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A GENE TO ORGANISM APPROACH—ASSESSING THE IMPACT OF ENVIRONMENTAL POLLUTION IN EELPOUT (*ZOARCES VIVIPARUS*) FEMALES AND LARVAE

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Abstract: A broad biomarker approach was applied to study the effects of marine pollution along the Swedish west coast using the teleost eelpout (*Zoarces viviparus*) as the sentinel species. Measurements were performed on different biological levels, from the molecular to the organismal, including measurements of messenger RNA (mRNA), proteins, cellular and tissue changes, and reproductive success. Results revealed that eelpout captured in Stenungsund had significantly higher hepatic ethoxyresorufin O-deethylase activity, high levels of both cytochrome P4501A and diablo homolog mRNA, and high prevalence of dead larvae and nuclear damage in erythrocytes. Eelpout collected in Göteborg harbor displayed extensive macrovesicular steatosis, whereby the majority of hepatocytes were affected throughout the liver, which could indicate an effect on lipid metabolism. Results also indicate that eelpouts collected at polluted sites might have an affected immune system, with lower mRNA expression of genes involved in the innate immune system and a higher number of lymphocytes. Biomarker assessment also was performed on livers dissected from unborn eelpout larvae collected from the ovary of the females. No significant differences were noted, which might indicate that the larvae to some extent are protected from effects of environmental pollutants. In conclusion, usage of the selected set of biological markers, covering responses from gene to organism, has demonstrated site-specific biomarker patterns that provided a broad and comprehensive picture of the impact of environmental stressors. *Environ Toxicol Chem* 2015;34:1511–1523.

Keywords: Biomarker Histopathology Genotoxicity Gene expression Eelpout

INTRODUCTION

Many coastal environments are contaminated with a variety of chemicals that are produced through industrial effluents, agricultural runoff, and domestic waste. Pollutants are found in different mixtures, and their concentration could vary over time because some are persistent and others only occasionally released into the environment. Different life stages, such as larvae development, can be more sensitive to exposure. There is a lack of understanding of the effects of chronic pollution on individual fish and fish populations as well as a large knowledge gap concerning the effects of complex mixtures of chemicals, such as those seen in most coastal environments. Assessment of the effects of chronic pollution is therefore important to be able to protect the organisms in the aquatic environment from disruption of reproductive status and to avoid possible decline in fish stocks and biodiversity.

To study and understand the effects of environmental pollutants, biological markers such as biomarkers often are used. Biomarkers indicate a change in biological response to exposure or effects of xenobiotics. These biomarker responses often are early warning signs that can pinpoint polluted sites and identify previously unknown sources of pollutants. Based on these results, appropriate measures can be taken to avoid adverse effects at higher biological levels. Biomarker responses in fish and other aquatic organisms have been employed in many environmental monitoring programs to assess the impact of pollutants. Studies using multibiomarker approaches provide the opportunity to correlate different effects, to guide biomarker selections, and to obtain a more holistic picture of the impact of contaminants [1–4].

The eelpout (*Zoarces viviparus*) is a marine fish species that is widespread in the coastal waters of northern Europe, and it used as sentinel species in the present study. It lives in relatively stationary populations, and because of this, contaminant loads and biological responses detected in the eelpout most likely reflect the environmental condition, including pollutants, within the area where it is captured. The eelpout has been used in environmental monitoring studies for several decades in Sweden, Denmark, and Germany [5]. The eelpout has been recommended as a suitable species for studying and monitoring pollutant effects in marine coastal waters by the International Council for the Exploration of the Sea, the Baltic Marine Environment Protection Commission, the Convention for the Protection of the Marine Environment of the North-East Atlantic, and the Swedish Environmental Protection Agency. Eelpout mate at the end of August, and the larvae hatch approximately 3 wk later, inside the female, where they develop for 4 mo to 5 mo [6]. Thus, in contrast to most fish species that lay eggs, the viviparous eelpout provides a unique opportunity to link responses in the individual female fish to ecologically

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relevant parameters, such as reproduction and larvae development. Guidelines for sampling and assessment criteria for the frequency of abnormal larvae in broods have been suggested by the International Council for the Exploration of the Sea [7].

Three polluted sites along the Swedish west coast (the Göteborg harbor, Stenungsund, and Brofjorden) were used in the present study. The Göteborg harbor is the largest general oil port in Scandinavia. The harbor has been involved in environmental monitoring conducted by our group for several years, in addition to studies conducted during dredging activities and after a big oil spill in 2003 [8–10]. Stenungsund is located in the vicinity of the largest petrochemical industry in Sweden. Brofjorden is situated close to an oil refinery and is Sweden's 2nd largest oil harbor. Earlier studies on eelpout collected at Stenungsund and Brofjorden revealed a higher prevalence of dead and malformed eelpout larvae in these sites compared with other sites along the Swedish west coast [11,12]. Two reference sites also were included in the present study: Fjällbacka, a national reference site used by the Swedish Environmental Protection Agency, and Billdal, located south of Göteborg. Both of these are considered to be sites with low anthropogenic impact. Eelpouts collected from Fjällbacka have been involved in annual biomonitoring since 1989 [13]. Many of the environmental contaminants found in Göteborg harbor, Stenungsund, and Brofjorden are commonly found in coastal areas and include chemicals such as polycyclic hydrocarbons (PAHs), metals (e.g., Cd, Cr, and Hg), organochlorinated contaminants (e.g., polychlorinated biphenyls [PCBs]), and organotins (e.g., tributyltin). These contaminants are known to have toxic effects in fish, including immunotoxic, genotoxic, and carcinogenic effects [14], which are measured in eelpout during our annual biomonitoring studies only to some extent.

The aim of the present study was to provide a comprehensive understanding of the impact of environmental pollutants on eelpout collected at different areas using a broad set of biomarkers. In addition to biomarkers used during our annual biomonitoring studies (biometric data, blood parameters, enzymatic measurements, and reproductive success), liver histopathology and genotoxic measurements were included. Furthermore, genes involved in the innate immune system and apoptosis/DNA, previously observed to be differentially regulated in Göteborg harbor compared with a reference site [15], were further verified in the present study. Measurements were thus performed on different biological organization levels (i.e., messenger RNA [mRNA], protein, cellular, and tissue levels, as well as reproductive success), all of which to varying degrees reflect functions known to be affected by pollutants. Biomarkers were also assessed in livers dissected from unborn eelpout larvae to study direct or indirect effects of environmental contaminants on developing larvae in the ovary.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, 2-mercaptoethanol, 7-ethoxyresorufin, 5,5′-dithio-bis (2-nitrobenzoic acid), 1-chloro-2,4-dinitrobenzene, reduced β-nicotinamide adenine dinucleotide 2′-phosphate, reduced β-nicotinamide adenine dinucleotide, glutathione and oxidized glutathione, trichloroacetic acid, (2,4-dinitrophenyl)hydrazine, guanidine hydrochloride, digitonin, ethylenediaminetetraacetic acid, and a protease inhibitor cocktail (P8340) were all purchased from Sigma-Aldrich. Rhodamine was obtained from Merck. The SYBR Green Supermix, iScript cDNA Synthesis Kit, and RNA StdSens Kit Reagents were products of Bio-Rad Laboratories; and the RNeasy Plus Mini Kit was purchased from Qiagen. All other chemicals were of analytical grade.

Sampling sites

Five different locations on the Swedish west coast were selected (Figure 1): Göteborg harbor (57.6866°N, 11.752°E), Stenungsund (58.0973°N, 11.8251°E), Brofjorden (58.3536°N, 11.4184°E), Billdal (57.5827°N, 11.9141°E), and Fjällbacka (58.6440°N, 11.2457°E). Fjällbacka was used as reference site for all measurements in the present study except for the genotoxicity study, for which Billdal also was included as a reference site. Göteborg harbor, located in the outlet of the Göta älv River, is the largest general oil port in Scandinavia. The harbor water and sediment are known to be contaminated with various anthropogenic substances and several toxic compounds, including PCBs, PAHs, tributyltin, dioxins, and heavy metals such as Hg [16–18]. Stenungsund is located in the vicinity of the largest petrochemical industry in Sweden. Many chemical industries are concentrated in the area including, a large naphtha cracker, and the area is known to be polluted with PAHs, hexachlorobenzene, octylphenol, and tributyltin [11,19]. Brofjorden is located close to an oil refinery and is Sweden's 2nd largest oil harbor. This site is also known to be contaminated with PAHs, tributyltin, PCBs, and polybrominated diphenyl ethers [19].

