Xenoestrogen exposure and effects in English sole (*Parophrys vetulus*) from Puget Sound, WA

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ABSTRACT

Vitellogenin, a yolk protein produced in the liver of oviparous animals in response to estrogens, normally occurs only in sexually mature females with developing eggs. However, males can synthesize vitellogenin when exposed to environmental estrogens, making the abnormal production of vitellogenin in male animals a useful biomarker for xenoestrogen exposure. In 1997–2001, as part of the Washington State's Puget Sound Assessment and Monitoring Program, we surveyed English sole from a number of sites for evidence of xenoestrogen exposure, using vitellogenin production in males as an indicator. Significant levels of vitellogenin were found in male fish from several urban sites, with especially high numbers of fish affected in Elliott Bay, along the Seattle Waterfront. Intersex fish were rare, comprising only two fish out of more than 2900 examined. Other ovarian and testicular lesions, including oocyte atresia, were also observed, but their prevalence did not appear to be related to xenoestrogen exposure. However, at the Elliott Bay sites where abnormal vitellogenin production was observed in male sole, the timing of spawning in both male and female English sole appeared altered. Sources of xenoestrogens and types of xenoestrogens present in Elliott Bay are poorly documented, but the compounds are likely associated with industrial discharges, surface runoff, and combined sewer outfalls.

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1. Introduction

Endocrine disrupting chemicals (EDCs) are natural or man-made substances capable of acting as hormone mimics or blocking hormone action. These substances can disrupt normal endocrine system responses and functions by altering the production, release, transport, metabolism, binding, action or elimination of an organism's natural hormones (Kavlock et al., 1996). Such interference may lead to adverse effects on development, growth, behavior and reproduction (Ankley et al., 1998). Xenoestrogens are one group of EDCs that have received particular attention because they are known to interfere with estrogen responses crucial for normal development and reproduction. Most of these compounds are structurally similar to estrogens that are produced naturally in the body, and include synthetic estrogens (e.g., ethinyl estradiol), surfactants (e.g., alkylphenol ethoxylates), plasticizers (e.g., bisphenol-A) and phytoestrogens (e.g., β-sitosterol). Some of these compounds are persistent and can bioaccumulate, and when they occur together, they may have additive effects. Xenoestrogens are often associated with sewage outfalls and industrial sites (Matthiessen and Sumpter, 1998), and studies suggest that they are widespread in freshwater systems in the United States (Kolpin et al., 2002). Less is known about their distribution in the marine waters of North America, such as Puget Sound, although recent studies suggest they are present at some nearshore and estuarine sites (Reddy and Brownawell, 2005; Schlenk et al., 2005; Zuo et al., 2006; King County, 2007).

Fish exposed to xenoestrogens in the wild and in laboratory studies have shown a variety of effects including inhibited testicular growth, reduced sperm production and quality, intersex gonads containing both male and female germ cells, reduced egg production, altered reproductive timing and behavioral changes (Matthiessen and Sumpter, 1998; Sumpter, 1998; Goksoyr, 2006). Perhaps the most widely documented biological response to xenoestrogens is abnormal vitellogenin (Vtg) production in male fish. Vtg is a sex-specific protein synthesized in the liver of oviparous animals in response to circulating estrogens. Although Vtg is normally produced only in sexually mature females, it can be induced in males exposed to estrogens, making it a useful biomarker for the presence of estrogenic substances in the environment. Although uncommon, Vtg production in wild male fish (both marine and freshwater) has been documented in several species, including

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rainbow trout, bream, roach, flounder, dab, and turbot (Purdom et al., 1994; Harries et al., 1996; Allen et al., 1999; Hashimoto et al., 2000; Roy et al., 2003; Kirby et al., 2004; Vethaak et al., 2005; Rempel et al., 2006; Deng et al., 2007; Scott et al., 2006, 2007), primarily in urban rivers and estuaries contaminated by sewage effluent and industrial discharges.

Vtg is relatively easy to measure in blood plasma using immunoassay techniques such as the enzyme-linked immunosorbent assay (ELISA) (Rotchell and Ostrander, 2003; Lequin, 2005). We developed an ELISA (Lomax et al., 1998) to measure plasma Vtg in English sole (*Parophrys vetulus*), a benthic marine fish that is a common target species in Puget Sound monitoring programs (Myers et al., 1994; Johnson et al., 1998; West et al., 2001). Previous studies had demonstrated that English sole inhabiting contaminated sites in the Sound were prone to various kinds of reproductive dysfunction, potentially due to exposure to xenoestrogens or anti-estrogenic compounds (Johnson et al., 1998, 1999). However, no data were available on exposure to xenoestrogens in this species, although uptake of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and other industrial compounds had been documented in Puget Sound English sole (Stein et al., 1992; Collier et al., 1993; Myers et al., 1994; Johnson et al., 1998, 1999; West et al., 2001).

The primary objective of the present study was to examine wild English sole in Puget Sound for evidence of exposure to xenoestrogens by comparing Vtg induction in male fish across sixteen locations where potential xenoestrogen sources vary widely. Additionally, we assessed the potential effects of xenoestrogen exposure by examining gonad histology and reproductive timing of male and female English sole from sites where Vtg screening was conducted. Histopathological abnormalities that have been reported in fish exposed to environmental estrogens include gonads containing both oogonia and spermatogonia (intersex), oocyte atresia, and other degenerative gonad lesions (Jobling et al., 2002; Weber et al., 2003). This survey was conducted in conjunction with Washington State's Puget Sound Assessment and Monitoring Program (PSAMP), as a collaboration between NOAA Fisheries and the Washington State Department of Fish and Wildlife.

