Reproductive Anomalies in Bottomfish from the Hylebos Waterway

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Introduction

One of the major environmental issues facing us today is the potential ecological and human-health impacts of chemicals that disrupt the endocrine system (Kavlock et al. 1996). The endocrine system is a network of glands and organs that regulates many bodily functions, including growth, metabolism, reproduction, and immune function, through chemical messengers known as hormones. Endocrine-disrupting chemicals include a broad range of compounds, such as aromatic hydrocarbons derived from fossil fuel products, polychlorinated biphenyls (PCBs), DDTs and other pesticides, surfactants present in detergents, phthalates present in plastic products, and food preservatives. Most of these chemicals interfere with endocrine function by either mimicking or blocking the effects of naturally occurring hormones.

There is considerable evidence that endocrine-disrupting chemicals are affecting the reproductive health of marine fish in the Puget Sound region. In previous studies (Johnson et al. 1988, 1993, 1997; Casillas et al. 1991; Collier et al. 1992), we examined the reproductive function of English sole *(Pleuronectes vetulus)* from selected urban areas in Puget Sound, WA, that were polluted with known or suspected endocrine disruptors, including PCBs and polycyclic aromatic hydrocarbons (PAHs) (Malins et al. 1980). These studies showed that sole from two heavily polluted sites, Eagle Harbor and the Duwamish Waterway, had a variety of reproductive problems, including depressed plasma levels of the female sex steroid, estradiol 17-β, inhibited gonadal development, alterations in egg size and number, spawning inhibition, and reduced egg and larval viability. Although the causative agents were not definitively identified, the aromatic and chlorinated hydrocarbons present in sediments at these sites were significant risk factors for the development of reproductive abnormalities. Laboratory investigations (Stein et al. 1991; Johnson et al. 1995) have subsequently shown effects of similar contaminants on circulating levels of sex hormones in flatfish, which are consistent with field results.

This paper summarizes the results of a field study, initiated in 1994, evaluating reproductive function in English sole from the Hylebos Waterway in Commencement Bay, WA. This waterway is one of the most polluted areas in southern Puget Sound; sediments from this site contain a variety of aromatic and chlorinated compounds, including PAHs and PCBs, as well as other chemicals such as hexachlorobenzene (HCB), which has been identified as a potential reproductive and developmental toxicant in mammals (National Library of Medicine 1992). The Hylebos flatfish reproduction study was one component of a larger effort by trustee agencies to assess the impacts of chemical contamination on several fish species inhabiting this waterway, in order to provide a scientific and legal basis for restoring this degraded habitat (Collier et al. 1998).

Methods

Fish and Sample Collection

Adult female English sole were collected by otter trawl from the Hylebos Waterway and a reference site in Colvos Passage (Figure 1) using the *R/V Harold W. Streeter*. Sampling was conducted approximately every 4–6 weeks from October 1994 through April 1995, during the season when vitellogenesis normally occurs in this species (Lassuy 1989; Johnson et al. 1991). Approximately 30 fish were collected at each site for every sampling period, and were maintained alive in holding tanks with

flowing sea water on board the research vessel until necropsies were performed. Fish were weighed (g) and measured (total length, mm), and otoliths were collected for age determination (Chilton and Beamish 1982). Samples collected included gonad for ovarian histopathology and determination of ovarian maturation stage; liver for organic contaminant and DNA adduct analyses; and bile for measurement of fluorescent aromatic compounds (FACs). Blood was also collected for measurement of plasma 17-β estradiol concentrations, and weights of ovaries, liver, and gutted bodies were collected to determine gonadosomatic, hepatosomatic, and condition indices.

Exposure Assessment

Selected individual liver samples were analyzed using rapid high-performance liquid chromatography with photodiode array detection (HPLC/PDA) for PCB congeners, DDTs and HCB (Krahn et al. 1994). Concentrations of 11 dioxin-like PCB congeners (PCBs 77, 81, 105, 118, 126, 156, 157, 169, 170, 180, and 189), other selected PCBs (PCBs 101, 128, 138, and 153) and chlorinated pesticides (e.g., DDTs, HCB) were determined. To estimate the relative toxicities of the dioxin-like PCBs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalents (TCDD TEQs) were determined using congener-specific toxic equivalent factors (TEFs) and concentrations of dioxin-like PCBs (Safe 1994).

