to ongoing environmental factors.

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