2. Methods

2.1. Fish and sample collection

The sampling for this study was conducted between 1997 and 2001 during the spring (April/May) of each year. Table 1 shows a list of the 16 sites sampled and an overview of site characteristics (West et al., 2001); the locations of these sites are shown in Fig. 1. All sites were sampled at least once during the study and some (Bell Harbor, Thea Foss Waterway, Bremerton, Port Gardner, Hood Canal, and Nisqually) were sampled as frequently as three times for blood and five times for gonad histology. Blood samples were collected from a subset of male English sole sampled from 1997 to 2001. The sixteen sites sampled during this study included English sole habitats ranging from well-known, highly polluted industrial or urban settings (termed "urban") to those far removed from known sources of pollution ("non-urban"). Intermediate sites were termed "near-urban". We classified five sites in Elliott Bay (Duwamish Waterway, Harbor Island, Bell Harbor, Myrtle Edwards, and Alki), Thea Foss Waterway in Commencement Bay, and two sites in Sinclair Inlet (Bremerton and Outer Sinclair) as urban. Three sites (Nisqually, Hood Canal, and Port Susan) were classified as non-urban, and the remaining sites were classified as near-urban.

All fish were collected by bottom trawl, following environmental sampling protocols developed by the Puget Sound Estuary Program (PSEP, 1990). Fish were weighed (to the nearest gram) and measured (total length, TL, to the nearest 1 mm). For measurement of Vtg, blood was withdrawn from the caudal vein of male English sole of adult size range (>230 mm TL; Lassuy, 1989) using heparinized syringes. Blood was centrifuged at 800 x g. Plasma was then collected, treated with the protease inhibitor, phenyl methyl sulfonyl sulfate (PMSF, 1 mM in plasma), and aliquoted. Samples were maintained on ice, then transported to the laboratory and transferred to freezers at the end of each day. Plasma samples were stored frozen at $-80\,°C$ until analyses were performed.

Interopercular bones were removed for age determination. Fish were then necropsied, and gonad samples were collected and preserved in Dietrich's fixative for histological examination. Details of necropsy procedures and histology sample collection and fixation are described in greater detail in Johnson et al. (1988, 1991), Sol et al. (1998) and Stehr et al. (1993).

2.2. Vitellogenin analyses

English sole plasma samples were analyzed for the presence of Vtg utilizing an ELISA developed for this species. This assay uses purified English sole Vtg as a standard and a polyclonal antibody developed in rabbits against the English sole Vtg. The ELISA is a competitive antibody capture assay and is described in detail elsewhere (Lomax et al., 1998). The number of samples analyzed for each site ranged from eight at Outer Sinclair Inlet to 55 at the Harbor Island site. Samples were analyzed over a 2 year period, in three major sets, corresponding to the dates of sample collection. As part of our laboratory quality assurance (QA) plan, a fish plasma control sample (pooled plasma from several vitellogenic female English sole) was analyzed in triplicate with the other plasma samples as part of each assay (Lomax et al., 1998). The average intraassay coefficient of variation (CV) for the QA sample was 9.6, while the interassay CV was 20.9. The average EC80 for these assays was 15.1 ng/ml and the samples were diluted to $100\times$. Thus, the average limit of detection was 1510 ng/ml. Because the assay uses a polyclonal antibody that could show some cross-reactivity with other plasma proteins similar to Vtg, and because we are unsure of how closely our results would match those from another laboratory performing a comparable assay, we consider these values to be semi-quantitative estimates of actual Vtg concentrations in sole plasma.

2.3. Age determination

Because the likelihood of reproductive development in fish is dependent on age, ages were determined in all sampled sole. Fish age was estimated to the nearest year by counting the number of clearly defined opaque zones in interopercular bones under a binocular dissecting microscope (Palmen, 1956).

2.4. Histology

Tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined microscopically. The developmental stages of the ovaries were classified according to criteria described in Johnson et al. (1991) into 6 stages: spent, regressed, previtellogenic, vitellogenic, ripe/hydrated, spawning, and spent. The developmental stages of the testes were classified according to criteria modified from Sol et al. (1998) into 5 stages: regressed, recrudescence, spermiogenesis, spawning, and spent.

Ovaries and testes were also examined to detect intersex fish (i.e., those whose gonads contained both ovarian and testicular tissue). Additionally, various other gonadal lesions were monitored. Ovaries were examined for: (1) follicular atresia of yolked and non-yolked oocytes, as well as late-stage atresia where the oocyte stage

Table 1

Locations and characteristics of sites where English sole were collected in April and May of 1997–2001, percentages of male English sole from those sites with detectable vitellogenin (Vtg) in plasma and the mean concentration of Vtg (ng/ml plasma) measured in those male sole. ND, not detected; concentration was below limits of detection

