Evaluation of reproductive endocrine status in hornyhead turbot sampled from southern California's urbanized coastal environments

Jesus A. Reyes[1], Doris E. Vidal-Dorsch, Daniel Schlenk[2], Steven M. Bay, Jeffrey L. Armstrong[3], Joseph R. Gully[4], Curtis Cash[5], Michael Baker[6], Timothy D. Stebbins[7], Gary Hardiman[6] and Kevin M. Kelley[1]

Abstract

As part of a region-wide collaboration to determine the occurrence of contaminants and biological effects in coastal ecosystems offshore of urban southern California, the present study characterized the reproductive endocrinology of an indigenous flatfish, the hornyhead turbot (*Pleuronichthys verticalis*), and compared groups sampled from different study sites representing varying degrees of pollution to screen for potential endocrine disruptive effects. Turbot were sampled from locations near the coastal discharge sites of four large municipal wastewater treatment plants (WWTPs) located between Los Angeles and San Diego, CA, and were compared with fish sampled from three far-field reference locations in the region. Despite environmental presence of both legacy contaminants and contaminants of emerging concern (CECs) and evidence for fish exposure to several classes of contaminants, both males and females generally exhibited coordinated seasonal reproductive cycles at all study sites. Patterns observed included peaks in sex steroids (17β-estradiol, testosterone, 11-ketotestosterone) in the spring and low levels in the fall, changes corresponding to similarly-timed gonadal changes and plasma vitellogenin (VTG) concentrations in females. Comparisons between fish captured at the different study sites demonstrated some regional differences in plasma levels of estrogens and androgens, indicative of location-associated effects on the endocrine system. The observed differences, however, could not be linked to the ocean discharge locations of four of the largest WWTPs in the world.

Introduction

The Southern California Bight (SCB) represents one of the most intensive interfaces in the world between a marine ecosystem and a substantial human

[1]*California State University, Environmental Endocrinology Laboratory, Long Beach, CA*

[2]*University of California, Department of Environmental Sciences, Riverside, CA*

[3]*Environmental Laboratory and Ocean Monitoring Division, Orange County Sanitation District, Fountain Valley, CA*

[4]*Sanitation Districts of Los Angeles County, Whittier, CA*

[5]*City of Los Angeles, Environmental Monitoring Division, Playa Del Rey, CA*

[6]*University of California, Department of Medicine, San Diego, CA*

[7]*City of San Diego Marine Biology Laboratory, Public Utilities Department, San Diego, CA*

population. In the region encompassing Los Angeles, Orange and San Diego Counties, a population of >20 million resides within 50 km of the coastline, in an area that includes extensive industrial, commercial, residential and other activities. Servicing these urban centers are four of the largest wastewater treatment plant (WWTP) facilities in the world, which together with some smaller WWTPs, release more than 1 billion gallons (3.8 billion liters) of treated wastewater each day into the coastal marine environment. In addition to the inputs of treated wastewaters, there are numerous other point and non-point sources of contaminants in the SCB ecosystem, including urban runoff, storm water, ports and harbors, and agriculture (Lyon and Stein 2009).

The impacts of southern California WWTP outfalls on environmental quality, benthic invertebrate and fish community structure, bioaccumulation of organic and metal contaminants in organisms, and toxicity to invertebrates and fish have been monitored and studied for more than 35 years by local municipalities and sanitation districts, and other agencies (OCSD 2010, CLAEMD 2010, Schiff *et al*. 2000, City of San Diego 2011, Allen *et al.* 2007). From this work, it has been well documented that many locales in the SCB, both near to and far from WWTP outfalls, have been contaminated by a wide variety of human-derived chemicals, particularly offshore of Los Angeles, Orange County, and San Diego.

A number of the contaminants known to be present in the SCB can act to disrupt endocrine and physiological systems in aquatic organisms such as fish. Such contaminants present in urban environments include chlorinated pesticides, industrial chemicals like polychlorinated biphenyls (PCBs), or polycyclic aromatic hydrocarbons (PAHs), but they also include so-called "contaminants of emerging concern" (CECs). The latter includes an increasing variety of pharmaceuticals and personal care product (PPCPs), current use pesticides (CUPs), various industrial and commercial compounds (ICCs), as well as natural and synthetic hormones (e.g., Gravel and Vijiyan 2006, Carr and Patino 2011, Alvarez-Cohen and Edlak 2003, Snyder 2008). Steroid hormones, derived principally from excretion by humans and domestic animals, are well established as endocrine disruptors present in aquatic ecosystems worldwide (Rempel and Schlenk 2008), while many other CECs are beginning to be linked to effects in organisms. Since many CECs are designed to be active in animals at relatively low concentrations, there is concern that increasing presence of CECs in urban aquatic environment may be leading to disrupted animal health, with possible relevance to ecosystem-level impacts.

In southern California, information is severely lacking on CEC inputs from municipal WWTP discharges and on whether there are associated effects in resident organisms, which would appear to be likely given the substantial interface between humans and the marine environment in this region (Roy *et al*. 2003, Reyes *et al*. 2002, Reyes 2006, Deng *et al*. 2007). Furthermore, there is limited study and understanding of the impacts of ocean-located WWTP outfalls worldwide on organism health Matthiessen 2003).

In the present study, an indigenous flatfish species, the hornyhead turbot (*Pleuronichthys verticalis*), was investigated to determine whether its residence in areas of WWTP discharges may be associated with disruptions in their reproductive endocrine system. Four of the WWTPs located from Los Angeles southward to San Diego are some of the largest facilities in the world, and discharge into the coastal marine environment. These sites and three far-field study sites were evaluated as part of a region-wide, multi-institution collaboration, which culminated in two chemistry studies measuring CECs and other contaminants present in WWTP effluents and receiving water samples Vidal-Dorsch *et al*. 2012) and in sediments and fish tissue samples (Maruya *et al*. 2012), and two biological studies centered on physiological measures of reproduction (Forsgren *et al*. 2012) and on endocrine system assessment and its relationship to reproductive function (present study).

Methods

Fish Sampling and Study Design

All fish analyzed in the present study were caught by otter trawling at specific coastal locations historically used in environmental monitoring in the SCB (Figure 1), which included the following seven sites (responsible agency in parentheses): outfall site "LA" located within Santa Monica Bay (City of Los Angeles, Bureau of Sanitation, Environmental Monitoring Division); outfall site "PV" and far-field site "PVF" located off the Palos Verdes Peninsula (Los Angeles County Sanitation Districts); outfall site "OC", far-field site "OCF", and a reference site near Dana Point ("DP"), all offshore of the Orange County coastline (Orange County Sanitation District); outfall