Sampling of fish and dissection

Eelpout (*Z. viviparus*) were caught using fyke nets by local fishermen in November 2011. In total, approximately 25 eelpout females were individually analyzed per site except for studies of reproductive success, for which 47 to 50 females per site were used. For logistical reasons, separate eelpouts were collected from Fjällbacka, Stenungsund, Göteborg, and Billdal for the genotoxicity studies on nuclear damage (14–15 individuals/site). Fish appeared normal, and no skin lesions were present among the caught fish. All fish collected were sexually mature females

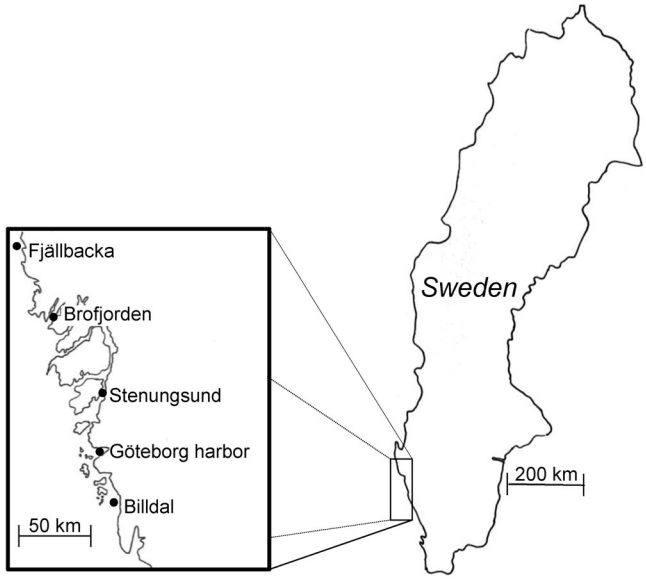

Figure 1. Eelpout sampling sites on the Swedish west coast in November 2011. Brofjorden is located close to a big oil refinery, Stenungsund is in the outlet of an area with chemical industries, and Göteborg harbor is Scandinavia's largest general oil port. Fjällbacka was used as a northern reference site. Billdal was used as a reference site for the genotoxic assays.

with the exception of fish from Fjällbacka used in genotoxicity studies, which were a mix of males and juveniles. Fish were killed with a sharp blow to the head, and blood was collected. Blood smears were prepared on glass slides, left to dry, and later used for blood cell counting. The rest of the blood was centrifuged for 90 s at 6000 *g*, and plasma was collected and then stored at –80 °C. Livers were dissected out of the mature females, and small slices (~2 mm) of adjacent liver pieces were taken from the same region of the liver on each individual. One piece was used for gene expression studies, 1 piece for protein carbonylation, and 1 piece for histopathology. The rest of the liver was used for enzymatic measurements. All liver pieces were snap-frozen in liquid nitrogen except for that used for histology, which was fixed in paraformaldehyde. Analysis of reproductive success in 47 to 50 females from each sampling site was performed (see *Reproductive success and embryo data*). Larvae (15 healthy larvae/female) were snap-frozen in liquid nitrogen and later used for ethoxyresorufin O-deethylase (EROD), mRNA expression, and protein oxidation measurements. Livers from these larvae were dissected using a dissecting scope. Ethical permission (no. 449-2008) for the samplings was approved by the local animal committee in Göteborg, Sweden.

Morphometric indices

Fish were measured and weighed, and weight measurements were noted for livers and gonads. After dissection, eelpouts were weighed once again for somatic weight (carcass weight). Condition factor, liver somatic index (LSI), and gonad somatic index (GSI) were calculated as follows: condition factor = (somatic weight [g] × 100)/length^3 (cm); LSI = (liver weight [g] × 100)/somatic weight (g); GSI = (gonad weight [g] × 100)/ somatic weight (g).

Blood parameters

Blood smears on glass slides were stained using May-Grünwald stain for 5 min followed by Giemsa stain solution for 18 min. Slides were then rinsed in water and left to dry. Glass slides were analyzed microscopically; approximately 2000 cells were counted per glass slide under magnification (×400). The numbers of immature red blood cells, thrombocytes, lymphocytes, and granulocytes were calculated and presented as a percentage of the total blood cells counted. The total amount of white blood cells was calculated as the sum of all thrombocytes, lymphocytes, and granulocytes and presented as a percentage of the total blood cells. The erythrocyte volume fractions (hematocrit) were estimated using hematocrit capillary tubes followed by centrifugation of the blood using a hematocrit capillary centrifuge for 2 min and a microhematocrit reader. The hemoglobin and glucose concentrations in blood were measured using a cuvette system from Hemocue, with assayed hemoglobin (HemoTrol; Eurotrol) and glucose (GlucoTrol-AQ; EuroTrol) as quality controls.

Reproductive success and embryo data

Analyses were performed on 47 and 50 females from each sampling site. Total weight of the brood was measured, larvae were counted, and the length of each individual larva was noted. Larvae were then grouped into 6 categories: 1) living larvae without malformation, 2) dead larvae without malformation a) with a size below 10 mm and b) with a size above 10 mm, 3) fully developed fertilized or unfertilized eggs, 4) living larvae with malformation, 5) dead larvae with malformation, 6) lumps of dead larvae, which could not be counted [20].

Enzymatic assays

Liver samples for EROD, glutathione *S*-transferase (GST), and glutathione reductase (GR) were prepared and analyzed as previously described by Förlin [21] and Sturve et al. [9] and references therein. The liver was homogenized (glass/Teflon) and centrifuged at 10 000 *g* for 20 min at 4 °C. The supernatant was recentrifuged at 105 000 *g* for 60 min at 4 °C to obtain the cytosolic and the microsomal fractions. Livers collected from larvae (a pool of 5 larvae livers/female) were homogenized through ultrasonication and centrifuged at 10 000 *g* for 20 min at 4 °C to obtain the S9 fraction. The activity of EROD was measured in eelpout females on the microsomal fraction using rhodamine as standard. The activity of EROD was also measured on larvae liver using the S9 fraction and a sample volume of 65 μL. The GST and GR activity were measured on liver cytosols. Catalase activity was measured in cytosol according to the method described in Aebi [22] adapted to the microplate reader by Sturve et al. [9].

Protein carbonyl measurement

Protein carbonyls were measured via a reaction with (2,4-dinitrophenyl)hydrazine followed by trichloroacetic acid precipitation as described by Carney Almroth et al. and references therein [10] on both female eelpout liver and larvae liver (a pool of 5 larvae livers/female) from Fjällbacka, Stenungsund, and Göteborg.

Protein measurement

Total protein concentration was measured according to the Lowry method.

Erythrocytic nuclear abnormalities assay

The assay was performed on 14 to 15 individual eelpouts per site collected from Stenungsund, Göteborg harbor, and the reference site in Fjällbacka. In addition, Billdal was used as a southern reference site. The fish used for the erythrocytic nuclear abnormalities assay were collected at the same sampling time point as all other fish used in the present study; for logistical reasons, however, separate fish were used for the erythrocytic nuclear abnormalities assay than for all other measurements. All fish were sexually mature except for the eelpout collected in Fjällbacka, which contained a mix of male and juvenile fish. Analyses were carried out on erythrocytes for the assessment of chromosomal damage using the micronucleus test. Blood smears were fixed and stained on 2 slides per individual as described previously [23]. The frequency of micronuclei was estimated by scoring of 4000 erythrocytes (2000/slide) from each individual. In addition to micronucleus frequency, the frequencies of other erythrocyte nuclear abnormalities, such as nuclear buds, blebbed, notched, lobed, circular nuclei, binucleated cell, and binucleated nuclei with bridges, were evaluated (Figure 2) [24,25].