Site no.	Water body	Site type	Site name	Latitude	Longitude	Depth (m)	N	% Vtg males	Mean Vtg (ng/ml) ± S.D.[a]	Range of Vtg in positive samples
6	Elliott Bay	Urban	Myrtle Edwards	47.622	122.378	49–55	49	46.9[*]	5,900 ± 12,000[**]	1,800–83,000
4			Bell Harbor	47.606	122.345	24–46	32	37.5[*]	4,400 ± 6,700[**]	2,500–36,000
5			Harbor Island	47.592	122.356	51–62	55	38.2[*]	3,800 ± 5,700[**]	2,200–35,000
7			Alki Point	47.584	122.411	42–59	17	11.8	2,700 ± 5,100	3,800–22,000
3			Duwamish Wwy	47.560	122.345	11	17	17.6	1,900 ± 1,600	3,000–7,500
2	Commencement Bay		Thea Foss Wwy	47.259	122.435	7–9	23	21.7[*]	2,300 ± 2,300	3,300–12,000
8	Sinclair Inlet		Bremerton	47.547	122.650	4–15	17	5.9	1,800 ± 1,200	6,400[b]
9			Outer Sinclair	47.571	122.589	24–31	8	0	ND	ND
15	Port Gardner	Near-urban	Port Gardner	47.985	122.244	11–60	16	18.8	2,900 ± 3,500	6,000–14,000
11	Port Orchard		N. Port Orchard	47.673	122.599	16–20	9	11.1	2,200 ± 1,100	5,100[b]
10			S. Port Orchard	47.629	122.583	33–38	9	0	ND	ND
13	Eagle Harbor		Eagle Harbor	47.620	122.511	9–13	25	0	ND	ND
12	Central Puget Sound		Blake Island	47.552	122.486	31–49	12	16.7	2,100 ± 1,200	2,500–5,700
14	Hood Canal	Non-urban	Hood Canal	47.836	122.637	31–46	35	5.7	1,800 ± 1,200	5,300–6,700
16	Port Susan		Port Susan	48.146	122.384	35–69	44	6.8	1,600 ± 1,200	2,600–8,700
1	Nisqually		Nisqually	47.161	122.669	73–139	17	0	ND	ND
	All non-urban							5.2	1,600 ± 1,100	2,500–8,700

[a] A value of one half the limit of detection was used in computing the mean, for samples with no detected Vtg. Detection limits ranged from 1720 to 5420 (ng/ml).

[b] Only one positive sample.

[*] Significantly different from combined non-urban sites (using modified Dunnett's compare to control test for proportions; $p \leq 0.05$).

[**] Significantly different from combined non-urban sites (using ANOVA and Dunnet's test; $p \leq 0.01$). For statistical analysis the values were log transformed to achieve a more normal distribution.
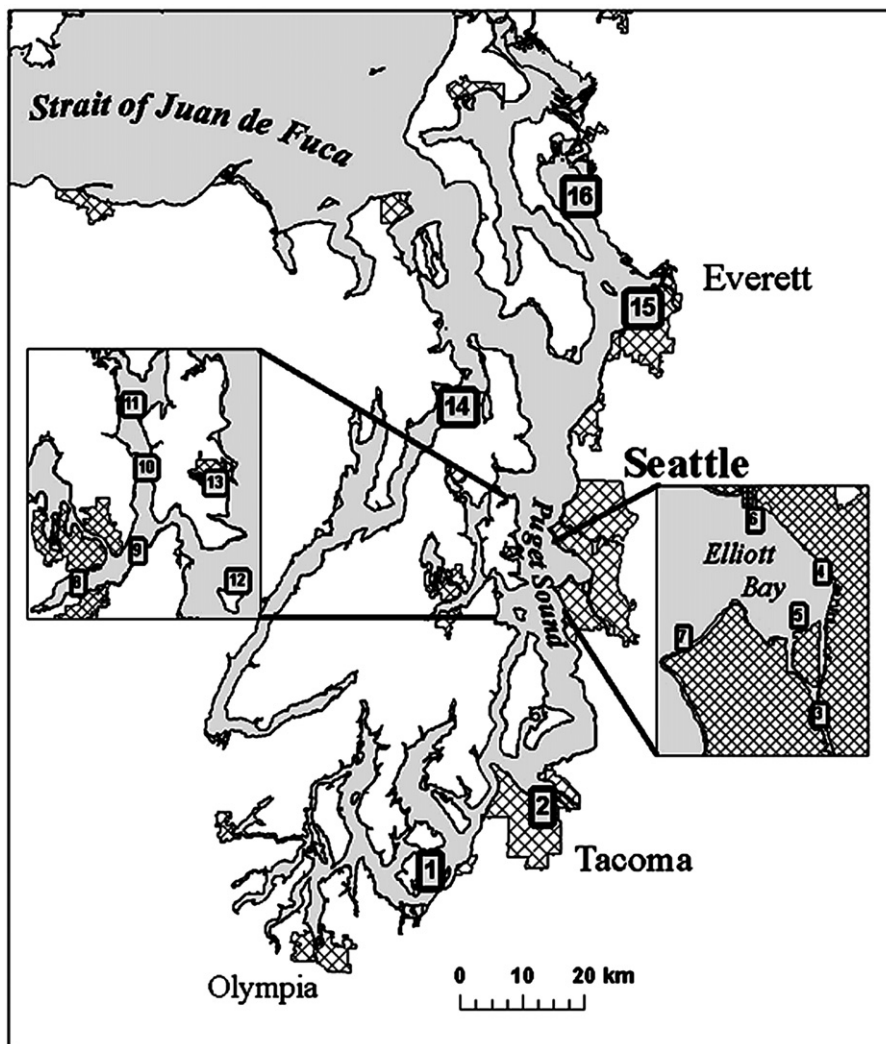
Fig. 1. English sole sampling sites for 1997–2001 Puget Sound ambient monitoring studies and vitellogenin survey. Insets show sites within Elliott Bay (3–7) and Sinclair Inlet (8–13). Site names and characteristics are provided in Table 1.

can no longer be determined; (2) ovarian macrophage aggregates, and other inflammatory lesions associated with oocyte resorption, including lymphoid or macrophage infiltrates; (3) vascular lesions; (4) proliferative lesions; and (5) neoplasms and related lesions, using criteria described in Johnson et al. (1991) and Blazer (2002). Atresia severity was rated on a scale of 1–7, where 1 = minimal, sparse or few; 2 = minimal to mild; 3 = mild, few, or small amount; 4 = mild to moderate, several; 5 = moderate, moderate amount, moderate number; 6 = moderate to severe; and 7 = severe, abundant, numerous. Because a certain amount of mild atresia (i.e., ranked 1–3 in severity) is normal in female English sole (Johnson et al., 1991), only moderate to severe atresia (i.e., ranked 4–7 in severity) was considered as constituting a lesion in this study. Testes were examined for inflammatory, necrotic, proliferative, vascular, and neoplastic lesions, as described in Sol et al. (1998) and Blazer (2002).