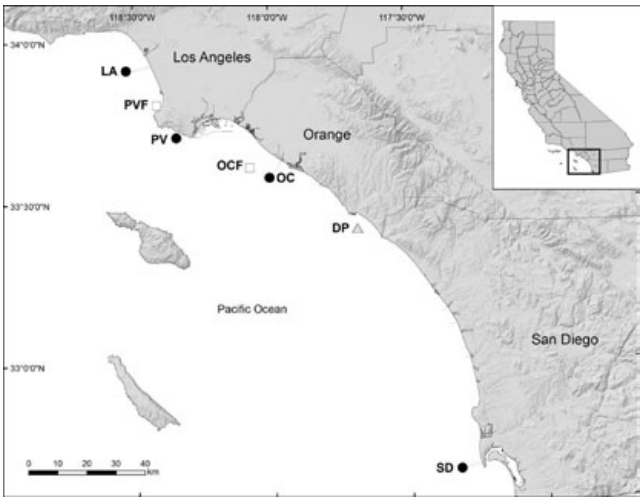

Figure 1. Map of the Southern California Bight (SCB) indicating the locations of the study sites. WWTP outfall locations (represented by open circles) include City of Los Angeles Hyperion Treatment Plant outfall in Santa Monica Bay (LA), Los Angeles County Sanitation Districts outfall at the Palos Verdes Peninsula (PV), Orange County Sanitation District outfall (OC), and City of San Diego Metropolitan Wastewater Treatment Plant outfall (SD). Far-field reference locations (represented by filled circles) include sites at Dana Point (DP), north Orange County (OCF), and north Palos Verdes Peninsula (PVF). Off-coast currents are predominantly northward in the inshore SCB region where the study sites are located.

site "SD" located offshore of Point Loma, San Diego (City of San Diego Public Utilities Department). All of the study sites were located at depths between 60 and 90 m (i.e., discharge depths of the WWTP outfalls) and exhibited temperatures consistently between 10 and 12°C. Trawling for fish at each site was guided by differential GPS and followed 500 to 600 m transects along the bottom at a speed of 1 to 1.5 m/second.

The reproductive seasonality in male and female hornyhead turbot was evaluated by quarterly sampling at five of the above sites (PV, PVF, OC, OCF, and DP), in spring (May-June, 2006), summer (August 2006), fall (November 2006) and winter (January and February, 2007). All seven study sites (LA, PV, PVF, OC, OCF, DP, SD) were sampled in May-June 2006 in order to assess potential site-associated differences in endocrine status across the entire region. Each trawl catch was released into on-board raceways with flow-through seawater, and adult hornyhead turbot ranging between 13 and 19 cm were selected for tissue sampling (the number of fish per each group is provided within tables and figures); the remaining fish were returned to the environment. Mean body weight (measured to nearest g) and standard length (measured to nearest mm) indicated that fish age (both sexes) did not significantly differ across study sites and did not correlate with endocrine measures (see Forsgren 2012) for body size and age data). Blood was collected from the caudal vein via heparinized 22 gauge needle and syringe, and immediately centrifuged for 5 minutes at 5,000 rpm to pelletize the blood cells; plasma was placed into heparinized microcentrifuge tubes (Fisher Scientific), frozen on dry ice, and then subsequently stored in a -80˚C freezer until analysis (described below). The sex of each fish was determined by inspection of the gonads, followed by removal of the right-side gonad for weight measurement (to the nearest mg). Gonad weight was then used to calculate ½ gonadosomatic index [GSI = right gonad weight (g)/body weight (g) x 100%], used as an indicator of relative reproductive status, as previously described Rempel *et al*. 2006, Deng *et al*. 2007). Analyses of seasonal and site-related differences in male and female gonadal histomorphology are reported by Forsgren *et al*. 2012. Whole liver was removed, weighed to the nearest mg, and used to calculate hepatosomatic index [HSI, liver weight (g)/body weight (g) x 100%], an indicator of relative hepatic fuel (glycogen, fat) storage in fishes (Haigwood *et al*. 2000).

In the associated chemistry studies, a variety of CECs and other contaminants were measured in effluent, receiving water, sediment, and in turbot liver composite samples derived from the four WWTP outfall locations and the DP reference site (Vidal-Dorsch *et al*. 2012, Maruya *et al*. 2012). Several PPCPs, ICCs, and hormones were frequently detected in effluents from each of the four WWTPs at relatively low µg/L concentrations, with only a small proportion detected in receiving waters at much lower (400 - 1000 times) concentrations (Vidal-Dorsch *et al*. 2012). Sediment also contained a variety of CECs and other contaminants (Maruya *et al*. 2012). While several steroid hormones were detected in effluents (E2, T, P and E1), they were not detected in water (Vidal-Dorsch *et al*. 2012) or sediment (Maruya *et al*. 2012); in fish liver samples, the synthetic estrogen, 17α-ethinylestradiol, was also not detected. The PPCP, diazepam (trade name Valium), was the sole PCPP detected in fish liver, present in 100% of the samples at a mean concentration of 56 µg/kg (wet weight), despite its infrequent detection in sediment at low concentrations (<1 µg/kg). The

ICC, 4-nonylphenol, was also frequently detected in fish tissue (mean concentration of 102 µg/kg), in addition to the polybrominated diphenyl ether (PBDE) congeners, BDE-47 (156 µg/kg), BDE 99 (69 µg/kg), BDE 100 (47 µg/kg) and BDE 154 (11 µg/kg). Legacy contaminants were frequently detected in fish tissues, including PCB congeners 138 and 153 (111 - 132 µg/kg) and notably high levels of 4,4'-DDE (12,200 µg/kg). When comparing across all study sites, there were generally lower concentrations and frequencies of detection in sediment and fish tissues from the DP reference site as compared with the WWTP sites, which was particularly evident for PBDEs, PCBs and organochlorine pesticides. The latter two classes (legacy contaminants) showed highest levels at the two northernmost outfalls (LA and PV), reflecting historic discharges from the Los Angeles region. In contrast to the above patterns, diazepam and 4-nonylphenol were present in liver of fish from the DP reference site at concentrations comparable to fish from WWTP sites. The results from these studies, which serve as a backdrop to the study sites used in this study, generally indicate that hornyhead turbot are exposed to a variety of CECs and legacy contaminants at all sites, with the DP reference site exhibiting relatively lower occurrences for several classes of contaminants (Vidal-Dorsch *et al*. 2012, Maruya *et al*. 2012).

Hormone Measurements

Plasma concentrations of 17β-estradiol (E2) and testosterone (T) were measured by radioimmunoassay utilizing ^{125}I-labeled E2 or T and specific polyclonal rabbit antisera obtained from Diagnostic Systems Laboratory, Inc. (Beckman Coulter, Inc.). Separation of free and bound antigen was achieved using a double antibody system (goat anti-rabbit gamma globulin serum) and polyethylene glycol as a precipitating aid. Pelleted ^{125}I was counted on a Perkin-Elmer Cobra II gamma counter (Packard Instruments Co.) and hormone concentrations were calculated from a standard curve of $\%B/B_o$ versus concentration of unlabeled ligand (0 - 750 pg/ml for E2; 0 - 25 ng/ml for T) using Sigma Plot v.11 software (SPSS, Inc.). Intra-assay precision was 7.4 and 7.5% (coefficients of variation = CV = SDEV/mean x 100%) for the E2 and T RIAs, respectively; inter-assay precision was 8.6 and 8.4%, respectively.

Plasma concentrations of the fish-specific androgen, 11-ketotestosterone (11-KT), were measured by enzyme immunoassay using an 11-KT-acetylcholinesterase conjugate as tracer and specific antisera obtained from Cayman Chemical Company. Antibody-bound tracer or endogenous hormone were bound to mouse monoclonal anti-rabbit IgG attached to microplate wells, washed, and then reacted in Ellman's Reagent. Absorbance at 412 nm was measured in a microplate spectrophotometer (Spectramax R250, Molecular Devices, Inc.) and hormone concentrations were calculated from a standard curve of $\%B/B_o$ versus concentration of unlabeled ligand (0 - 100 pg/ml) using Sigma Plot v.11 software (SPSS, Inc.). Intra-assay precision was 11.2% (CV); inter-assay precision was 8.1%.