Biliary fluorescent aromatic compounds (FACs) were analyzed by HPLC as described by Krahn et al. (1986). Biliary FACs were monitored by fluorescence at excitation/emission wavelength pairs for naphthalene (NPH) (290/335 nm), phenanthrene (PHN) (260/380 nm) and benzo[a]pyrene (BaP) (380/430 nm). Levels of FACs were reported as equivalents of known concentrations of BaP, NPH, or PHN standards on the basis of biliary protein (i.e., as ng PAH equivalents per mg biliary protein) because recent studies (Collier and Varanasi 1991) have shown that such normalization can account for variation in FAC levels associated with the feeding status of the sampled fish. Concentrations of biliary protein were determined by the method of Lowry (1951) using bovine serum albumin as the standard. Hepatic xenobiotic DNA adducts were measured using the ^{32}P-postlabelling technique as described by Reichert and French (1994).

Assessment of Reproductive Function

Ovaries were preserved in Deitrichs' fixative for histological assessment. Tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined microscopically. The ovary was classified into one of the following developmental stages: regressed, late regressed, pre-vitellogenic, vitellogenic, hydrated, spawning, and spent, based on criteria outlined in Johnson et al. (1991) Ovaries were also examined for follicular atresia, hermaphroditism, ovarian macrophage aggregates, and other inflammatory lesions associated with oocyte resorption, including lymphoid or macrophage infiltrates.

For measurement of plasma17-β estradiol concentrations, 1–3 mL blood samples were taken from each fish with a heparinized syringe. Blood samples were centrifuged at 800 G, and the plasma was collected and stored at –80 _C until analyses were conducted. Plasma 17-β estradiol levels were determined by radioimmunoassay (Sower and Schreck 1985).

Somatic indices (gonadosomatic index, GSI; hepatosomatic index, HSI; and condition factor) were calculated as follows:

GSI = (ovary weight [g]/gutted body weight [g]) x 100

HSI = (liver weight [g]/gutted body weight [(g]) x 100

Condition factor = (gutted body weight [g]/length^3 [(cm]) x 100

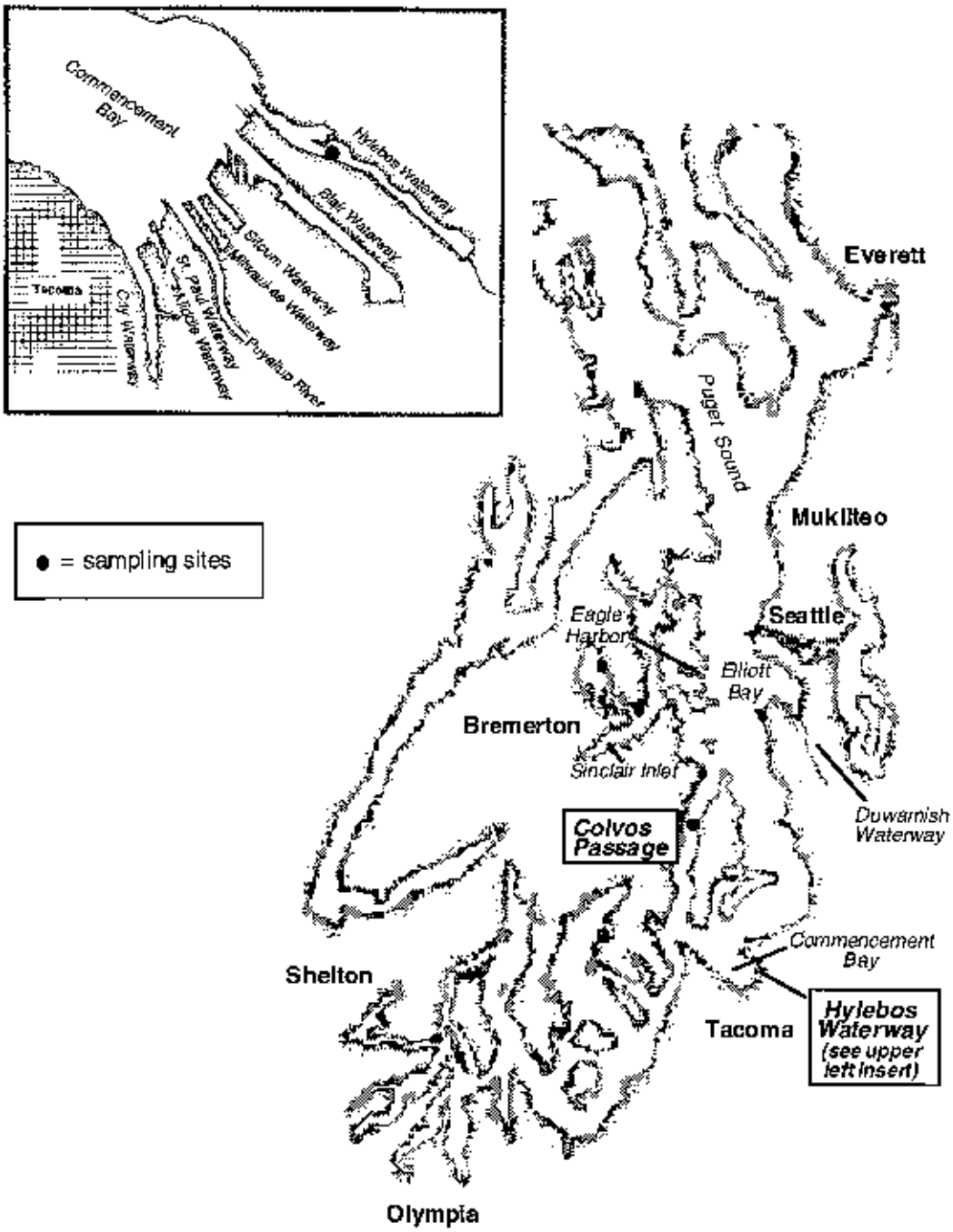

Figure 1. Sampling sites for the flatfish reproductive injury study.

Statistical Analyses

Analysis of variance (ANOVA) was used to identify statistically significant inter-site differences in chemical concentrations and biological parameters. Linear regression analyses and/or Spearman-Rank non-parametric correlation analysis were used to evaluate relationships between bioindicators of exposure (e.g., tissue contaminant concentrations, biliary FACs, DNA adducts) and indicators of biological effects (e.g., plasma estradiol concentrations, GSI, and HSI). For parametric statistical tests such as ANOVA and linear regression, data were normalized through log-transformation prior to analysis. Logistic regression analysis was used to evaluate relationships between binomial or proportional outcome variables (e.g., absence or presence of vitellogenic eggs in the ovary, lesion occurrence). Significance levels (i.e., *p*-values) for individual statistical analyses are reported below in the Results section.

Results

English sole from Hylebos Waterway showed considerably higher exposure to xenobiotic compounds than fish from Colvos Passage. Biliary FAC-BaP, FAC-NPH, and FAC-PHN concentrations were 2–5 times as high in Hylebos Waterway sole as in sole from Colvos Passage; these differences were statistically significant at $p \leq 0.0001$ (Table 1). Levels of DNA adducts in liver tissue, which are an indicator of long-term exposure to aromatic compounds, were also significantly higher in Hylebos Waterway sole than in Colvos Passage sole (Table 1). Concentrations of several classes of chlorinated hydrocarbons, including HCB, DDTs, and PCBs were significantly higher in Hylebos Waterway sole than in Colvos Passage sole (Table 1). In addition to the summed PCB concentration, the mean TCDD TEQ concentration of PCBs (i.e., the concentration of dioxin-like PCBs) was much higher in liver of Hylebos Waterway English sole than in sole from Colvos Passage.

Table 1. Contaminant exposure in English sole from Colvos Passage and the Hylebos Waterway. Significant inter-site differences (1-way ANOVA, $\alpha = 0.05$) are indicated.

	Colvos Passage	Hylebos Waterway	p-value
AH exposure			
FACs-BaP (ng/mg bile protein)	300 ± 70 (59)	770 ± 80 (92)	p = 0.0001
FACs-NPH (ng/mg bile protein)	32,900 ± 2500 (59)	106,800 ± 5500 (92)	p = 0.0001
FACs-PHN (ng/mg bile protein)	8970 ± 2700 (59)	39,500 ± 2200	p = 0.0001
DNA adducts (nmol/mole bases)	5 ± 0.3 (32)	36 ± 5 (48)	p = 0.0001
CH exposure			
SPCBs (ng/g wet wt)	99 ± 8 (48)	1270 ± 240 (56)	p = 0.0001
TCDD equivalents (ng/g wet wt)	3.7 ± 0.3 (48)	36.8 ± 7.1 (56)	p = 0.0001
HCB (ng/g wet wt)	0.9 ± 0.1 (48)	43 ± 5 (56)	p = 0.0001
DDTs (ng/g wet wt)	2.6 ± 0.3	36 ± 9	p = 0.0001