RNA isolation and quantitative polymerase chain reaction analysis

Quantitative polymerase chain reaction (qPCR) was performed on mRNA isolated from livers from 6 individual eelpout females per site and a pool of livers from 5 embryos from each female (resulting in analysis of 6 individual female livers and 6 individual embryo liver pools per site). Quantitative PCR was performed on mRNA from larvae to study possible correlations between mRNA expression levels in a female and her corresponding larvae. Isolation of RNA and complementary DNA synthesis were performed as described elsewhere [15]. The following genes were selected for the expression studies:

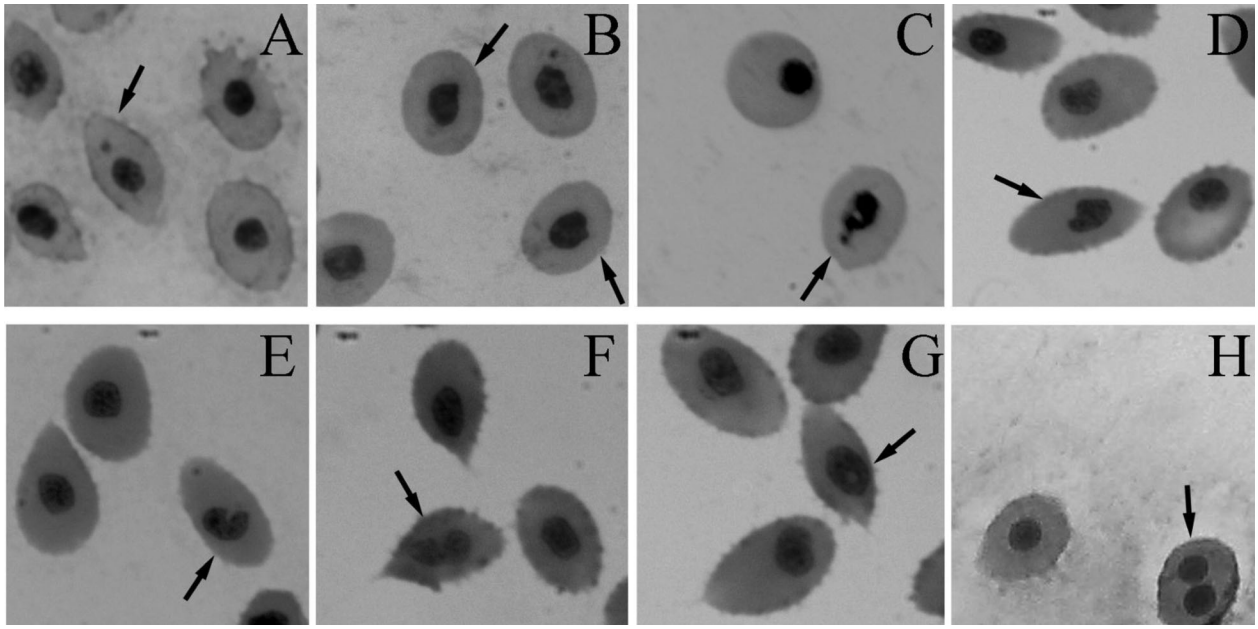

Figure 2. Erythrocytic nuclear abnormalities assay in eelpout erythrocytes: Images of different cell nucleus types. Erythrocyte with (**A**) micronucleus, (**B**) bleb, (**C**) bud, (**D**) lobed nucleus, (**E**) notched nucleus, (**F**) binucleated with bridge, (**G**) circular nucleus, and (**H**) binucleated erythrocyte.

cytochrome P450 1A (*CYP1A*), DNA damage–inducible transcript (*DDIT4*), diablo homolog (*Diablo*), hepcidin (*HAMP*), complement component 7 (*C7*), and lysozyme C (*LysC*), as well as 2 reference genes (ubiquitin and α-tubulin). The qPCR assay was then performed using the fluorescent dye iQ SYBR Green Supermix (Bio-Rad) as described elsewhere [15]. For information regarding primer sequences, primer concentrations, and annealing temperatures, see Supplemental Data, Figure S1. Primers were designed using the sequenced eelpout liver transcriptome data [26] except for the eelpout CYP1A qPCR primers, which were a gift from B. Wassmur and M. Celander (University of Gothenburg, Göteborg, Sweden). Quantitative PCR data were analyzed using the $2^{\Delta Ct}$ method [27] with the average of the reference genes which were assessed as stable with an analysis of variance (ANOVA; $p = 0.332$).

Histopathology

In total, histopathological lesions were analyzed in 24 to 25 eelpouts per site from Göteborg harbor, Stenungsund, Brofjorden, and the reference site Fjällbacka. Liver pieces were fixed in neutral buffered formalin and transferred to 70% ethanol after 24 h. Tissues were processed for formalin-fixed, paraffin-embedded histology using standard histological protocols. Sections of 3 μm to 4 μm were obtained using a rotary microtome and subsequently stained with hematoxylin and eosin for histological examination. Slides were examined using a Nikon Eclipse Ni-U microscope. Micrographs were captured using the Nikon NIS Elements BR image analysis software. Lesions indicative of contaminant exposure were recorded according to Biological Effects Quality Assurance in Monitoring Programmes and International Council for the Exploration of the Sea criteria [28]. These criteria are also listed in the baseline study by Fricke et al. [29], where eelpouts were collected from different areas along the Baltic Sea.

Statistical analyses and clustering

Statistical analyses were performed using SPSS Statistics 20 (IBM). To test for differences between sites, a one-way ANOVA followed by a Tukey post hoc test was used in cases where data fulfilled the homogeneity criteria assessed by Levene's test of equality of variances. When the criteria were not met, the Kruskal-Wallis ANOVA and Mann-Whitney test were used. For hypothesis testing, the significance level was set to $\alpha = 0.05$. Pearson's correlations were used to compare parameters on the individual level (within each site). For hierarchical clustering, the function hclust in R with Euclidean distance and complete linkage was used. Clustering was performed on 31 biological markers measured in the 4 sites: Fjällbacka (reference), Brofjorden, Stenungsund, and Göteborg harbor. Because the parameters had different scales, they were standardized before the mean values for each site were calculated, and these were used for clustering. Histological findings occurring in fewer than 15% of the individuals were not used in the clustering. Genotoxic data were not included in the clustering because they were measured in only 3 of the above sites.

RESULTS

Biometric data

Biometric data from eelpout sampled in 4 different sampling sites on the Swedish west coast indicate that the eelpouts captured at Stenungsund and the reference site Fjällbacka were on average the smallest (in both length and weight; Table 1). The eelpout collected at Göteborg harbor differed significantly from those at other sites, showing the lowest condition factor, as well as the highest LSI, compared with all other sites.

Blood parameters

The relative percentage of immature red blood cells, thrombocytes, lymphocytes, and granulocytes was evaluated in female eelpout (Table 1). The number of white blood cells (sum of thrombocytes, lymphocytes, and granulocytes) was significantly higher in eelpout from Göteborg harbor than in fish from Stenungsund and the reference site Fjällbacka. The number of lymphocytes was higher in eelpouts from Göteborg harbor than both Stenungsund and Fjällbacka and higher in Brofjorden compared with the reference site Fjällbacka. A significantly higher number of immature red blood cells was observed in eelpout

Table 1. Biometric data, blood parameters, and enzymatic biomarker measurements in female eelpout[a]