Vitellogenin Measurement

Plasma concentrations of vitellogenin (VTG) were measured by enzyme-linked immunosorbent assay as described in Rempel *et al*. (2006) and Forsgren *et al*. (2012), using rabbit anti-turbot VTG primary antiserum from Cayman Chemical Co. and alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit, BioRad Labs, Inc.). Absorbance at 405 nm was measured and hormone concentrations in the samples were calculated from a standard curve of $\%B/B_o$ versus concentration of unlabeled purified California halibut VTG as standard, as described above, and expressed as ng/ml (females) or µg/ml (males). In the paper by Forsgren *et al*. (2012), location-related differences are reported, while in this paper correlative relationships with the endocrine system and overall seasonality were reported.

Data Analysis

Individual hormone concentrations (ng/ml, pg/ml), HSI (%), GSI (%) and VTG concentrations (ng/ml) were organized according to sex, study site, and season, and used to calculate mean ± SEM values, as provided in tables and figures. Potential differences in mean values (within each sex) across the study sites were evaluated by one-way ANOVA using Sigmaplot v.11 software (SPSS, Inc.). Differences in mean values according to season (Fall, Winter, Spring, Summer) were evaluated by ANOVA using all quarterly sampled fish (i.e., from sites PVF, PV, OCF, OC, DP); analysis of only non-outfall quarterly sampled fish (from PVF, OCF, DP) did not elucidate any significant differences (the former analysis is illustrated). Differences among groups were compared by the Holm-Sidek ad hoc multiple comparisons test. The non-parametric Kruskall-Wallis ANOVA on ranks test was used to evaluate datasets that were not

normally distributed. Spearman Rank Order correlation tests were carried out using the same software to examine relationships between parameters (e.g., hormone concentration vs. GSI). Differences in mean ± SEM values or in correlation coefficient (R) were considered significant when $p<0.05$.

Results

Overall Seasonal Changes in Reproductive Endocrine Status in Males

Males from the five sites sampled quarterly (DP, PVF, PV, OCF, OC) exhibited significant seasonal differences in plasma androgens. Concentrations of 11-ketotestosterone (11-KT) increased in spring to >4 ng/ml ($p <0.001$) as compared with fall, winter and summer during which concentrations were 0.2 to 0.7 ng/ml (Figure 2a). Concentrations of testosterone (T) exhibited a similar seasonal profile, with a spring peak of 1.45 ±0.09 ng/ml and concentrations of 0.25 to 0.70 ng/ml in the other seasons ($p <0.05$; Figure 2a). The spring peak in 11-KT was nearly 3-fold greater in concentration than that of T ($p <0.001$), while there were no differences between the two androgens in the other seasons. T and 11-KT were

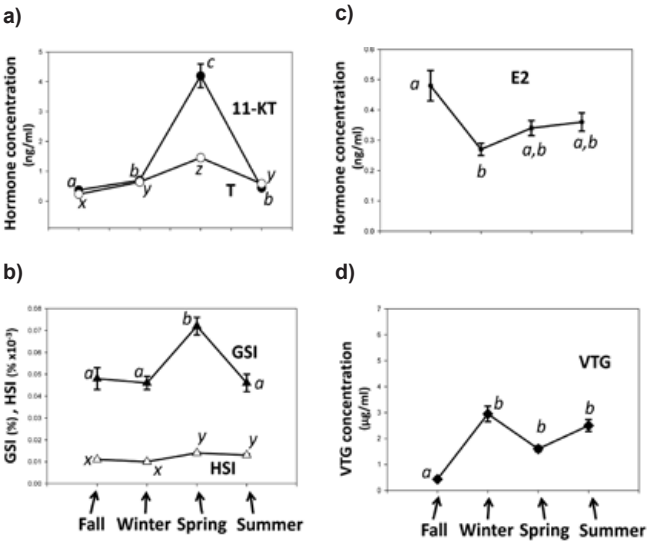

Figure 2. Reproductive seasonality in southern California hornyhead turbot males. Males from study locations sampled quarterly were measured for testosterone (T; a), 11-ketotestosterone (11-KT; a), gonadosomatic index (GSI; b), hepatosomatic index (HSI; b), 17β-estradiol (E2; c), and vitellogenin (VTG; d), as described in Methods. Different a-csuperscripts and x-zsuperscripts denote significantly different means ($p <0.05$); n = 81 (fall), 82 (winter), 106 (spring) or 69 (summer).

positively correlated with each other when analyzed among all males ($R = 0.72$, $p <0.00001$, $n = 383$); in fall and winter, T and 11-KT exhibited strong positive correlations (0.6 - 0.9, $p <0.05$), while in spring and summer they were generally not correlated (Table 1).

Testicular GSI exhibited a modest increase in spring as compared with the other seasons ($p <0.05$; Figure 2b). GSI correlated with the androgens overall ($R = 0.33$ - 0.36, $p <0.0001$, $n = 387$) and within some subgroups ($R = 0.3$ - 0.9; Table 1). Hepatosomatic index (HSI) increased by ~30% in spring and summer as compared with fall and winter ($p <0.05$; Figure 2b). HSI was positively correlated with T overall ($R = 0.42$, $p <0.0001$, $n = 387$), also evident in some sub-groups (Table 1), but a weaker overall relationship existed between 11-KT and HSI ($R = 0.27$, $p <0.0001$, $n = 387$).

In contrast to the distinct spring peaks in androgens, male E2 concentrations were unchanged in the winter, spring and summer (0.27 - 0.36 ng/ml; Figure 2c). In fall, mean E2 concentration (0.47 ±0.05 ng/ml) was slightly higher than in winter, but not in the other seasons (Figure 2c). E2 was not correlated with either GSI or HSI in the males. E2 exhibited a weak but significant correlation with T overall ($R = 0.21$, $p = 0.007$, $n = 387$), which was seen in some sub-groups ($R = 0.4$ - 0.6; Table 1), however it was not correlated with 11-KT. Plasma concentrations of VTG (Figure 2d) did not follow the seasonal patterns of the steroid hormones in males (T, 11-KT, E2); VTG exhibited a small number of significant correlations with E2 (in some sub-groups; Table 1), however half were negative.