2.5. Statistical analyses

Because the likelihood of reproductive development in fish is dependent on size and age, we measured and compared these parameters in sole from our sampling sites. The primary objective of this analysis was to document that the fish used in this study were of adult size and age, and there were no major intersite dif-

ferences in size and age distribution that would confound other results. Analysis of variance (ANOVA) and Dunnett's test for comparing a control mean to other group means (Zar, 1998) were used to identify statistically significant intersite differences in length and age. To determine the control mean, data from the three non-urban sites were pooled to form a presumed unexposed reference group. Similarly, ANOVA and Dunnett's test for comparing a control mean to other group means (Zar, 1998) were used to identify statistically significant differences in plasma Vtg levels in male sole. For parametric tests such as ANOVA, data were normalized as necessary through log transformation prior to analysis. Dunnett's test to compare a proportion to a control (Zar, 1998) was used to identify significant differences in proportions of male sole with Vtg, female sole with ovarian lesions, and male sole with testicular lesions at the urban and near urban sites, as compared to the three non-urban sites (pooled to form a presumed unexposed reference group).

3. Results

3.1. Vitellogenin induction

Twelve of the sixteen sites sampled had male fish with quantifiable levels of Vtg, with concentrations ranging from 1800 to

83,000 ng Vtg/ml of plasma. Elliott Bay, in which five sites were sampled, was the most urbanized area and had the largest number of male sole with detectable levels of Vtg (Table 1). Within Elliott Bay, the Myrtle Edwards site at the north end of the Seattle Waterfront had both the highest percentage of animals affected (47%) and the highest average Vtg concentration (5900 ng/ml). The Bell Harbor site in the middle of the Seattle Waterfront and the Harbor Island site at the southern end of the Seattle Waterfront also had high percentages (37–38%) of fish with Vtg and high Vtg levels. The two remaining sites in Elliott Bay (Duwamish Waterway entering Elliott Bay and Alki in outer Elliott Bay) showed lower proportions of male fish with Vtg (18 and 12%, respectively).

Outside of the Elliott Bay sites, the Thea Foss Waterway in Commencement Bay was the only urban station where more than 20% of male fish had Vtg. At Sinclair Inlet, the third urban/industrial embayment sampled, 6% of males from the Bremerton site had Vtg, but Vtg was not detected in male fish from the Outer Sinclair site. At the near-urban sites, the percentage of males with Vtg ranged from 0 to 19%, with the highest proportion of vitellogenic males at the Port Gardner site, just outside the industrially-developed Everett Harbor. The presence of Vtg was also detected in a small proportion (6–7%) of fish from non-urban stations like Port Susan and Hood Canal. Vtg was not detected in any fish from the Nisqually site.

3.2. Fish length and age

Surveys of English sole from the Pacific Coast of North America indicate sexual maturity in female English sole typically occurs at 3–4 years of age while in male sole it occurs at 2–3 years of age (Lassuy, 1989). Depending on the population of sole sampled, the reported length at 50% maturity ranges from 23 to 31 cm for females (Lassuy, 1989; Sampson and Al-Jufaily, 1999), while the typical length at 50% maturity is ∼22 cm for males (Lassuy, 1989).

The average length of female English sole sampled in this study ranged from 263 mm at the Port Susan site to 354 mm at the S. Port Orchard site, while the average age ranged from 4.5 years of age at the Nisqually and Outer Sinclair sites to 7.4 years of age at the Harbor Island site (Table 2). Thus, female sole sampled from all sites were within what would be considered an adult size and age range. However, the fish collected from non-urban sites tended to be smaller and younger than fish collected from urban or non-urban areas. Female sole from six of the eight urban sites and four of the five near-urban sites were significantly larger than female sole from

the non-urban areas, while female sole from five of the eight urban sites were significantly older than female sole from the non-urban areas. Female sole from the near-urban areas were generally similar in age to those from the non-urban areas. The Blake Island site was the only near-urban site where female sole were significantly older than those from the non-urban areas.

The male sole sampled as part of this study tended to be slightly older than female sole, as their average age ranged from 6 years at the Blake Island site to 8.7 years at the Harbor Island site (Table 2). The average length of male English sole ranged from 239 mm at the Port Susan site to 287 mm at the Bremerton site (Table 2). Like female sole, the male sole from all sampling sites were within the adult age and size range, although sole collected from non-urban sites tended to be smaller and younger than sole collected from urban or near-urban areas. Male sole from four of the eight urban sites and three of the five near-urban sites were significantly larger than male sole from the non-urban areas, and male sole from two of the eight urban sites, Bell Harbor and Harbor Island, were significantly older than male sole from the non-urban areas. However, no significant differences in age were found between male sole from the near-urban sites and the non-urban areas.