Exposure to chemical contaminants was associated with striking changes in patterns of reproductive development in female English sole sampled from the Hylebos Waterway. Although the timing of the seasonal reproductive cycle was similar in sole from both sites (Figure 2), the proportion of adult females that produced yolked eggs during the peak period of vitellogenesis tended to be lower in the Hylebos Waterway than at Colvos Passage. Overall, approximately 80% (n=44) of adult females (≥300 mm in length or five years of age) from Colvos Passage collected between October and February were undergoing ovarian development, whereas only 58% (n=52) of females of comparable size were developing in the Hylebos Waterway. Both mean GSI and mean plasma 17β-estradiol concentration were significantly lower in adult sole sampled from the Hylebos Waterway during the peak period of gonadal development (October–February) than in sole from Colvos Passage (Table 2). Logistic regression and Spearman-Rank correlation analysis indicated that exposure to aromatic hydrocarbons was the strongest risk factor for inhibited ovarian development (p = 0.0008), reduced GSI and plasma estradiol concentrations (p = 0.02), and ovarian atresia in adult sole (p = 0.004). These findings were very similar to the effects we observed in earlier studies of adult female English sole from the Duwamish Waterway and Eagle Harbor in Puget Sound (Johnson et al. 1988, 1993).

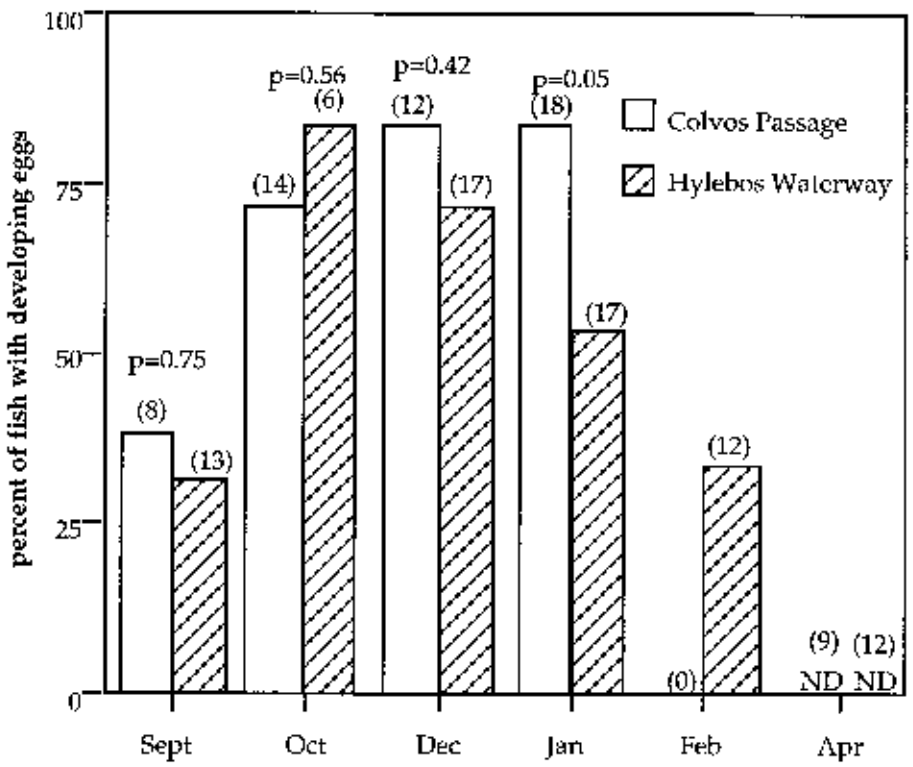

Figure 2. Percentages of female English sole with developing yolked eggs at Colvos Passage and Hylebos Waterway during the 1994 reproductive season. Only adult females of reproductive size (≥300 mm in length) are included in this summary. The number of animals sampled at each time point is given in parentheses. ND = not detected, i.e. no animals with developing eggs were observed. In February no adult female sole were found at Colvos Passage, probably because at this time the fish had migrated to their spawning grounds. The p-values for the statistical comparisons of prevalences of vitellogenic females for at Hylebos and Colvos Passage for each sampling time are shown on the graph.