Comparisons between Study Locations in Male Reproductive Endocrine Status

The seven study sites compared in spring 2006 included three far-field reference sites (DP, OCF, PVF) and four WWTP outfall sites (OC, PV, LA, SD). Plasma 11-KT concentrations among males were not significantly different across locations (Figure 3a); however, a non-significant trend of reduced 11-KT in was noted at OCF and OC (2 - 2.5 ng/ml vs. 5 - 6 ng/ml at the other locations). Within each of the five stations sampled seasonally (DP, PV, PVF, OC, OCF), 11-KT consistently showed higher springtime concentrations (3- to 12-fold higher, $p <0.01$). During the spring at each site, concentrations of 11-KT were ~3-fold higher than for T ($p <0.01$), a difference which was not observed in the other seasons. Seasonal patterns in T, on the

Table 1. Correlation coefficients (R) and p-values of the relationships among reproductive parameters measured in hornyhead turbot males sampled from different study sites (site codes identified in Figure 1).

	males	Orange County			Palos Verdes		San Diego	Santa Monica Bay
		far-field	*far-field*	*outfall*	*far-field*	*outfall*	*outfall*	*outfall*
	Season:	DP	OCF	OC	PVF	PV	SD	LA
	Fall	**0.78**[c] (23)	**0.87**[c] (22)	**0.86**[c] (18)	**0.75**[d] (6)	**0.73**[b] (15)	--	--
T	**Winter**	**0.82**[c] (14)	**0.74**[c] (26)	**0.74**[c] (19)	**0.56**[d] (5)	**0.77**[b] (12)	--	--
vs.	**Spring**	ns (32)	ns (5)	**0.31**[d] (24)	ns (13)	ns (31)	ns (34)	ns (23)
11-KT	**Summer**	ns (20)	**0.67**[b] (19)	ns (9)	ns (8)	ns (10)	--	--
	all seasons	**0.80**[c] (89)	**0.60**[c] (72)	**0.77**[c] (70)	ns (27)	**0.69**[c] (68)	--	--
	Fall	ns (23)	ns (22)	ns (18)	ns (6)	**0.40**[a] (15)	--	--
T	**Winter**	**0.60**[a] (14)[#]	**0.39**[a] (27)	ns (19)	ns (5)	ns (13)	--	--
vs.	**Spring**	ns (32)	**0.90**[d] (5)	ns (24)	**0.35**[d] (13)[#]	ns (31)	ns (34)	**0.32**[d] (23)
GSI	**Summer**	**0.41**[a] (20)	ns (20)	ns (10)	ns (8)	ns (10)	--	--
	all seasons	**0.33**[b] (89)[#]	ns (74)	ns (71)	ns (27)	**0.23**[a] (69)[#]	--	--
	Fall	ns (23)	**0.40**[a] (22)*	ns (18)	ns (6)	ns (15)	--	--
T	**Winter**	ns (14)	ns (27)	ns (19)	ns (5)	ns (13)	--	--
vs.	**Spring**	**0.38**[a] (32)	ns (5)	**0.67**[b] (24)	ns (13)	ns (31)	**0.40**[a] (34)	**0.31**[d] (23)
HHSI*	**Summer**	ns (20)	ns (20)	**0.66**[a] (10)	ns (8)	ns (10)	--	--
	all seasons	**0.30**[b] (89)	**0.31**[b] (74)	**0.41**[b] (71)	ns (27)	**0.31**[b] (69)	--	--
	Fall	ns (23)	**-0.48**[a] (22)[x]	**0.38**[d] (18)	ns (6)	**0.35**[d] (15)	--	--
T	**Winter**	**0.52**[a] (14)	**0.44**[a] (27)	**0.45**[a] (19)	ns (5)	ns (13)	--	--
vs.	**Spring**	ns (32)	ns (5)	ns (24)	**0.41**[d] (13)	ns (31)	ns (34)	**0.61**[b] (23)
E2†H	**Summer**	**0.35**[d] (20)	ns (20)	ns (10)	ns (8)	ns (10)	--	--
	all seasons	ns (89)	ns (74)	ns (71)	**0.31**[d] (27)	ns (69)	--	--
	Fall	**-0.36**[d] (23)	ns (23)	ns (18)	ns (6)[‡]	**0.51**[a] (15)[‡]	--	--
E2	**Winter**	ns (15)	ns (28)	ns (20)	ns (5)	ns (13)	--	--
vs.	**Spring**	**-0.29**[d] (32)	**0.80**[d] (5)	ns (24)	ns (13)	ns (31)	**-0.45**[b] (34)	**-0.39**[d] (23)
VTGH	**Summer**	ns (20)	**0.45**[a] (20)	ns (10)	**0.91**[c] (8)	ns (10)	--	--
	all seasons	**-0.52**[b] (90)	ns (76)	ns (72)	ns (27)	ns (69)	--	--

[a-d]Indicates significance level of correlation coefficient ([a]p=0.05-0.01; [b]p=0.01-0.001; [c]p<0.001; [d]p=0.06-0.10)

[#]11-KT was also correlated with GSI in these groups (R=0.24-0.62, p<0.05)

*11-KT was not significantly correlated with HSI, with two exceptions (OCF-fall, R=0.64; PV-all, R=040, p>0.05)

[†]E2 was not significantly correlated with 11-KT, with one exception (OCF-fall, R=-0.49, p<0.05)

[‡]E2 was not significantly correlated with GSI, except in these groups.

[x]11-KT was also negatively correlated with E2 (R=-0.52, p=0.001, n=23)

other hand, were more variable, although T was most often lowest in the fall and relatively elevated in spring (Figure 3b). Spring concentrations of T were significantly lower in males from the OCF and PVF far-field locations as compared with the OC and SD outfall sites ($p < 0.05$; Figure 3b). Fish at PVF and PV generally had relatively higher T concentrations outside of the spring season (Figure 3b).

As reported in Forsgren *et al*. (2012), mean testicular GSI values were not significantly different in fish across study sites, nor did GSI exhibit obvious seasonality at most stations. HSI also did not differ among the sites, with the exception of one location (PV) in which it was slightly increased (from ~1.2 to 1.7% in other groups, $p < 0.05$; data not illustrated). HSI did not exhibit any significant seasonal

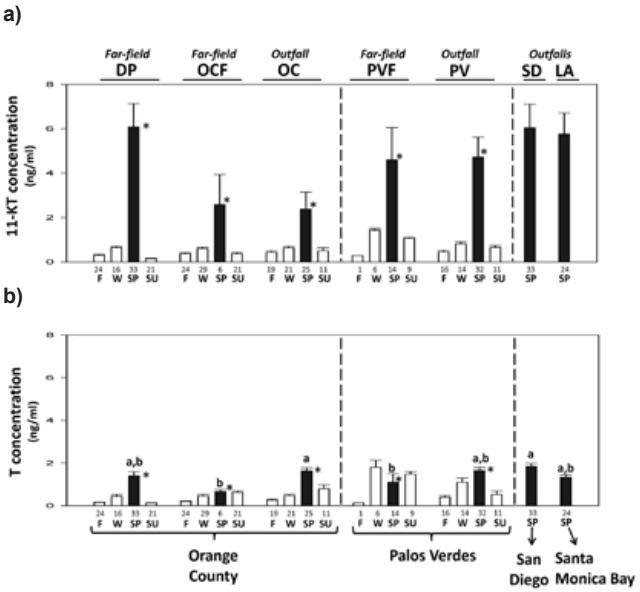

Figure 3. Location-associated differences in androgen levels in male hornyhead turbot. Seven southern California locations were compared in spring 2006 (DP, OCF, OC, PVF, PV, SD, and LA; see map in Figure 1) and are represented as filled bars. Open bars represent the other three seasons, which were evaluated only at sites DP, OCF, OC, PVF and PV. Data are presented as mean ±SEM ng/ml values for 11-ketotestosterone (11-KT; a) and testosterone (T; b), with n shown below each bar. F = Fall, W = Winter, SP = Spring, SU = Summer. Asterisk indicates spring mean is significantly higher than in one or more of the other seasons at that site (p <0.05); a and b superscripts denote significantly different means between sites (p <0.05).