3.3. Stage of gonadal development

The three urban locations that exhibited the greatest Vtg induction in male fish also exhibited an unusual pattern of maturity in females. Under normal conditions, gonadal recrudescence in Puget Sound English sole begins in the late summer or early fall, with females entering vitellogenesis and sperm production beginning in males at this time (Johnson et al., 1991; Sol et al., 1998). Gonadal development proceeds throughout the winter, and spawning typically occurs in February and March. By April or May, when our sampling occurred, females have generally completed spawning, and typically exhibit a spent or regressed condition. Males may continue to exhibit a spermiogenic or spawning condition for a somewhat longer period (e.g., through the month of May). In the present study, female English sole conformed to this typical pattern of reproductive cycling at all but the three urban Elliott Bay sites, where Vtg was most frequently detected in males (Fig. 2). The majority of female sole (87–100%) from thirteen locations had completed spawning, and their ovaries were in a spent or regressed to pre-vitellogenic state. However, at the three Seattle Waterfront sites (Myrtle Edwards, Bell Harbor, and Harbor Island), only 5–35% of fish were spent or regressed; the majority of females from these

Table 2
Mean lengths and ages of male and female English sole collected from Puget Sound sites in April and May of 1997–2001

Site no.	Site name	N	Females length (mm) ± S.D.	Age (years) ± S.D.	N	Males Length (mm) ± S.D.	Age (years) ± S.D.
6	Myrtle Edwards	49	289 ± 41	6.4 ± 2.0	67	267 ± 62**	6.7 ± 2.3
4	Bell Harbor	107	312 ± 55**	7.0 ± 2.9**	190	266 ± 28**	8.5 ± 2.7*
5	Harbor Island	23	337 ± 25**	7.4 ± 1.9**	58	276 ± 24**	8.7 ± 2.3*
7	Alki Point	39	313 ± 38**	6.8 ± 2.4**	21	259 ± 28	8.0 ± 3.0
3	Duwamish Wwy	25	313 ± 25**	6.6 ± 2.4	34	264 ± 21	8.5 ± 2.7
2	Thea Foss Wwy	146	300 ± 153**	6.6 ± 3.3**	136	253 ± 28	6.9 ± 3.1
8	Bremerton	195	351 ± 51**	5.9 ± 2.6*	103	287 ± 35**	7.2 ± 3.5
9	Outer Sinclair	49	280 ± 44	4.5 ± 1.4f	11	264 ± 29	7.0 ± 3.4
15	Port Gardner	214	279 ± 36	5.7 ± 2.2	85	254 ± 21	8.1 ± 2.4
11	N. Pt. Orchard	35	319 ± 47**	5.1 ± 2.3	25	271 ± 28*	6.8 ± 3.6
10	S. Port Orchard	35	354 ± 44**	6.3 ± 2.2	25	281 ± 23**	6.8 ± 2.5
13	Eagle Harbor	25	297 ± 38**	5.1 ± 1.8	99	264 ± 28*	6.6 ± 3.4
12	Blake Island	48	316 ± 42**	7.3 ± 2.6**	12	266 ± 22	6.0 ± 2.7
14	Hood Canal	235	293 ± 37	6.1 ± 2.2	64	276 ± 24	7.8 ± 3.0
16	Port Susan	40	263 ± 21	4.9 ± 1.3	47	239 ± 21f	6.8 ± 3.0
1	Nisqually	222	272 ± 38	4.5 ± 2.0	76	258 ± 24	7.0 ± 3.3
	All non-urban	497	281 ± 38	5.3 ± 2.2	187	252 ± 23	7.3 ± 3.2

* Significantly different from combined non-urban sites (using ANOVA and Dunnett's test, $p \leq 0.05$).
** Significantly different from combined non-urban sites (using ANOVA and Dunnett's test, $p \leq 0.01$).
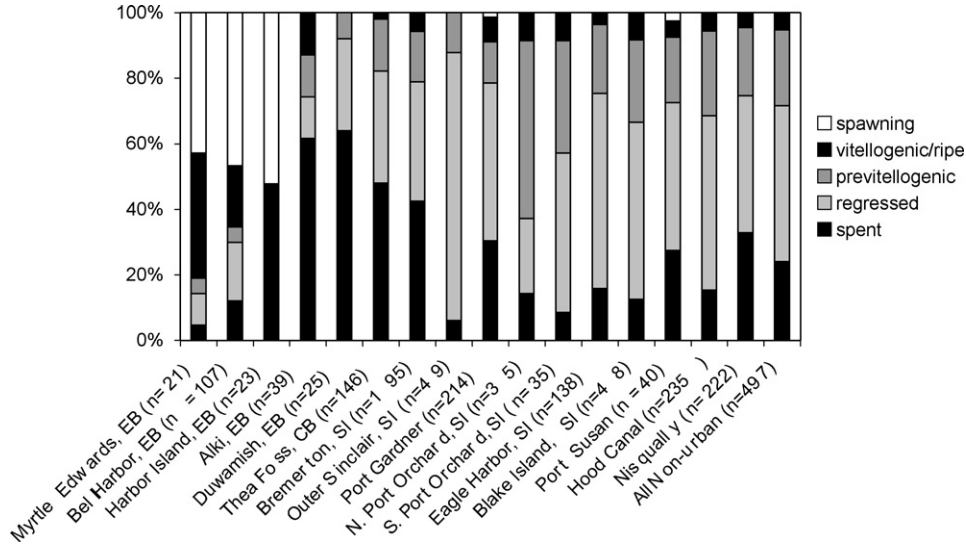
Fig. 2. Percentages of female English sole in different stages of ovarian development at Puget Sound sites sampled in April and May of 1997–2001. EB, Elliott Bay; CB, Commencement Bay; SI, Sinclair Inlet.

three sites (65–95%) were still either vitellogenic to ripe, or in a spawning condition. Male English sole from the three high Vtg sites also showed an unusual pattern of gonadal maturation (Fig. 3). At most sampling sites the majority of males were spent, but at the Seattle Waterfront sites (Myrtle Edwards, Bell Harbor, and Harbor Island), the majority of males (52–78%) were still in spawning condition.