	Sampling site			
	Fjällbacka	Brofjorden	Stenungsund	Göteborg harbor
Length (cm)	24.0 ± 4.6 A	25.8 ± 4.2 B	23.5 ± 5.4 A	28.3 ± 4.8 C
Total weight (g)	72.6 ± 4.7 A	101.4 ± 5.1 B	70.8 ± 5.1 A	106.7 ± 4.7 B
Somatic weight (g)	52.6 ± 3.4 A	66.5 ± 3.2 B	47.8 ± 3.4 A	75.8 ± 3.9 B
Liver weight (g)	0.90 ± 0.07 A	1.05 ± 0.06 A	0.80 ± 0.07 A	1.50 ± 0.11 B
LSI	1.65 ± 0.05 A	1.55 ± 0.06 A	1.63 ± 0.05 A	1.91 ± 0.06 B
Gonad weight (g)	10.2 ± 0.8 A	18.5 ± 1.2 B	12.1 ± 1.4 A	16.3 ± 0.8 B
GSI	19.1 ± 09 A	27.3 ± 1.3 C	24.1 ± 1.3 BC	21.7 ± 0.9 AB
Condition factor	0.372 ± 0.05 B	0.376 ± 0.09 B	0.356 ± 0.05 B	0.329 ± 0.06 A
Lymphocytes (%)[b]	1.33 ± 0.06 A	1.66 ± 0.10 BC	1.49 ± 0.08 AB	1.90 ± 0.09 C
Granulocytes (%)[b]	0.82 ± 0.05 A	0.93 ± 0.07 A	0.86 ± 0.07 A	0.97 ± 0.09 A
Thrombocytes (%)[b]	1.05 ± 0.07 A	1.18 ± 0.08 A	1.07 ± 0.06 A	1.15 ± 0.06 A
WBC (%)[b]	3.21 ± 0.14 A	3.77 ± 0.18 AB	3.43 ± 0.15 A	4.03 ± 0.17 B
iRBC (%)[b]	0.411 ± 0.02 A	0.594 ± 0.05 B	0.420 ± 0.02 A	0.521 ± 0.04 AB
Hemoglobin (g/L blood)	46.9 ± 3.3 A	34.2 ± 1.9 B	39.5 ± 1.8 AB	41.7 ± 2.9 AB
Hematocrit (%)	20.4 ± 2.0 A	14.9 ± 1.1 A	15.1 ± 0.7 A	26.5 ± 1.8 B
Hemoglobin/hematocrit	2.37 ± 0.66 B	2.49 ± 0.78 B	2.61 ± 0.63 B	1.62 ± 0.76 A
EROD (nmol/mg protein × min)	0.070 ± 0.01 A	0.083 ± 0.01 A	0.190 ± 0.02 B	0.077 ± 0.01 A
GR (nmol/mg protein × min)	17.9 ± 1.0 A	18.0 ± 1.5 A	17.9 ± 1.0 A	19.8 ± 0.8 A
GST (μmol/mg protein × min)	0.364 ± 0.02 A	0.326 ± 0.03 A	0.370 ± 0.02 A	0.381 ± 0.02 A
Catalase (mmol/mg protein × min)	136.3 ± 9.8 A	108.3 ± 7.4 A	124.3 ± 15.5 A	119.9 ± 9.8 A

[a]Assessment performed on 20 to 25 individuals per site and shown as mean ± standard error. Uppercase letters indicate significant differences between sites ($p < 0.05$).
[b]Frequency (%) of lymphocytes, granulocytes, thrombocytes, WBC, and iRBC of total number of blood cells counted.
LSI = liver somatic index; GSI = gonad somatic index; WBC = the sum of white blood cells (lymphocytes, granulocytes, and thrombocyte); iRBC = immature red blood cells; Hematocrit = red blood cell volume; EROD = ethoxyresorufin *O*-deethylase (CYP1A activity); GR = glutathione reductase; GST = glutathione *S*-transferase.

captured at Brofjorden compared with Stenungsund and Fjällbacka. No significant differences were observed for thrombocyte and granulocyte relative percentages between the sites.

The highest levels of hematocrit (26.5%) were measured in eelpout captured in Göteborg harbor (Table 1), and a significantly higher concentration of hemoglobin was seen in eelpout from the reference site compared with Brofjorden (Table 1). The ratio hemoglobin to hematocrit (mean corpuscular hemoglobin concentration) was calculated, and the lowest ratio was detected in eelpout collected in Göteborg harbor compared with fish from the rest of the sites.

Reproductive biology

The fecundity (total amount of healthy larvae) was higher in Brofjorden and Göteborg harbor compared with the reference site in Fjällbacka (Table 2). This was expected because eelpout from Brofjorden and Göteborg also had the highest gonad weight and were on average the largest fish (Table 1). Eelpouts collected in Stenungsund and Fjällbacka were shortest in length (Table 1) and had the larvae with the shortest length (Table 2). The number of larvae that had died during development (category 2 [a and b]) was highest in eelpout captured at Stenungsund, and both subcategories were at an elevated level according to the suggested International Council for the Exploration of the Sea guidelines (Table 2). Also, the category including the total frequency of abnormal fry (sum of categories 2 [a and b], 4, and 5) was at an elevated level in larvae from female eelpout collected in Stenungsund. The frequency of larvae that died at a late stage of development (category 2b) was also found at an elevated level in Göteborg harbor (Table 2). The proportion of female eelpouts with more than 5% dead, but developed larvae in the brood was 20% in Stenungsund. This

Table 2. Reproductive success in eelpout females collected along the Swedish west coast[a]

	Brood size[b] (no.)	Length of larvae (mm)	Early dead[c] (%)	Late dead[d] (%)	Malformed (%)	Total abnormal fry[e] (%)
Fjällbacka (ref)	43 ± 20 A	38.6 ± 2.8 A	0.84	0.84	0.56	2.25
Brofjorden	60 ± 34 B	44.6 ± 3.8 B	0.69	1.31	0.31	2.31
Stenungsund	55 ± 28 AB	37.2 ± 3.1 A	2.52	2.31	0.53	5.36
Göteborg harbor	56 ± 25 B	43.5 ± 3.0 B	1.14	2.56	0.21	3.90
Thresholds for assessment[f]						
Background levels			0–2.5	0–2	0–1	0–5
Elevated levels			>2.5–5	>2–4	>1–2	>5–10
Levels of concern			>5	>4	>2	>10

[a]Assessment performed using 47 to 50 females per site. Brood size and length of larvae are shown as mean ± standard error. Uppercase letters indicate significant differences between sites ($p < 0.05$).
[b]Brood size, excluding dead or malformed larvae.
[c]Frequency (%) of early dead larvae (<10 mm in length).
[d]Frequency (%) of late dead larvae (>10 mm in length).
[e]Frequency (%) of total abnormal fry (sum of early dead, late dead, and malformed larvae).
[f]Threshold for assessment of reproductive success according to the International Council for Exploration of the Sea guidelines [7].

proportion of females was 8% for Göteborg and 4% for Brofjorden. Fjällbacka had no females with a brood with more than 5% developed but dead larvae. The number of malformed larvae (categories 4 and 5) was below the background threshold (0–1%) in all sites.

Enzymatic assays

Eelpout females collected in Stenungsund had a significantly higher level of EROD activity compared with those from all other sites (Table 1). No significant difference was observed in EROD activity between the larvae from the different sites (data not shown). However, there was significantly lower activity in the larvae compared with the females. No significant difference between the sites was seen in GST, GR or catalase activity in female liver (Table 1).

Protein carbonyl levels

The mean protein carbonyl levels were higher in eelpout females collected in Stenungsund and Göteborg harbor compared with Fjällbacka; however, the results were not significant (results not shown). There were no significant differences in protein carbonyl levels when comparing larvae from the different sites (results not shown). The protein carbonyl levels were significantly lower in larvae than in females, however, and this was seen at all 3 sites.

Genotoxic responses

For logistical reasons, separate eelpouts were collected from Fjällbacka (reference), Stenungsund, Göteborg, and Billdal (reference) for genotoxicity. All fish were sexually mature females with the exception of fish from Fjällbacka, which were a mix of males and juveniles. Because no significant differences were noted in genotoxic responses between the 2 reference sites, data from the Fjällbacka site also were included in Figure 3. Nuclear abnormalities (Figure 2) scored as notched, lobed, budded and circular showed significantly higher frequencies in fish sampled in proximity to Stenungsund (Figure 3) compared with both reference sites. The frequency of budded was higher in Göteborg harbor compared with both reference sites. The frequency of blebbed nuclei also was higher in eelpout collected at Stenungsund compared with the reference site in Fjällbacka (Figure 3). No significant difference was seen between the frequency of binucleated nuclei (Figure 3). Because the frequency of binucleated nuclei with bridges was very low, no test for differences between the sites were performed. The highest frequency of micronuclei was found in individual eelpouts collected in Göteborg harbor. However, this difference was not statistically significant (Kruskal Wallis test, $p = 0.513$; Figure 3).

mRNA expression levels

Results from mRNA expression levels of selected genes in a total of 6 females per site are shown in Figure 4. Hepcidin and C7 had significantly lower mRNA expression levels in female eelpout from Brofjorden and Stenungsund compared with the reference site. Diablo homolog had a significantly higher mRNA expression in eelpout from Stenungsund compared with all other sites. For CYP1A, the females with the highest expression level were collected in Stenungsund. The mRNA expression levels for lysozyme C and DDIT4 did not show any significant difference between groups.