The strong correlation between PAH exposure and inhibited gonadal development in female sole is consistent with recent reports that these compounds have anti-estrogenic activity because of their properties as Ah-receptor agonists (Gillesby and Zacharewski 1998), as well as with laboratory studies showing inhibited gonadal development and atresia in fish exposed to PAHs (Thomas and Budiantara 1995). Preliminary studies in our laboratory (Anulacion et al. 1997) suggest that extracts of Duwamish Waterway sediments containing high concentrations of PAHs, as well as significant concentrations the TCDD-like and anti-estrogenic PCBs congeners, are effective at suppressing vitellogenin production in English sole pretreated with estradiol. Overall, our observations on Hylebos fish provide strong confirmation of our earlier findings concerning the effects of chemical contaminant exposure on reproductive development in adult female English sole.

Perhaps the most striking finding of this study, however, was the tendency of the Hylebos fish to show precocious maturation (Figure 3). No fish below three years of age showed signs of sexual development at the Colvos Passage site, whereas in the Hylebos Waterway 40–50% of these fish were maturing. Approximately 20% of three-year-old Colvos females were maturing, as compared to 50% of Hylebos females. Logistic regression analysis indicated that Colvos Passage English sole were more likely to undergo reproductive development as they grew older ($p = 0.0001$), at least up to the age of about six or seven, when the proportion of maturing fish stabilized. In Hylebos fish, however, no association between age and probability of reproductive development was found ($p = 0.205$); approximately 50% of sampled females developed, regardless of age class. In sole less than five years of age, mean GSI was significantly higher in Hylebos sole than in Colvos Passage sole (Table 2). Plasma estradiol concentrations also tended to be higher in young Hylebos fish than in young Colvos fish ($p = 0.095$), although the difference was not statistically significant at $\alpha = 0.05$. Because our previous studies of English sole reproductive function in Puget Sound focused specifically on adult sole, we did not observe precocious maturation in these investigations; however, similar effects may be occurring at other sites with sediment contaminant profiles similar to that of the Hylebos Waterway.

Table 2. Mean GSI, plasma estradiol 17-β (E2) concentration, and condition factor in female English sole collected during the 1994 reproductive season (October–February) from Colvos Passage and Hylebos Waterway. Fish are divided into two groups: subadult fish less than five years of age that would be unlikely to mature sexually, and adult fish five years of age and above, which should normally undergo reproductive development.

	Subadult fish (< 5 years old)			Adult fish (> 5 years old)		
	GSI	plasma E2 (pg/mL)	condition factor	GSI	plasma E2 (pg/mL)	condition factor
Colvos Passage	1.82 ± 0.30 (n = 34)	1360 ± 450 (n = 27)	0.73 ±.01 (n = 35)	4.07± 0.42 (n = 45)	3800 ± 580 (n = 45)	0.74 ± 0.01 (n = 44)
Hylebos Waterway	3.12 ± 0.47 (n = 43)	2240 ± 470 (n = 39)	0.79 ± 0.01 (n = 42)	3.49 ± 0.55 (n = 53)	2700 ± 540 (n = 53)	0.74 ± 0.03 (n = 53)
	p = 0.006	p = 0.095	p = 0.0001	p = 0.045	p = 0.003	p = 0.625

The precocious maturation exhibited by Hylebos sole may be partially related to their size, nutritional status, or growth rate, all of which are important cues for the onset of puberty in fish (Le Gac et al. 1993). In fish age five and under, Hylebos fish tended to be about 5–15% larger and have a 5–10% higher condition factor than Colvos fish of the same age (Table 2, Figure 4). However, the average size of Hylebos fish at one and two years of age (240–260 mm) was still smaller than the average length at first reproduction in English sole, which is generally 280–300 mm (Lassuy 1989).