3.4. Prevalence of intersex fish

As part of the histological examination of the gonads, all fish were examined for the presence of male reproductive tissue in the female gonads and vice versa. Among the 2921 English sole examined between 1997 and 2001, two intersex fish were observed, one at Port Gardner in Everett, and one in Commencement Bay at Thea Foss Waterway site (Table 3). The Port Gardner fish was a masculinized female, while the Thea Foss fish was a feminized male. Both

of these sites were sampled for five consecutive years, but intersex fish were observed only once at each site.

3.5. Other ovarian and testicular lesions

Overall, 14% of female English sole had one or more idiopathic ovarian lesions (i.e., atresia, inflammatory lesions, and vascular lesions) with prevalences ranging from 0 to 43% at individual sites (Table 3). Ovarian lesions were most prevalent (and significantly greater than at the non-urban reference sites) in sole from the Thea Foss Waterway, Port Orchard, Alki Point, and Blake Island. The primary ovarian lesion was oocyte atresia, affecting over 40% of females at two sites. Atresia of both yolked and non-yolked (regressed and previtellogenic) oocytes was observed, as well as late stage atresia in which the stage of the resorbing oocyte could no longer be identified. In addition to ovarian atresia, some female sole exhibited inflammatory or vascular lesions in the ovary,
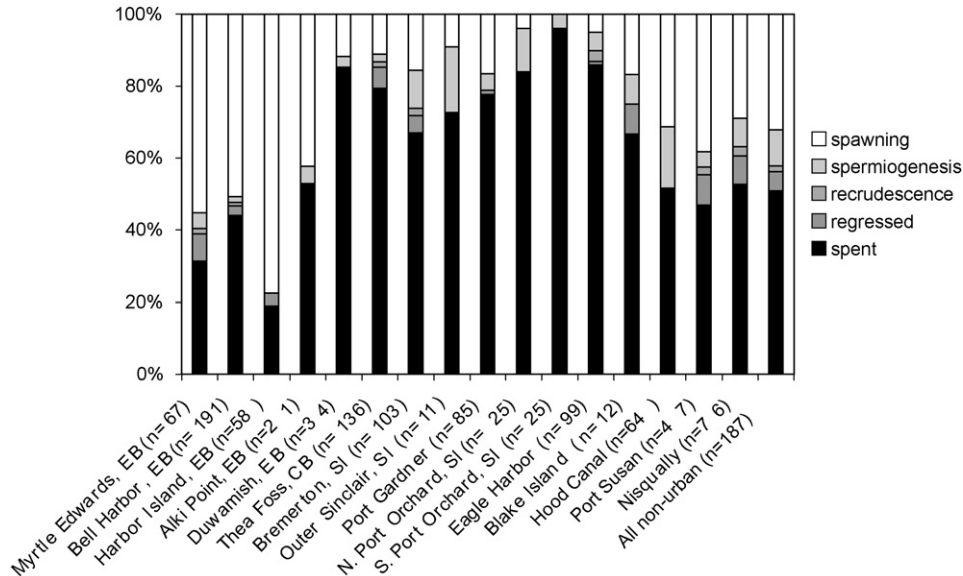

Fig. 3. Percentages of male English sole in different stages of testicular development at Puget Sound sites sampled in April and May of 1997–2001. EB, Elliott Bay; CB, Commencement Bay; SI, Sinclair Inlet.

Table 3
Percentages of ovarian and testicular lesions in English sole sampled from Puget Sound in April and May of 1997–2001

Site no.	Site name	Females						Males				
		N	Oocyte atresia	inflammatory lesions	Vascular lesions	Intersex (spermatogonia in ovary)	One or more ovarian lesions	N	Neoplasms	inflammatory lesions	Intersex (oocytes in testis)	One or more testicular lesions
6	Myrtle Edwards	21	5	0	0	0	5	67	0	1.5	0	1.5
4	Bell Harbor	140	5	0	0	0	5	191	0	0.5	0	0.5
5	Harbor Island	23	0	0	0	0	0	58	0	0	0	0
7	Alki Point	39	15*	5.1**	0	0	18*	21	0	0	0	0
3	Duwamish Wwy	25	8	0	0	0	8	34	0	2.9*	0	2.9*
2	Thea Foss Wwy	170	17**	1.2	2.4	0	19**	136	0.7	0	0.7	1.5
8	Bremerton	267	9	0	0.7	0	10	103	0	1.0	0	1.0
9	Outer Sinclair	35	8	0	5.7*	0	11	11	0	0	0	0
15	Port Gardner	312	12**	1.9	0.3	0.3	14	83	0	1.2	0	1.2
11	N. Pt. Orchard	35	17**	0	0	0	17	25	0	0	0	0
10	S. Port Orchard	35	40**	2.9	0	0	43**	25	0	0	0	0
13	Eagle Harbor	138	9	2.2	0	0	11	99	0	0	0	0
12	Blake Island	48	40**	0	0	0	40**	12	0	0	0	0
14	Hood Canal	283	6	0	2.5	0	8.1	64	0	0	0	0
16	Port Susan	40	10	0	0	0	10	47	0	0	0	0
1	Nisqually	327	11	2.1	0.3	0	14	76	0	0	0	0
	All non-urban	650	8.8	1.1	1.2	0	11	187	0	0	0	0

* Significantly different from combined non-urban sites (using modified Dunnett's compare to control test for proportions, $p \leq 0.05$).
** Significantly different from combined non-urban sites (using modified Dunnett's compare to control test for proportions, $p \leq 0.01$).

but these were found only at low prevalences ($\leq 6\%$). The prevalence of inflammatory lesions at Alki Point, and the prevalence of vascular lesions at Outer Sinclair Inlet, were significantly different from prevalences at the non-urban reference sites, but no other significant intersite differences were observed. Ovarian lesion prevalences were not significantly elevated in any of the Seattle Waterfront sites, where Vtg induction was most frequently observed.