Histopathology

Macrovesicular steatosis (fatty liver) was the most commonly observed histological finding, whereby single large vacuoles were seen to occupy affected hepatocytes. In the eelpouts collected at Göteborg harbor, this lesion was present in 83% of the individuals (Table 3). In addition, 46% of the eelpouts from Göteborg harbor demonstrated extensive macrovesicular steatosis, whereby the majority of hepatocytes were affected throughout the liver (Figure 5). Macrovesicular steatosis also was observed in Brofjorden, Stenungsund, and the reference site Fjällbacka (Table 3) but to a lesser extent. Macrophage aggregates were relatively common in the sampled eelpouts and were observed at a frequency of 80%, 58%, 46%, and 25% of the individuals at Brofjorden, Stenungsund, Göteborg harbor, and Fjällbacka, respectively (Table 3). Hepatocellular and nuclear polymorphisms were observed at a prevalence between 13% and 21%. Other hepatic conditions observed at low prevalence and severity included hypertrophy of the connective tissue associated with blood vessels, basophilic follicular carcinoma, hepatocellular necrosis, hepatocellular regeneration, hepatocellular apoptosis, lymphocytic/monocytic infiltration, granuloma, chronic inflammatory lesion, and infection with nematodes. Hepatocellular hemosiderosis and phospholipoidosis were not detected, and no confirmation using further staining and histochemical analysis was conducted. No neoplastic lesions were observed.

Correlations and clustering of parameters

Within-site correlations were calculated to estimate the correlations coefficients (r) between pairs of parameters for each site separately. Correlations with p value less than 0.05 for at least 3 of the 4 sites are listed. At all sites, length and weight had strong correlations ($0.861 < r < 0.956$; $p < 0.001$), as had white blood cells and lymphocytes and thrombocytes as well as white blood cells and granulocytes ($0.534 < r < 0.844$; $p < 0.001$). Strong correlations between early and dead larvae were noted for all sites ($0.758 < r < 0.945$; $p < 0.00001$). The enzyme activities of GR and GST, GR and catalase, and GST and catalase correlated in Fjällbacka, Stenungsund, and Brofjorden ($0.554 < r < 0.868$; $p < 0.001$). Göteborg harbor only showed correlation between GR and GST ($r = 0.484$; $p = 0.014$). Strong correlations between the mRNA levels of C7 and hepcidin were noted in Fjällbacka, Stenungsund, and Göteborg harbor ($0.914 < r < 0.980$; $p < 0.011$). No correlations with p value less than 0.05 were noted between females and larvae for the parameters EROD and oxidative stress. Messenger RNA levels for the selected genes showed no correlation with p value less than 0.05, except for correlations between mRNA levels of lysozyme C measured in a female and in her larvae, captured in Göteborg harbor ($r = 0.84$; $p = 0.035$).

Hierarchical clustering was performed on 31 biological markers based on mean values for each site for the parameters measured in the 4 sites: Fjällbacka (reference), Brofjorden, Stenungsund, and Göteborg harbor. Clustering analysis revealed potential groupings of the biological markers based on similarity of their patterns at site level (Figure 6). This reflects at site level how the biological markers in relation to each other have responded to the environment and contamination at the sites. The biological markers were divided into 7 clusters. Cluster 1 was formed by EROD and the mRNA levels of CYP1A and diablo homolog. Cluster 2 contained frequencies of late and early dead fry, frequency of total abnormal fry, and the mRNA level of DDIT4. The enzymes involved in oxidative stress (GST, GR, and catalase) were all found in cluster 3 together with the mRNA level of LysC, frequency of hepatocellular and nuclear pleomorphisms, as well as frequency of malformed fry and

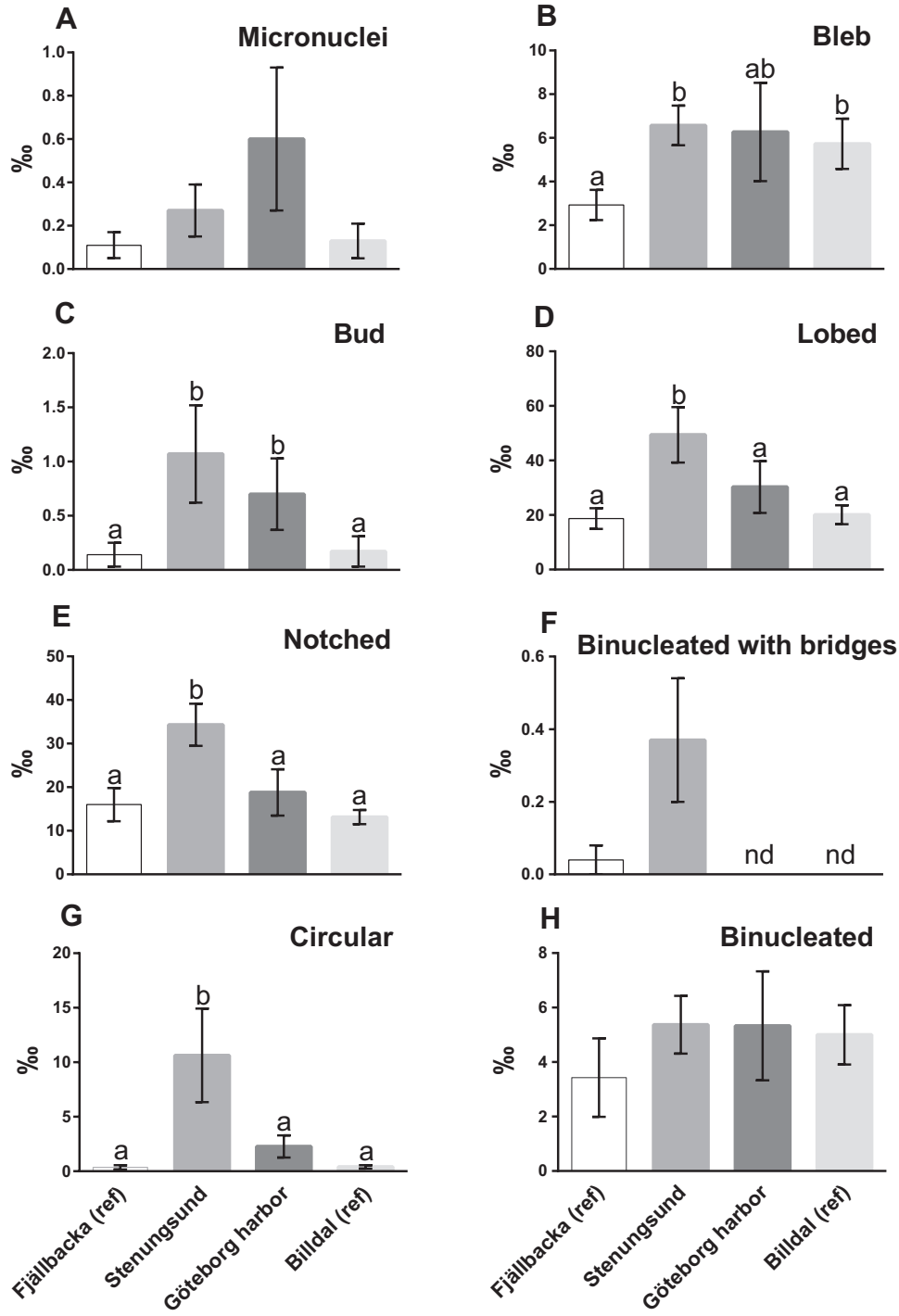

Figure 3. Frequency of (**A**) micronuclei, (**B**) blebbed nuclei, (**C**) budded nuclei, (**D**) lobed nuclei, (**E**) notched nuclei, (**F**) binucleated nuclei with bridges, (**G**) circular nuclei, and (**H**) binucleated nuclei (mean ± standard error) in erythrocytes from eelpouts collected in Fjällbacka, Stenungsund, Göteborg, and Billdal (14–15 individuals per site). All fish used were sexually mature females with the exception of fish from Fjällbacka, which were a mix of males and juveniles. Fjällbacka and Billdal are considered as reference sites. Lower-case letters indicate significant differences between sites ($p < 0.05$). nd = nuclear abnormalities not detected.

hemoglobin. In cluster 4, the blood cell counts for immature red blood cells, granulocytes, and thrombocytes were clustered together. The GSI and macrophage aggregates were also found in cluster 4. Cluster 5 contained condition factor, mean corpuscular hemoglobin concentration, and hepatocellular regeneration. The mRNA level of C7 and hepcidin, involved in the innate immune system, formed cluster 6. In cluster 7, the subcluster containing length and weight as well as the blood cell count for lymphocytes and white blood cells were found. The subcluster for LSI and macrovesicular steatosis was also contained in cluster 7 together with hematocrit.