Both aromatic and chlorinated hydrocarbons emerged as potential risk factors for precocious maturation. Of the compounds measured, the strongest risk factor was HCB ($p = 0.0004$), a chemical that is found at particularly high concentrations in the Hylebos Waterway. Although HCB is generally considered a reproductive toxicant (Jarrel et al. 1993), there are cases where it has been shown to stimulate reproductive development. In gastropods, HCB exposure stimulated early egg production (Baturo et al. 1995). At certain stages in the reproductive cycle, it may also increase ovarian weight in rats (Foster et al. 1992). However, other chlorinated hydrocarbons, such as DDTs and PCBs were also significant risk factors for precocious maturation ($0.002 \leq p \leq 0.01$) and could play a role in the development of this abnormality. Indicators of exposure to ACs (biliary FACs and DNA adducts) also showed strong associations with precocious maturation ($0.003 \leq p \leq 0.004$). Most of these indicators of contaminant exposure were also significantly positively correlated with GSI and plasma estradiol concentrations in sole under age five ($0.001 \leq p \leq 0.04$). Because all of these classes of chemicals co-occur in Hylebos Waterway sediments, and fish are exposed to them simultaneously, it is difficult to establish clear cause-and-effect relationships between specific individual chemicals and the reproductive effects observed.

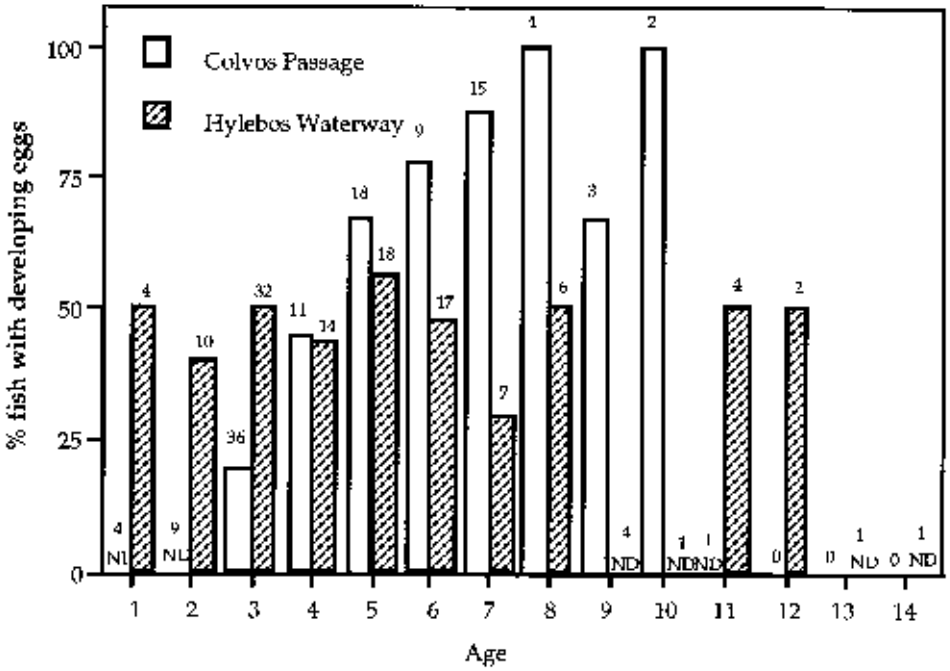

Figure 3. Percentage of maturing female English sole (i.e., sole with developing or vitellogenic eggs) by age class in the Hylebos Waterway and at Colvos Passage. The proportion of fish maturing at ages 1–3 was significantly higher at the Hylebos Waterway than at Colvos Passage, but the proportion maturing at ages five and above was significantly lower.