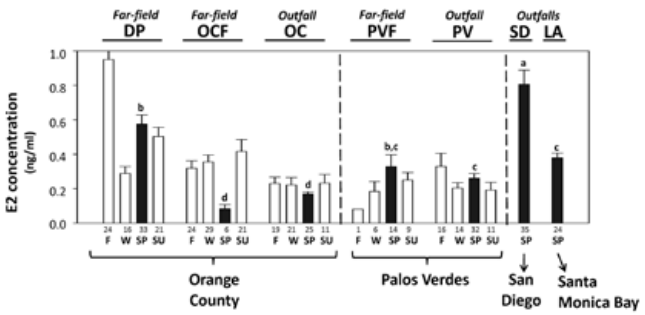

Figure 4. Location-associated differences in 17β-estradiol (E2) levels in male hornyhead turbot. Seven southern California locations were compared in spring 2006 (DP, OCF, OC, PVF, PV, SD, and LA; see map in Figure 1) and are represented as filled bars. Open bars represent the other three seasons, which were evaluated only at sites DP, OCF, OC, PVF, and PV. Data are presented as mean ±SEM ng/ml values for E2, with n shown below each bar. F = Fall, W = Winter, SP = Spring, SU = Summer. Different a through d superscripts denote significantly different means between sites (p <0.05).

differences across sites, but in general tended to be higher in spring and summer at most sites (data not illustrated), consistent with the data shown in Figure 2b.

Plasma concentrations of E2 in the males (Figure 4a) were lowest at the OC and OCF sites (83 - 170 pg/ml, p <0.05 vs. other locations), intermediate in Santa Monica Bay (LA) and Palos Verdes (PVF & PV), and highest at the DP and SD locations (575 - 800 pg/ml, p <0.05 vs. other locations). Among the seasonally sampled locations, DP tended to have the highest E2 in males. There were no significant seasonal patterns in male E2 levels within any site (Figure 4).

Overall Seasonal Changes in Female Reproductive Endocrine Status

Among females from the five quarterly sampled sites, overall E2 concentrations ranged between 150 - 230 pg/ml and were significantly higher in spring as compared with the other seasons (p <0.05; Figure 5). Ovarian GSI similarly peaked in spring as compared with the other seasons (p <0.05), and was also relatively elevated in summer as compared with fall and winter (p <0.05; Figure 5b). Plasma concentrations of VTG were considerably higher in females than in males (e.g., 1.7 mg/ml vs. 1.6 µg/ml, respectively, in spring; p <0.0001) and followed a seasonal pattern similar to that observed for E2 and GSI (Figure 5B). E2 concentrations showed overall positive correlations with both GSI (R = 0.27, p <0.0001, n = 353) and VTG (R = 0.34, p <0.0001, n = 353); when evaluated within subgroups (e.g., within season or site), some similar and stronger correlations were observed (R = 0.4 - 0.7; Table 2). GSI and VTG were strongly correlated with each other, both overall (R = 0.61, p <0.00001, n = 353) and within a majority of subgroups (Table 2).

HSI in females exhibited a seasonal profile comparable to that observed for E2, GSI and VTG, with higher values in spring and summer (p <0.05, Figure 5c). Unlike in the males, HSI was positively correlated with GSI and plasma VTG in females, both overall (R = 0.54 and 0.44, respectively, p <0.00001, n = 353) and within several subgroups (R = 0.4 - 0.8; Table 2). HSI was not correlated with E2 or 11-KT overall, but showed a weak overall relationship with T (R = 0.24, p = 0.005, n = 343).

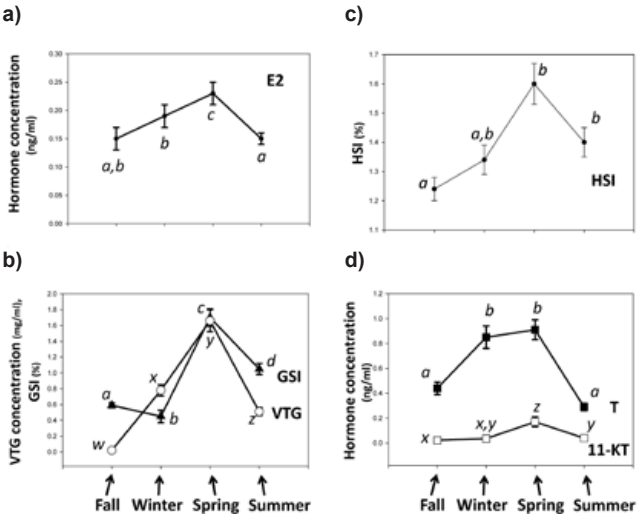

Figure 5. Reproductive seasonality in southern California hornyhead turbot females. Females from study locations sampled quarterly were measured for 17β-estradiol (E2; a), gonadosomatic index (GSI; b), vitellogenin (VTG; b), hepatosomatic index (HSI; c), testosterone (T; d), and 11-ketotestosterone (11-KT; d), as described in Methods. a through d superscripts and w through z superscripts denote significantly different means (p <0.05); n = 71 (Fall), n = 70 (Winter), n = 90 (Spring), and n = 83 (summer).

Plasma concentrations of T in females (Figure 5d) were less than 50% those observed in males ($p < 0.05$). Female T concentrations were elevated in winter and spring (>0.85 ng/ml) as compared with summer and fall (≤0.4 ng/ml; $p < 0.05$). Concentrations of 11-KT (Figure 5d) were yet lower (0.02 - 0.17 ng/ml, $p < 0.05$ in each season vs. T values), but were also elevated in spring ($p < 0.05$ vs. each other season) and in summer as compared with fall ($p < 0.05$). T, which is a precursor for E2 biosynthesis, was positively correlated to E2, both overall ($R = 0.33$, $p < 0.0001$, $n = 343$) and in several subgroups ($R = 0.4$ - 0.8), while 11-KT was mostly unrelated to E2 (Table 2). T and 11-KT levels were positively correlated overall ($R = 0.577$, $p < 0.0001$, $n = 342$) and within several subgroups ($R = 0.4$ - 0.9; Table 2).

Comparisons between Study Locations in Female Endocrine Status

Females exhibited spatial differences in plasma E2 concentrations reminiscent of that seen in the males (Figure 6a), with the highest E2 concentrations at the DP and SD sites ($p < 0.05$ vs. all sites except PV) lowest values at the OC and OCF sites. Within each season except winter, E2 was consistently higher in females from DP as compared with other sites. In contrast to the overall spring peak in E2 (see Figure 5a), seasonal patterns in E2 concentrations were mostly not detectable when evaluated within each location (Figure 6a). GSI and VTG generally showed spring increases within each location, except OCF (Forsgren *et al*. 2012. Correlations between these indices and E2 were not detectable in some sub-groups (e.g., OCF, DP) but detectable in others (e.g., OC, PV, PVF; Table 2).