DISCUSSION

In the present study, biomarkers were selected from different biological levels, including molecular, protein, cellular, and tissue changes, as well as on the level of reproductive success, to gain a broad understanding of the impact of environmental

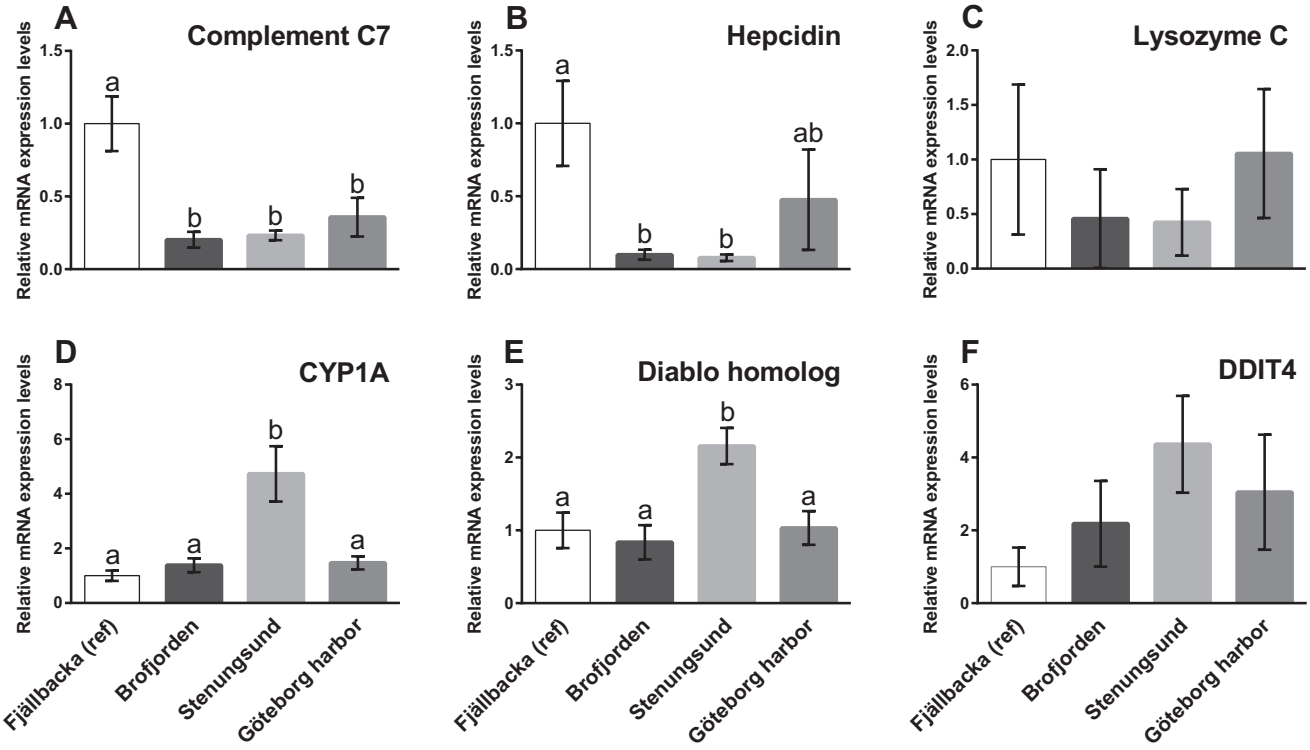

Figure 4. Quantitative polymerase chain reaction results for (**A**) complement component C7, (**B**) hepcidin, (**C**) lysozyme C, (**D**) CYP1A, (**E**) diablo homolog, and (**F**) DDIT4. Gene expression levels in Fjällbacka were set to 1, and levels in polluted sites were set accordingly (mean ± standard error). Lower-case letters indicate significant differences between sites ($p < 0.05$). CYP1A = cytochrome P4501A; DDIT4 = DNA damage transcript 4.

stressors in eelpout. Three polluted sites along the Swedish west coast were studied. Alterations were found at all biological levels, each of which will be discussed below.

mRNA levels

In a recent study on eelpout using global gene expression analysis [15], we showed that eelpout from Göteborg harbor had lower mRNA expression of genes coding for proteins involved in the innate immune system and higher expression of genes coding for proteins involved in apoptosis and DNA damage. In the present study, we wanted to verify some of these differentially regulated mRNA levels in eelpout collected at other polluted sampling sites, such as Brofjorden and Stenungsund.

Table 3. Frequency (%) of histological liver alterations observed in eelpout female collected along the Swedish west coast[a]

	Fjällbacka	Brofjorden	Stenungsund	Göteborg harbor
No abnormalities detected	4	8	8	0
Phospholipoidosis	0	0	0	0
Fibrillar inclusions	0	0	0	0
Hepatocellular and nuclear pleomorphism	21	16	13	21
Hydropic degeneration	0	0	0	0
Spongiosis hepatis	0	0	0	0
Clear-cell FCA	0	0	0	0
Vacuolated FCA	0	0	0	0
Eosinophilic FCA	0	0	0	0
Basophilic FCA	0	0	4	4
Mixed-cell FCA	0	0	0	0
Coagulative necrosis	13	8	8	13
Apoptosis	0	4	8	0
Microvesicular steatosis	0	0	0	0
Macrovesicular steatosis	42	24	38	83
Extensive macrovesicular steatosis[b]	4	4	13	46
Hemosiderosis	0	0	0	0
Variable glycogen content	0	0	0	0
Macrophage aggregates	25	80	58	46
Lymphocytic/monocytic infiltration	8	8	13	4
Granuloma	0	16	0	4
Fibrosis	0	0	0	0
Hepatocellular regeneration	29	36	4	0

[a]No neoplastic lesions were observed in any of the individuals. Assessment performed using 24 to 25 individuals per site.
[b]Individuals who exhibited an increased severity of macrovesicular steatosis characterized by a large majority of affected hepatocytes throughout the liver.
FCA = follicular carcinoma.

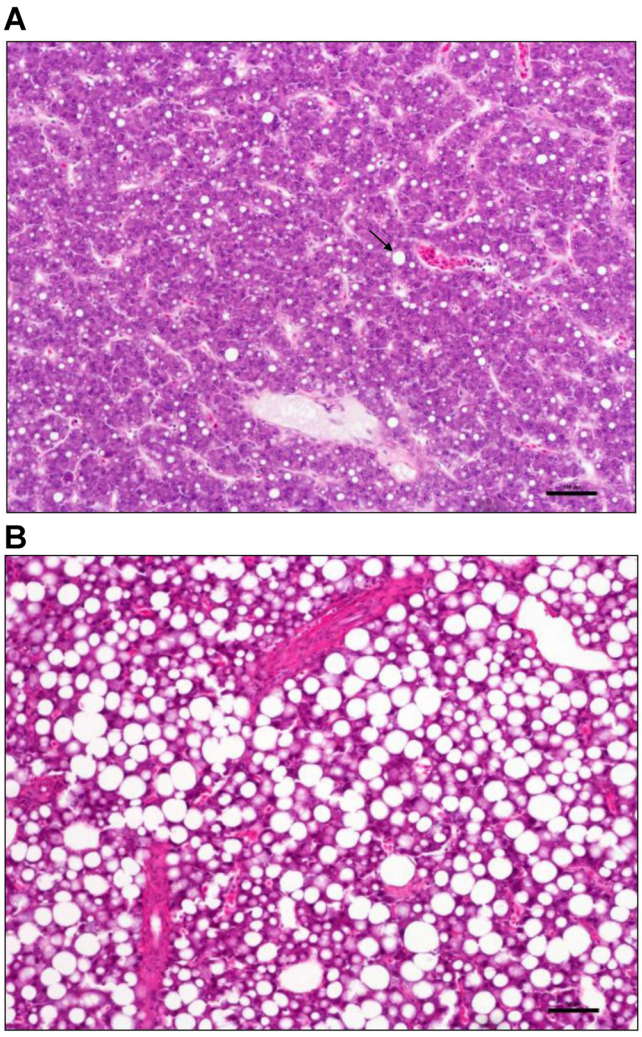

Figure 5. (**A**) Reference liver section demonstrating mild macrovesicular steatosis (arrow). Scale bar = 100 μm. (**B**) Liver section demonstrating extensive macrovesicular steatosis characterized by the presence of elevated numbers of singular large vacuoles within hepatocytes. Vacuoles were often seen displacing nuclei within affected hepatocytes. Scale bar = 50 μm.