The prevalences of male sole with testicular lesions were generally low. Lesions were only observed in sole from urban and near-urban sites (Thea Foss Waterway, the Duwamish Waterway, Bell Harbor, Myrtle Edwards, Port Gardner, and Bremerton) at prevalences ranging from 0.5 to 2.9%. Sole from the Duwamish Waterway, one of the most industrialized sites (West et al., 2001) had significantly higher prevalences of inflammatory lesions than sole from the non-urban reference sites, but no other significant differences were observed. One fish from the Thea Foss Waterway had a testicular neoplasm, a seminoma or tumor of the testicular epithelium, and, as noted above, one intersex fish, with oocytes in the testis, was observed at this site. Although lesions were more common in sole from urban sites, prevalences were not significantly higher in sole from the Seattle Waterfront sites (Myrtle Edwards, Bell Harbor, and Harbor Island), where male sole expressing Vtg were most abundant.

4. Discussion

The results of this study indicate that exposure of flatfish to xenoestrogens in Puget Sound is widespread, and provide one of the few reported cases of altered reproductive cycling in association with xenoestrogen exposure in a wild marine fish. Vtg induction, an indicator of estrogen exposure, was observed in male English sole at three-fourths of the sites we sampled, and exposures were greatest near urban centers with high inputs of stormwater and industrial discharges, and at sites near combined sewer overflows (CSOs) and/or sewage treatment plant (STP) discharges. The Myrtle Edwards station in Elliott Bay in Seattle (the area with the highest population density in the region) had the highest overall percentage of male fish exhibiting Vtg induction, and is near several CSOs and storm drain overflows. The same characteristics apply to other sites with high percentages of fish with Vtg or high Vtg levels, such as the other Seattle waterfront stations, Harbor Island and Bell Harbor, as well as the Thea Foss Waterway station in Commencement Bay near the city of Tacoma. Although the comparability of Vtg measurements in assays conducted by different laboratories is limited (Andersen et al., 1999), the levels of Vtg measured in Puget Sound English sole are within the range of levels found in other flatfish species where similar studies on endocrine disruption in fish have been conducted (Folmar et al., 1996; Jobling et al., 1998; Allen et al., 1999; Hashimoto et al., 2000; Kirby et al., 2004; Kleinkauf et al., 2004; Vethaak et al., 2005). In some of these studies, additional biological effects such as intersex and altered testis shape were observed, while in others, biological effects were minimal.

We observed only two intersex fish out of nearly 3000 fish examined, and their occurrence was not clearly associated with xenoestrogen exposure, although the fish were found at an urban and a near-urban site (Thea Foss and Port Gardner) where some Vtg induction in males was observed. Similarly, intersex fish were rare in large-scale surveys of xenoestrogen exposure and effects in Europe, except in a few highly contaminated estuaries in the United Kingdom (Allen et al., 1999; Kleinkauf et al., 2004; Kirby et al., 2004). In our study, prevalences of other gonadal lesions were also low (<5%), with the exception of oocyte atresia, which was found in up to 40% of females at some sampling sites. While some of these lesions, including tumors, were more prevalent at urban sites,

their occurrence showed no clear relationship with Vtg induction. However, they may be associated with exposure to other industrial contaminants (e.g., PCBs, polycyclic aromatic hydrocarbons, and heavy metals) known to be present at these sites (West et al., 2001).

The co-occurrence of Vtg induction in males and altered reproductive timing in the females suggests that exposure to estrogenic compounds may have altered the spawning cycle in female English sole from some sites in Puget Sound. Many of these fish appeared to remain in the vitellogenic or ripe condition for a more extended period than normal (i.e., through late April or early May, as compared to late February or early March under typical conditions; Johnson et al., 1991; Sol et al., 1998). This may have been because final maturation and spawning were blocked or delayed, because the timing of the reproductive cycle was altered, or because exposed sole did not engage in normal migration behavior and so failed to spawn. Year-round sampling would be needed to distinguish among these alternatives. Male fish also appeared to show alteration in their spawning time at the Seattle Waterfront sites, possibly in response to altered timing in maturation of females.

Although sole from all sites were of adult size and age (Van Cleve and El-Sayed, 1969; Lassuy, 1989), both male and female English sole from urban sites, including Bell Harbor and Harbor Island on the Seattle waterfront, tended to be older than sole from non-urban areas. While some studies suggest that younger flatfish, especially those less than 3 years of age, tend to be reproductively active for a shorter seasonal period, and complete spawning earlier than older fish (Bromley, 2000), studies of English sole populations in British Columbia suggest that, within a spawning season, smaller and younger sole tend to mature later and spawn later than larger sole (Fargo and Tyler, 1994). Thus, if anything, sole from the non-urban sites would be expected to mature and spawn later than sole from the Elliott Bay sites, whereas we observed the opposite. Therefore, the alterations in reproductive timing in fish from urban versus non-urban sites are unlikely to be due to differences in fish age.

Inhibition of final maturation and spawning related to xenoestrogen exposure has been shown in other fish species. For example, Noaksson et al., 2005 observed that while gonadal growth and Vtg synthesis appeared normal in female perch (*Perca fluviatilis*) and brook trout (*Salvelinus fontinalis*) living near sewage treatment plants in Sweden, these fish were less likely to be in spawning condition than fish from reference areas where such plants were not present. In the United Kingdom, Kleinkauf et al. (2004) observed greater variability in timing of gonadal growth and spawning in flounder from estuaries where a high proportion of males showed Vtg induction compared to flounder from reference estuaries. There are also several reports of inhibition of spawning and egg production in zebrafish and fathead minnow exposed to estrogenic compounds in laboratory studies (e.g., Van den Belt et al., 2003; Brion et al., 2004; Parrott and Blunt, 2005). However, the laboratory studies typically involved exposures initiated during the embryonic stage, and in some cases not only final maturation and spawning, but also gonadal growth itself was suppressed.