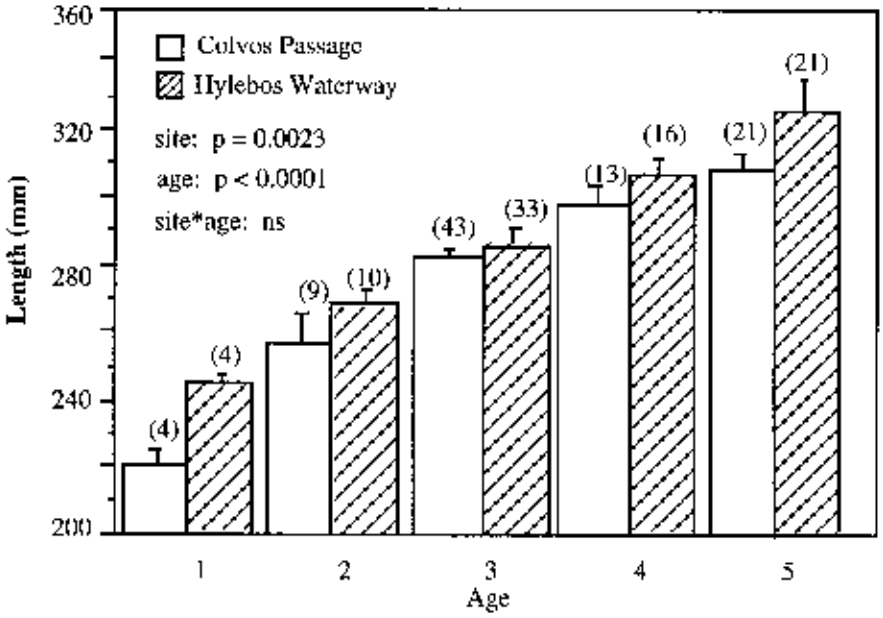

Figure 4. Mean length (+ SE) by age class in female English sole from Colvos Passage and the Hylebos Waterway for animals age 1–5. Younger fish were significantly larger at the Hylebos Waterway than at Colvos Passage. No significant inter-site differences in length at age were observed for fish six years and older (data not shown).

The seemingly contradictory effects of sediment contaminants in the Hylebos Waterway on reproductive development in subadult and adult fish is puzzling, and we have no clear explanation for this phenomenon at the present time. However, the ability of chemical contaminants to stimulate processes such as growth, sexual maturation, and fecundity at low levels of exposure, and inhibit the same processes at high levels of exposure has been demonstrated for a variety of compounds and taxa (Laughlin et al. 1981, Calabrese and Baldwin 1998). For example, although PAHs generally inhibit gonadal development, in starry flounder, exposure to very low concentrations of oil appeared to accelerate egg maturation (Whipple et al. 1978). Similarly, Aroclor (PCB) mixtures at low to moderate concentrations stimulated growth in fetal mice (Marks et al. 1989) and chick embryos (Gould et al. 1997), but inhibited growth at higher doses, probably because of the higher concentration of dioxin-like PCBs in the high dose Aroclor mixtures. Because PCBs are bioaccumulated by English sole, concentrations of PCBs, as well as PCB TEQs tend to increase with fish age. This suggests that the apparent differences in the effects of PCBs on younger and older sole might be due, at least in part, to higher concentrations of dioxin-like PCBs in the older fish.

Several of the contaminants present in the Hylebos Waterway are recognized as endocrine-disrupting compounds, and their stimulatory effects on growth and reproduction in subadult Hylebos sole might be mediated in part through the endocrine system. Several studies have shown that HCB and PCBs can affect thyroid function (van Raaij et al. 1993; Byrne et al. 1987; Ness et al. 1993; Van den Berg et al. 1994; Seo et al. 1995), growth hormone synthesis (Gould et al. 1997), and lipid metabolism (Billi de Catabbi et al. 1997; Bell et al. 1994; Griffin et al. 1994), causing physiological changes that could affect growth, body fat accumulation, and the timing of sexual development. Additionally, some of the chemicals present in the Hylebos Waterway, including DDTs and certain PCB congeners such as PCB 153, have estrogenic activity (Safe 1994; McKinney and Waller 1994) and have been shown to trigger early puberty in rats (Heinrich 1987). There is evidence that other chemicals, such as the dioxin-like PCBs, might stimulate release of gonadotropic hormones in immature females (Li et al. 1997). Studies with mammals indicate that exposure to HCB can alter plasma progesterone levels (Foster et al. 1992, 1995).

In summary, the results of this study indicate that reproductive function is significantly altered in female fish from the Hylebos Waterway, and these changes are associated with exposure to contaminants present in Hylebos Waterway sediments. Aromatic hydrocarbons were most closely associated with inhibited gonadal development in adult sole, while both chlorinated and aromatic compounds are potential risk factors for precocious maturation. Because a complex of factors can influence fish population growth rates, it is difficult to predict with high certainty the consequences of contaminant-related injury to female English sole on sole populations in the Hylebos Waterway and Commencement Bay. However, the altered pattern of reproductive development in Hylebos fish would clearly have an impact on the reproductive output of fish from this site, and could potentially reduce the overall resilience and productivity of the English sole population in Puget Sound in the face of multiple environmental stressors such as overfishing, climate change, or destruction or alteration of nursery or other critical habitats.

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