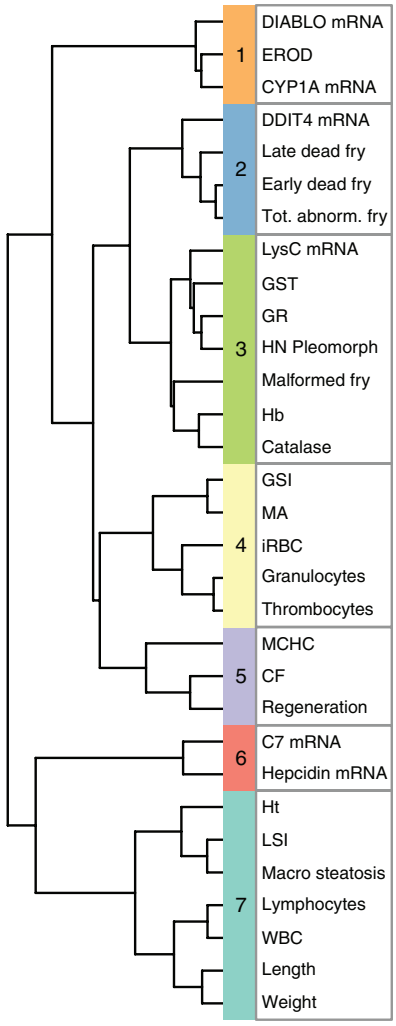

Figure 6. Dendrogram showing unsupervised hierarchical clustering (Euclidean distance) of 31 biological markers. The clustering was based on mean values for the markers measured in each of the 4 sites: Fjällbacka (reference), Brofjorden, Stenungsund, and Göteborg harbor. The biological markers were divided into 7 clusters, marked by color. DIABLO = diablo homolog; EROD = ethoxyresorufin *O*-deethylase (CYP1A activity); CYP1A = cytochrome P4501A; DDIT4 = DNA damage transcript 4; tot. abnorm. fry = total abnormal fry; LysC = lysozyme C; GST = glutathione *S*-transferase; GR = glutathione reductase; HN Pleomorph = hepatocellular and nuclear pleomorphism; Hb = hemoglobin; GSI = gonad somatic index; MA = macrophage aggregates; iRBC = immature red blood cells; MCHC = mean corpuscular hemoglobin concentration (ratio of Hb/Ht); CF = condition factor; regeneration = hepatocellular regeneration; C7 = complement component C7; Ht = hematocrit; LSI = liver somatic index; WBC = white blood cells.

The role of the innate immune system is to respond to bacterial and viral infection. Several pollutants are also known to have a suppressive and/or an activating effect on the immune system [14]. In the present study, 3 genes involved in the innate immune system were selected: complement component C7, hepcidin, and lysozyme C. The expression of the complement component C7 and the antimicrobial peptide hepcidin was high in the reference site, Fjällbacka, and low in the polluted sites (not significant for hepcidin mRNA levels in eelpout from Göteborg harbor). This could suggest a suppression of the innate immune response in fish captured in polluted sites or a response to bacterial infection in eelpouts captured in the reference site. These results would, however, need to be further evaluated both on the protein/enzymatic level and as possible responses to pathogen infection. A relationship in the expression of hepcidin and C7 on the individual level within each site was indicated by positive correlations between the mRNA levels (p value <0.05 in 3 out of 4 sites), possibly suggesting a coexpression of hepcidin and C7. Similarities in expression patterns were also noted on the site level as C7 and hepcidin clustered together (Figure 6). The mRNA expression level for lysozyme C was low in Stenungsund and Brofjorden but not significantly.

Diablo homolog and *DDIT4* are genes involved in apoptosis and DNA damage and were selected to verify their expression in polluted sites. Diablo homolog is a mitochondrial protein known to be transported to the cytosol to activate caspases during initiation of apoptosis [30]. The mRNA levels of the diablo homolog have been suggested to be a biomarker of pollutant exposure in flounder [31]. A significantly higher mRNA level of the gene coding for diablo homolog was found in eelpouts collected at Stenungsund in the present study. The *DDIT4* gene has been postulated to be induced during hypoxia conditions and apoptosis [32]. The mRNA expression levels were higher in the polluted site but not significantly (Stenungsund vs Fjällbacka, $p = 0.076$). The mRNA expression levels of the detoxification enzyme CYP1A were measured as a marker for exposure to aryl

hydrocarbon receptor ligands such as PAHs and were significantly higher in Stenungsund.

Enzymatic measurements

Induction of the hepatic CYP1A enzyme is a commonly used biomarker to indicate exposure to specific xenobiotics such as PAHs and halogenated dioxins. Stenungsund was the only polluted site with a significantly higher CYP1A/EROD activity in eelpout compared with the reference site, which would indicate an exposure to, for example, PAHs or dioxins in this area. Eelpout from this site also had the highest mRNA levels of CYP1A and diablo homolog. There was higher EROD activity as well as higher diablo homolog mRNA expression level in the eelpouts collected in the harbor compared with Fjällbacka in 2006 and 2010, which could indicate that higher EROD activity is accompanied by a higher expression of diablo homolog [15]. The mRNA levels of diablo homolog, EROD, and CYP1A all were found in the same cluster when clustering mean values for each parameter from each site. This could indicate that a site with high expression of the CYP1A enzyme, measured as high activity (EROD) and/or high mRNA levels of CYP1A, would also be a site where fish express high levels of diablo homolog mRNA. This could additionally support the need for further evaluation of diablo homolog as an indicator of a pollutant effect.

Oxidative stress parameters

Oxidative stress is used commonly as a biomarker of exposure to different pollutants and may lead to oxidative damage in cellular molecules, including proteins, lipids, and nucleic acids [33]. When antioxidant defenses are insufficient, 1 form of irreparable damage that can occur in proteins is carbonylation. A higher level of protein carbonylation is an indicator of oxidative stress and has been demonstrated in several studies with fish [10,34,35]. Although oxidative stress can be assessed using measurements of antioxidant enzymes, these responses tend to be transient. In the present study, no significant differences were noted for enzymes involved in oxidative stress (GR, GST, and catalase) between the sites (Table 1). Damage products such as protein carbonyls can provide a more integrated and physiologically relevant assessment of oxidative stress. In the present study, no statistically significant differences between sites were noted, but levels of protein carbonyls were highest in Stenungsund and Göteborg harbor (results not shown) and significantly higher in females than in larvae.

Blood parameters

There was a significantly lower concentration of hemoglobin in blood from eelpout captured at Brofjorden compared with the reference site, Fjällbacka. Eelpouts from Brofjorden also had the highest immature red blood cell levels compared with Stenungsund and Fjällbacka. A high level of immature red blood cells could indicate anemia and the need for initiating erythropoiesis to compensate for the low oxygen uptake, which was also indicated in the low hemoglobin level at this site. Whether contaminant exposure contributes to this process has to be evaluated in future studies.

Measurement of red blood cell parameters showed that the ratio between hemoglobin concentration and hematocrit (red blood cell volume), also called the mean corpuscular hemoglobin concentration, in eelpouts collected in Göteborg harbor had a significantly lower value compared with all other sites. This difference was observed previously in eelpout from Göteborg harbor compared with Fjällbacka [11,15]. It was suggested that this could be attributable to a difference in iron homeostasis and/or oxygenation of the blood, because genes known to be involved in these processes were expressed differently in eelpout collected in Göteborg harbor compared with Fjällbacka [15]. The health status of the eelpouts collected in the harbor was lower compared with fish collected at other sites, as noted by a significantly lower condition factor in the harbor eelpouts (Table 1). Clustering analysis found similar patterns for condition factor and mean corpuscular hemoglobin concentration at the site level.