The endocrine control of gonadal growth and spawning in teleost fish is complex (Nagahama et al., 1995; Schulz et al., 2001), and there are various mechanisms through which xenoestrogens might alter normal cycling. In the perch and trout examined by Noaksson et al. (2005), a delay in spawning in fish residing near STPs was associated with a delay in the steroidogenic shift from the synthesis of sex steroids (e.g., 17-β estradiol) towards the production of maturation inducing steroids (various C17 progestins), that normally triggers final maturation and spawning (Nagahama et al., 1995; Schulz et al., 2001; Jalabert, 2005). Exposure to xenoestrogens can also interfere with production of sex steroid-derived pheromonal signals that are important in coordinating the timing of egg and sperm release (Stacey et al., 2003). Another possibility is that exposure to xenoestrogens might be stimulating female fish to mature on a more continuous basis, so the window of time in which fish were capable of spawning would be longer. At this point, we are uncertain which, if any, of these mechanisms are responsible for the alterations in the spawning cycle that we observed in English sole.

The consequences of altered spawning time in English sole are uncertain, but larval and juvenile survival may be compromised if gametes are broadcast during periods of suboptimal environmental conditions. The sperm quality of male English sole might also be affected by exposure to xenoestrogens. Although we did not measure sperm quality in male English sole in this study, a number of reports indicate reductions in sperm production, sperm quality, and fertilization success in male fish exposed to exogenous estrogens (e.g., ethinyl estradiol, nonylphenol) at concentrations comparable to those associated with Vtg induction (Brion et al., 2004; Lahnsteiner et al., 2005; Parrott and Blunt, 2005).

As yet, we have not identified the specific compounds that are responsible for Vtg induction and other biological effects that we have observed in Puget Sound English sole, and information on concentrations of estrogenic compounds in Puget Sound is limited. Sediment concentrations of nonylphenol and the phytoestrogen, β-sitosterol, were measured throughout Puget Sound in a study conducted by NOAA's National Ocean Service and the Washington State Department of Ecology (Long et al., 1999, 2000, 2002; Partridge et al., 2005). Reported concentrations of both nonylphenol ($\leq$30 ng/g dry wt) and β-sitosterol ($\leq$3500 ng/g dry wt) were in the lower range of values reported for other industrial sites in the United States, Asia, and Europe (Koh et al., 2002; Petrovic et al., 2002; Kannan et al., 2001; Ferguson et al., 2001; Isobe et al., 2001; Mudge et al., 1999; Martins et al., 2002; Seguel et al., 2001; Chan et al., 1998; Mudge and Norris, 1997). The few available sediment-exposure studies with xenoestrogens suggest that exposure to these compounds at levels reported in sediments alone would be unlikely to account for Vtg induction in English sole (Schlenk et al., 2005). Synthetic and natural estrogens such as E2 and EE2 were not measured by Long et al. (1999, 2000, 2002) or Partridge et al. (2005), but these substances have been detected in marine sediments elsewhere (Braga et al., 2005; Reddy and Brownawell, 2005; Isobe et al., 2006).

While sediment data are limited, various other studies demonstrate that estrogenic compounds are present in stormwater and in watersheds that feed into Puget Sound. The King County Department of Natural Resources and Parks has conducted some surveys of natural and synthetic estrogens (17-β estradiol or E2, and ethinyl estradiol, or EE2) and other estrogenic industrial contaminants (i.e., bisphenol A or BPA, and nonylphenol or NP) in marine and fresh waters in Puget Sound receiving treated sewage effluent, stormwater, and other industrial discharges, and in a limited number of stormwater samples (King County, 2007). All treated municipal effluent in the study area is discharged to marine waters. In some of the stream and point-source stormwater samples, reported concentrations of EE2, NP, and BPA were comparable to those associated with estrogenic effects in fish such as Vtg induction, behavioral alterations, and reproductive and developmental abnormalities in laboratory exposures (Kwak et al., 2001; Länge et al., 2001; Tabata et al., 2001; Kashiwada et al., 2002; Parrott and Wood, 2002; Örn et al., 2003; Honkanen et al., 2004). Limited data suggest that stormwater may be a source of some of these compounds.

In summary, this survey of English sole has revealed previously unappreciated inputs of endocrine-disrupting compounds to marine waters along developed coastlines in the Pacific Northwestern United States, inputs that could be affecting many other species within this ecosystem. More work is needed to characterize the

concentrations and distribution of xenoestrogens in Puget Sound, and the biological impacts of these compounds on English sole, as well as other Puget Sound biota, should also investigated more thoroughly. Xenoestrogen exposure appears to be having some biological effects on sole, particularly on the timing of reproductive cycling, and other sensitive endpoints not examined in this study, such as sperm quality, could also be affected. However, some of the conditions we identified, including gonadal lesions, showed little correlation with xenoestrogen exposure. This finding highlights the potential for factors other than xenoestrogens, such as environmental conditions and other classes of contaminants, to act as modulators of reproductive and endocrine function. Future research should focus on developing and applying methods to monitor concentrations of estrogenic substances in sediments, in the water column, and in the fish themselves, and on assessing exposure to and impacts of these compounds on other species of concern in Puget Sound.

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