Female T concentrations were mostly not different between sites, except at the LA site at which relatively lower values were observed (Figure 7a). At the seasonally sampled sites, T showed higher levels in spring compared with one or more other seasons, except at OCF ($p < 0.05$; Figure 7a). Although present at an order of magnitude less concentration than T, 11-KT was also generally higher in springtime across all sites; at OCF, spring 11-KT concentrations were lower than at several other sites (Figure 7b). T and 11-KT showed weak overall correlations with plasma VTG and ovarian GSI ($R = 0.2$ - 0.3, $p < 0.001$, $n = 343$ -3 52) and selected subgroups also reflected these correlations ($R = 0.4$ - 0.8, $p < 0.05$; Table 2), except at OCF. HSI exhibited no seasonal or spatial patterns in females when evaluated within or across study sites (data not illustrated). Consistent with its overall relationship to GSI and VTG in females (reported above), HSI showed several positive correlations with GSI and VTG within site and/or season sub-groups (Table 2); however, HSI had few relationships with E2 and T and no relationship with 11-KT in females, and there were no discernable patterns due to study site.

Discussion

In the present study, hornyhead turbot residing offshore of urban southern California were investigated for possible environment-associated effects on their endocrine physiology, particularly in relation to the discharge locations of four regional WWTP facilities. These four facilities are some of the largest in the world, and they use outfalls that discharge directly into the coastal ocean habitat of flatfish including turbot. While the hornyhead turbot has a long history of being monitored in southern California for contaminant accumulation and population measures by the discharge agencies (e.g., OCSD 2010, CLAEMD 2010, Schiff *et al*. 2000, City of San Diego 2011) and by regional monitoring

Table 2. Correlation coefficients (R) and p-values of the relationships among reproductive parameters measured in hornyhead turbot females sampled from different study sites (site codes identified in Figure 1).

	Females	Orange County			Palos Verdes		San Diego	Santa Monica Bay
	Season:	***far-field***	***far-field***	***outfall***	***far-field***	***Outfall***	***outfall***	***outfall***
		DP	**OCF**	**OC**	**PVF**	**PV**	**SD**	**LA**
	Fall	ns (7)	ns (7)	ns (12)	ns (29)	**0.58**[a] (15)	--	--
E2	**Winter**	ns (15)	ns (1)	ns (10)	ns (25)	ns (17)	--	--
vs.	**Spring**	ns (18)	ns (9)	**0.40**[d] (26)	ns (17)	**0.41**[d] (19)	ns (16)	**0.39**[a] (27)
GSI	**Summer**	ns (10)	ns (10)	ns (20)	**0.56**[a] (22)	ns (20)	--	--
	all seasons	ns (50)	ns (28)	**0.46**[c] (68)	**0.45**[a] (93)	ns (71)	--	--
	Fall	ns (7)	ns (7)	ns (12)	ns (29)	**0.50**[a] (15)	--	--
E2	**Winter**	ns (15)	ns (1)	**0.60**[a] (10)	**0.67**[c] (25)	**0.37**[d] (17)	--	--
vs.	**Spring**	**0.56**[a] (18)	ns (9)	**0.53**[a] (26)	**0.50**[a] (17)	**0.41**[d] (19)	ns (16)	ns (27)
VTG	**Summer**	ns (10)	ns (10)	ns (20)	**0.45**[a] (22)	ns (20)	--	--
	all seasons	ns (50)	ns (28)	**0.45**[c] (68)	**0.65**[c] (93)	**0.34**[b] (71)	--	--
	Fall	**0.82**[b] (7)	**0.61**[d] (7)	ns (12)	**0.64**[c] (29)	**0.66**[b] (15)	--	--
GSI	**Winter**	**0.90**[c] (15)	ns (1)	**0.67**[a] (10)	**0.40**[d] (25)	ns (17)	--	--
vs.	**Spring**	**0.57**[a] (18)	ns (9)	**0.80**[c] (26)	ns (17)	**0.60**[b] (19)	**0.42**[d] (16)	ns (27)
VTGH	**Summer**	ns (10)	**0.86**[c] (10)	**0.82**[c] (20)	**0.75**[c] (22)	**0.45**[a] (20)	--	--
	all seasons	**0.80**[c] (50)	**0.50**[a] (28)	**0.87**[c] (68)	**0.38**[c] (93)	**0.40**[b] (71)	--	--
	Fall	ns (7)	ns (7)	**0.76**[b] (11)[#]	ns (29)	**0.65**[b] (15)	--	--
E2	**Winter**	**0.52**[a] (13)	ns (1)	ns (10)	**0.59**[b] (25)	**0.62**[b] (17)	--	--
vs.	**Spring**	**0.42**[d] (18)	**0.73**[a] (9)[#]	ns (26)	ns (17)	**0.37**[d] (19)	**0.45**[d] (16)	**0.33**[d] (27)
T	**Summer**	ns (10)	ns (10)	**0.55**[a] (20)	**0.63**[b] (21)	ns (20)	--	--
	all seasons	**0.40**[a] (46)	**0.40**[a] (28)	**0.46**[c] (67)	**0.40**[a] (92)	**0.69**[c] (67)[#]	--	--
	Fall	**0.75**[a] (7)	ns (7)	ns (11)	ns (29)	**0.60**[a] (15)	--	--
T	**Winter**	**0.56**[a] (13)	ns (1)	**0.69**[a] (10)	ns (25)	ns (17)	--	--
vs.	**Spring**	ns (18)	ns (9)	ns (26)[†]	**0.41**[d] (17)	ns (19)[†]	ns (16)	**0.47**[a] (27)
GSIH	**Summer**	ns (10)	ns (10)	ns (20)	ns (21)	ns (20)	--	--
	all seasons	**0.37**[b] (46)	ns (28)	**0.36**[b] (67)	**-0.30**[a] (92)	ns (67)[†]	--	--
	Fall	**0.93**[c] **(7)**	ns (7)	**0.89**[c] **(11)**	**0.60**[b] **(29)**	**0.63**[a] **(15)**		
T	**Winter**	**0.65**[a] **(13)**	ns (1)	**0.67**[a] **(10)**	**0.38**[d] (25)	**0.61**[b] **(17)**		
vs.	**Spring**	ns (18)	ns (9)	**0.42**[a] **(26)**	ns (17)	**0.50**[a] (19)	ns (16)	ns (27)
11-KT	**Summer**	ns (10)	**0.62**[a] (10)	**0.73**[c] **(20)**	**0.57**[b] **(21)**	ns (20)		
	all seasons	ns (46)	**0.45**[b] **(28)**	**0.70**[c] **(67)**	**0.49**[c] (92)	**0.76**[c] **(66)**		
	Fall	ns (7)	**0.75**[a] (7)	ns (12)	**0.44**[a] (29)	**0.60**[a] (15)	--	--
HSI	**Winter**	**0.78**[c] (15)	ns (1)	**0.65**[a] (10)	ns (25)	ns (17)	--	--
vs.	**Spring**	**0.64**[b] (18)	ns (9)	**0.65**[c] (26)	ns (17)	ns (19)	**0.46**[d] (16)	**0.56**[b] (27)
GSIH	**Summer**	ns (10)	**0.44**[d] (10)	**0.62**[b] (20)	**0.57**[b] (22)	**0.51**[a] (20)	--	--
	all seasons	**0.55**[c] (50)	ns (28)	**0.65**[c] (68)	ns (93)	**0.37**[b] (71)	--	--
	Fall	ns (7)	**0.64**[d] (7)	ns (12)	ns (29)	**0.52**[a] (15)	--	--
HSI	**Winter**	**0.80**[c] (15)	ns (1)	**0.91**[c] (10)	**0.52**[b] (25)	ns (17)	--	--
vs.	**Spring**	**0.70**[c] (18)	ns (9)	**0.73**[c] (26)	ns (17)	ns (19)	ns (16)	ns (27)
VTGH	**Summer**	ns (10)	ns (10)	**0.45**[a] (20)	**0.58**[b] (22)	ns (20)	--	--
	all seasons	**0.60**[c] (50)	**0.31**[d] (28)	**0.69**[c] (68)	**0.30**[b] (93)	**0.35**[b] (71)	--	--