Blood cell count

The highest number of white blood cells was found in eelpout collected in Göteborg harbor compared with fish collected in Stenungsund and the reference site in Fjällbacka. The difference in total white blood cell counts was primarily attributable to a higher number of lymphocytes in eelpout from Göteborg harbor in comparison with the 2 other sites. In addition, a higher number of lymphocytes was detected in eelpout collected in Brofjorden compared with Fjällbacka. This indicates that a part of the adaptive immune system is modulated in eelpout collected at these 2 sites and could signal some sort of inflammatory response in these fish but also could indicate overall response to stressors [36]. An increase in white blood cell count has been noted previously for sábalo (*Prochilodus lineatus*) captured in 3 sites along the Salado River in Argentina, an area impacted with industrial, agricultural, and domestic waste along with high levels of heavy metals [2]. In contrast to the present study, however, the increase in numbers of white blood cells was mainly the result of an increase in neutrophils as the lymphocyte count instead decreased. Wolf fish (*Hoplias malabaricus*) exposed daily via diet to methylmercury for more than 70 d showed an increase in white blood cells as well as number of neutrophils and differential lymphocyte/monocyte count [37]. The concentration of heavy metals, especially mercury, is known to be high in sediments from Göteborg harbor [18,19] and could possibly be responsible for the increased levels of lymphocytes seen in the present study.

Genotoxic parameters

The micronucleus test is commonly used to test for genotoxic responses and chromosomal damage and is considered to be a good biomarker for assessing genotoxic pollutants in the marine environment [38]. In contrast to single-strand breaks, which are rapidly repaired, chromosomal damage and development of micronucleus occur after breaking of both DNA strands. The assessment of the frequency of micronucleus has been suggested as a genotoxic biomarker for detection of genotoxic effects in both mussels and fish in the Baltic and North Seas. The background level for micronucleus frequencies in eelpout erythrocytes captured in the Baltic Sea has recently been established as <0.38 micronucleus/1000 erythrocytes [39]. The highest frequency of micronucleus in the present study was found in eelpouts collected in Göteborg harbor (0.6 ± 0.33), though this was not significantly different from the reference sites (Figure 3). High levels of damaged DNA paralleled by a peak in bile PAH metabolites were also detected in eelpout collected at sites impacted by an oil spill in the Göteborg harbor area [40].

Apart from micronucleus, other nuclear alterations in piscine erythrocytes have been considered as possible indicators of genotoxicity [41–43]. A significantly higher prevalence of nuclear damage was found in erythrocytes in eelpouts collected

in Stenungsund compared with the reference sites, which could be interpreted as a response to the pollutants (Figure 3). Because the turnover time for erythrocytes is approximately 3 mo, the exposure causing the genotoxic effects is likely to be recent. Exposure to PAHs found in the area could be a reason as PAH exposure is known to cause nuclear abnormalities [44].

Histological parameters

Histopathological assessment revealed that macrovesicular steatosis was the most prominent category of liver lesion observed in eelpouts in the present study. Steatosis refers to an imbalance in lipid metabolism and storage within a cell. This imbalance involves enlargement of lipid-containing vesicles/vacuoles that eventually could distort the nucleus and, in severe cases, also disrupt the cell [45]. Macrovesicular steatosis was found in eelpouts collected from all sites, although generally the condition was observed at relatively low severity; that is, relatively small numbers of hepatocytes within discrete regions were affected. However, 46% of the eelpouts collected at Göteborg harbor exhibited an increased severity as characterized by a large majority of affected hepatocytes throughout the liver. A significantly higher LSI also was found in eelpouts collected in the harbor, which could be because of the high lipid content (steatosis) in the liver of these fish as a positive correlation between the level of severity of macrovesicular steatosis and LSI was noted on the individual level in this site ($r = 0.626$, $p < 0.001$). Both macrovesicular steatosis and LSI were found in the same cluster, further supporting an eventual link between the parameters. Earlier studies reported a higher prevalence of macrovesicular steatosis in sharptooth catfish [45] and flounder [46] collected at polluted sites. Macrovesicular steatosis has also been noted in eelpouts collected at polluted sites along the coast of Estonia [3] but not at a frequency as high as the levels found in eelpouts in the Swedish west coast sites collected in the present study. Although previous studies demonstrated increased macrovesicular steatosis in livers of fish exposed to contaminants under controlled experimental conditions [45,47], it is important to note that different fish species may possess alternative mechanisms for lipid storage under normal conditions, particularly concerning seasonal or reproductive conditions [28].

Histopathological alterations categorized as cellular immune responses include macrophage aggregates. Macrophage aggregates are known to increase in size and frequency as a result of environmental stressors and are suggested as a biomarker when assessing effects of chemical pollution in the aquatic environment [48]. In a baseline study on 1070 eelpouts collected from 34 different areas distributed along the Baltic Sea, the mean prevalence of macrophage aggregates was 69% [29]. In the present study, the highest prevalence of macrophage aggregates was found in eelpout collected in Brofjorden, with a prevalence of 80%. The frequency of macrophage aggregates was 58% of individuals in Stenungsund, 46% in Göteborg, and 28% in the reference site Fjällbacka. Although the prevalence of macrophage aggregates may appear high in the present study, their occurrence is commonplace and, as such, they are often observed in fish livers sampled from both clean and polluted locations. In the present study, macrophage aggregates did not exhibit characteristics that might be considered to be related to environmental pollution, such as increased size, frequency, and lipofuscin (brown pigment) intensity. Macrophage aggregates also are known to have an age-dependent accumulation [28,49]; because age was not determined in eelpout in the present study, it cannot be excluded that eelpout with a larger number of macrophage aggregates are actually older. Despite the fact that macrophage aggregates were relatively common in the eelpouts collected, they were not considered to be at a severity above baseline levels [29].

Effects on reproduction

The viviparity of eelpouts provides a unique opportunity to link responses in the individual female fish to reproduction and larvae development. The eelpout is being used in monitoring studies to assess the effects of pollutants on reproductive success in several areas around the Baltic Sea [5]. Eelpouts collected in the Stenungsund area had elevated levels of abnormal fry in their broods, which included both early dead and late dead larvae (Table 2). Elevated levels of late dead larvae also were noted for eelpouts collected in Göteborg harbor (Table 2). The levels of early and dead larvae correlated in all 4 sites, and both parameters were found in the same cluster (Figure 6). Hypoxic conditions previously have been proposed as causative in the death of eelpout larvae in the ovary prior to birth [20,50]. In the present study, the level of malformed larvae was low and below the background levels set by the International Council for the Exploration of the Sea for eelpouts. In contrast, earlier studies in Stenungsund and Brofjorden [11,12] reported high levels of malformation in developing larvae. The result in the present study could therefore indicate an improved quality of the environment at these sites; this, however, needs to be confirmed in future studies.

Biomarker assessment in larvae

During development in the ovary, eelpout larvae are dependent on nutrients and oxygen from the mother fish [51]. Laboratory studies have demonstrated that when pregnant female eelpouts are exposed to chemicals, these compounds can be transferred from the female to the developing larvae [52,53]. This was not evident in the parameters measured in the present study, in which we attempted to study effects on unborn eelpout larvae using biomarkers of effect. Very few correlations with p value less than 0.05 were found between females and larvae for the parameters EROD, oxidative stress, and mRNA levels for the selected genes. This lack of correlation could indicate that females protect their unborn offspring from external stressors. It is also possible that the biomarker responses in larvae differ from adult fish.

Site-specific responses

An elevated anthropogenic chemical burden is known to be found in eelpouts from the selected sites according to previous studies [54]. No chemical analysis was performed in the present study; thus, no direct link between pollutant and biomarker assessment was established. Taken together, however, the results demonstrate that different sites were identified as polluted depending on the type of biomarkers used in the assessment. Histological assessments identified Göteborg harbor as the site where eelpouts had the highest frequency of extensive macrovesicular steatosis. Reproductive success assessment identified both Göteborg harbor and Stenungsund as sites with an environment that had an effect on the frequency of dead larvae in broods. Blood parameter assessment, on the other hand, pointed out Göteborg harbor and Brofjorden as sites were pollutants might affect organisms in these areas. Using measurements for genotoxic damage and enzymatic activity of detoxification enzymes pointed out Stenungsund as the only site with significantly higher responses in these parameters. The discrepancy between the sites identified as polluted depending

on the biomarker used again points to the importance of using a broad approach and combining different biomarkers during environmental monitoring. Differences in biotic and abiotic conditions, including chemical mixtures between sites, will result in unique effects in living organisms and result in unique patterns that can provide a more comprehensive picture of the impact of environmental stressors.

SUPPLEMENTAL DATA

Table S1. (16 KB DOC).

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Data availability—All data, metadata, and calculation tools are available on request from the authors (noomi.asker@bioenv.gu.se).

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