[a-d] Indicates significance level of correlation coefficient ([a]p=0.05-0.01; [b]p=0.01-0.001; [c]p<0.001; [d]p=0.06-0.10)

[#]E2 was also correlated with 11-KT in these three groups (R= 0.35-0.74, p<0.05)

[†]GSI was also correlated with 11-KT in these three groups (R=0.53-0.54, p<0.05)

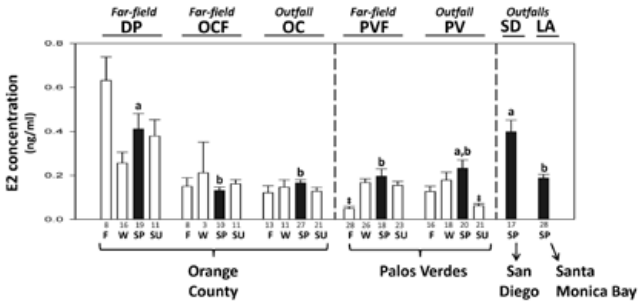

Figure 6. Location-associated differences in 17β-estradiol (E2) levels in female hornyhead turbot. Seven southern California locations were compared in spring 2006 (DP, OCF, OC, PVF, PV, SD, and LA; see Figure 1) and are represented as filled bars. Open bars represent the other three seasons, which were evaluated only at sites DP, OCF, OC, PVF, and PV. Data are presented as mean ±SEM ng/ml values for E2, with n shown below each bar. F = Fall, W = Winter, SP = Spring, SU = Summer. ‡ indicates mean is significantly lower than in other seasons at that site (p <0.05); a and b superscripts denote significantly different means between sites (p <0.05).

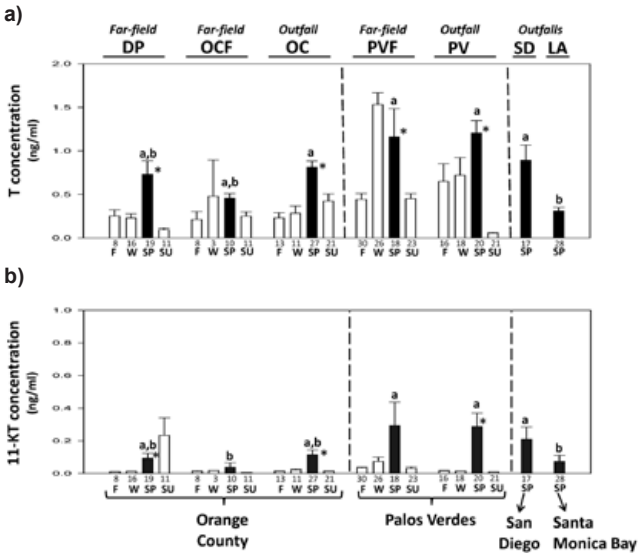

Figure 7. Location-associated differences in androgen levels in female hornyhead turbot. Seven southern California locations were compared in spring 2006 (DP, OCF, OC, PVF, PV, SD, and LA; see Figure 1) and are represented as filled bars. Open bars represent the other three seasons, which were evaluated only at sites DP, OCF, OC, PVF, and PV. Data are presented as mean ±SEM ng/ml values for 11-ketotestosterone (11-KT; a) and testosterone (T; b), with n shown below each bar. F = Fall, W = Winter, SP = Spring, SU = Summer. * indicates spring mean is significantly higher than in one or more of the other seasons at that site (p <0.05); a and b superscripts denote significantly different means between sites (p <0.05).

programs (e.g., Carr and Patino 2011), this species has received only limited biological study to date, particularly with respect to its endocrine physiology and reproduction (Deng *et al*. 2007, Goldberg 1982, Cooper 1997).

It was therefore of initial interest to establish how endogenous hormone levels change throughout a given year in this species. The results of the present study indicated that spring is the season during which there are elevated sex steroid levels in both male (T and 11-KT) and female (E2 and androgens) hornyhead turbot, while fall was the principal season during which the lowest hormone levels occurred. In both sexes, the spring peaks in these hormones were associated with coincident changes in other reproductive parameters, including GSI or plasma VTG concentrations in females.

Across the five seasonally-sampled study sites (PV, PVF, OC, OCF, DP), distinct spring peaks in 11-KT were observed in male hornyhead turbot at each site, and T showed similar seasonality, although more variably. Preceding its spring peak (in fall and winter), 11-KT concentrations were strongly correlated with T concentrations (R = 0.68 - 0.84); however, in spring and summer, the two androgens were generally not correlated. Since T serves as the precursor in the synthesis of 11-KT by the fish testis (Kusakabe et al. 2002), this finding indicates that during the period of increasing androgen synthesis, 11-KT production is linked to T production, whereas during the peak and post-peak periods, the steroidogenic pathways leading to 11-KT appear to decouple. Across all sites and seasons, significant differences in 11-KT were not detected, although the androgen tended to be lower at the OCF and OC sites. In contrast, T concentrations were lower at the OCF far-field site and to a lesser degree at PVF far-field site, as compared with WWTP outfall sites. While the latter observation could imply an effect associated with the outfall discharges, T levels were similar in fish at the outfall sites and the DP reference site located farthest from any of the outfalls (and containing the lowest levels of contaminants among the study sites, as discussed further below). Therefore, the findings do not support a linkage between the spatial differences in T concentrations in males and their presence at the WWTP discharge locations.

While the T and 11-KT concentrations in male hornyhead turbot were within normal ranges seen

in males of other fish species and also showed comparable seasonal changes (Reyes 2006, Kusakabe *et al*. 2002, Garcia-Lopez *et al*. 2006, Scott *et al*. 2006, Petschauer 2008, Gemmell *et al*. 2011), plasma concentrations of E2 in male hornyhead turbot were relatively high. This finding is in agreement with previous work by Reyes *et al*. (2002, 2006), who measured E2 concentrations ranging between 300 and 1400 pg/ml in hornyhead turbot males sampled offshore of Los Angeles and Orange Counties, as compared with ≤50 pg/ml in males of several other fish species tested. In the present study, plasma E2 concentrations ranged between 200 and 900 pg/ml in males, but did not show any seasonal patterns. The apparently normal seasonality in androgen levels in hornyhead turbot males suggests that these E2 levels do not impair gonadal steroid production or its seasonality. Whether the high E2 levels in males of this species reflects a normal or abnormal condition is not yet understood. In another species of flatfish, *Platichthys flesus*, Scott *et al*. (2006) reported male plasma E2 concentrations up to 220 pg/ml, which approaches the levels observed in this species; similarly, they found no seasonality in E2 in male flounders, despite apparently normal levels and seasonality for 11-KT.

E2 concentrations in male hornyhead turbot exhibited significant location-associated differences. In accordance with Reyes *et al*. (2002, 2006), male E2 concentrations were lowest in fish from Orange County (OC and OCF) and relatively higher in fish from Palos Verdes (PV and PVF) and Santa Monica Bay (LA). However, yet higher E2 concentrations were observed in males from the DP reference site and at the SD outfall site. The basis for these environment-associated differences is not clear, but again did not coincide with residence of fish at WWTP discharge locations. Despite the different E2 levels by location, there were no detectable correlations with testicular GSI, nor did plasma VTG concentrations exhibit any consistent relationships with E2; in a few sub-groups, E2 was negatively correlated with VTG while in other sub-groups the opposite was observed. The latter findings may suggest that either estrogenic or androgenic effects may predominate at certain locations in the SCB, presumably dependent upon the specific environmental constituents present at each site. In addition, it was noted that the spatial patterns in E2 and 11-KT were similar, in that their levels were relatively lower in Orange County and highest at DP and SD. These two steroids showed a significant positive correlation overall, and both use T as a precursor in their biosynthesis. Therefore, the spatial differences observed in this study are possibly associated with factors that influence steroidogenic pathways leading to conversion of T into 11-KT as well as into E2.

Female hornyhead turbot exhibited a similarly timed reproductive cycle as compared to males, characterized by a spring peak and a fall decline in the reproductive indices measured. In addition to an overall spring peak in E2 concentrations, female fish showed significant springtime increases in ovarian GSI as well as in plasma VTG concentrations. GSI and VTG were strongly correlated with each other, and they were also positively correlated with E2. These findings point to a coordinated seasonal female reproductive system regulated by E2. When each of the study sites was evaluated separately, however, seasonality in E2 concentrations was not evident. Instead, the androgens showed more prominent seasonality, overall and within study sites. Although at significantly lower concentrations than in males, T levels were significantly increased in winter and peaked in spring while 11-KT showed a similar pattern. Both androgens showed some modest positive correlations with ovarian GSI. It was also noticed that T and 11-KT were correlated with each other in the females, which was particularly strong in the fall and winter seasons. Recent work by Forsgren and Young (2012) indicates a potential role for androgens in regulating ovarian follicular development in salmon. Increasing levels of T and 11-KT in the winter-to-spring periods in hornyhead turbot (as seen in Figure 5) would be consistent with such actions.

Across the study sites, mean E2 levels in female hornyhead turbot exhibited significant location-associated differences. The highest E2 levels were observed in females from DP and SD, while relatively lower levels were seen in females from Orange County (OC and OCF) and PVF, a spatial pattern similar to that observed in the males E2 levels. The spatial pattern in both sexes did not coincide with the locations of WWTP outfall discharges, but the findings do appear to point to a common environmental or biological factor(s) influencing gonadal steroidogenesis and E2 production in both sexes. It was also observed that correlations between E2 and GSI and VTG in females were detectable in some sub-groups (e.g., PV sites) but not detectable in others (e.g., OCF, DP), indicating possible spatial differences in E2 action (e.g., on

VTG production). Further research will be required to understand these differences.

While there were no significant differences in age among all study sites for both female and male hornyhead turbot, Forsgren *et al*. (2012) noted smaller mean length and lower springtime gonadal maturity stage in fish sampled from the OCF study site as compared with fish from the other sites which all had similar springtime maturity (true in both sexes). Therefore, the sample of fish collected at the OCF site may have been slightly less mature which may help to explain the relatively lower springtime concentrations of E2 in females and T and E2 in males from this site. With the exception of the OCF group, however, endocrine measures did not correlate with fish size and age, likely a result of the limited range of fish size chosen for evaluation in the study. Forsgren *et al*. (2012) speculated that the proximity of petroleum-drilling platforms near OCF and relatively higher levels of PAHs could be related to reduced maturity, which would require additional studies. It is also possible that that the turbot population age structure is different in the area where the OCF site is located, as compared with other study sites.

As part of their annual cycle, both male and female hornyhead turbot exhibited increases in HSI in spring and summer. While this may represent seasonal differences in food availability, and therefore relatively increased hepatic fuel storage (Haigwood *et al*. 2000) during these months, it was also noted that T concentrations were significantly correlated with HSI in both sexes. In females, GSI and VTG concentrations were also correlated with HSI and T. These findings suggest a role of androgens in adapting hepatic metabolism during the reproductive season in hornyhead turbot, actions which have been documented in studies of other fish species (Holloway *et al*. 1999, Sangiao-Alvarellos *et al.* 2006).

A large number of published studies to date have reported that fish residing in WWTP-impacted environments may exhibit reproductive abnormalities, including altered levels of sex steroids and VTG, as well as gonadal changes such as ovo-testis formation or aberrant expression of steroidogenic genes (Hassanin *et al*. 2002, Jobling *et al*. 2006, Woodling *et al*. 2006, Scott *et al*. 2007, Baker *et al*. 2009, Vajda *et al*. 2011). While most research to date has been carried out in relatively confined aquatic locations (e.g., rivers or streams), the present study was undertaken in an open coastal marine environment. Matthiessen (2003) previously noted that endocrine disruption effects are likely to be more difficult to detect in marine environments, given that dispersion and dilution rates are typically greater (e.g., WWTP effluents). Offshore of Orange County, previous research has been unable to demonstrate unequivocal effects of the OCSD outfall on plasma VTG or reproductive steroid levels, GSI, or in gonadal histology in flatfish (Reyes *et al*. 2002, Rempel *et al*. 2006, Reyes 2006, Deng *et al*. 2007, Petschauer 2008, Baker *et al*. 2009). Yet, fish sampled from the OCSD outfall area are well known to exhibit higher tissue concentrations of several kinds of contaminants (e.g., PCBs) typical at the discharge site.

In the paper by Maruya *et al*. (2012), hornyhead turbot liver composite samples were measured for a variety of contaminants, in parallel to our biological measures of hornyhead turbot from the same study sties. Their results indicate that hornyhead turbot experience exposures to contaminants such as PCBs, DDTs, and PBDEs, which are greater in sediments and tissue of fish sampled from WWTP discharge locations as compared with the DP reference location. As described earlier, the observed spatial differences in the endocrine system of hornyhead turbot did not reflect this pattern. Although present in effluents, E2, estrone, T or 17α-ethinylestradiol were not detectable in receiving waters or sediments (Vidal-Dorsch *et al*. 2012, Maruya *et al*. 2012) and therefore environmental delivery of steroids to the fish (leading to altered endogenous E2 or androgens) is not supported by the data. Among the list of analytes measured in liver, the only PPCP detectable in hornyhead turbot was diazepam (trade name Valium), which was present in 100% of the samples at a mean concentration of 56 µg/kg (Maruya *et al*. 2012). The ICC, 4-nonylphenol, was also frequently detected, at a mean concentration of 102 µg/kg. Based upon previous experimental studies, both diazepam (Calvo *et al*. 1991) and 4-nonylphenol (Kortner and Arukwe 2007) may impair gonadal steroidogenesis. However, their liver concentrations in hornyhead turbot from the DP reference location were comparable to those from all four of the WWTP sites. Therefore, while some environment-associated differences in endocrine function were documented in hornyhead turbot in this study, it is not yet clear the degree to which environmental contaminants or WWTP discharges in the coastal habitat are related. Further research will be needed,

particularly evaluations that can combine measurements of contaminant exposures and effects in the same subjects, to enable direct linkages to be made.

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