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A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*)

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Abstract

As a first stage in developing a DNA array-based approach to investigating the effects of pollutants on an environmentally relevant European fish species, we have constructed a 160-gene custom microarray for European flounder. Degenerate primers were used to amplify 110 different fragments of stress-related and other genes from European flounder cDNA and genomic DNA. Additionally, 22 fragments were obtained by suppressive subtractive hybridisation (SSH). These fragments were cloned and sequenced, then, with additional control genes, used to create a cDNA microarray for flounder. After optimisation of the arraying process, hepatic mRNA was isolated from flounder caught in the polluted Tyne and relatively unpolluted Alde estuaries. Fluorescent cDNA probes were synthesised from the mRNA and used in dual-colour hybridisations to the microarray. A number of transcripts were differentially expressed between Tyne and Alde female flounder but these changes were not significant, due to high inter-individual variation. However, in comparisons between Tyne and Alde male flounder, 11 transcripts were found to significantly differ in expression ($P < 0.05$). Seven transcripts were more highly expressed in the Tyne male fish (CYP1A, UDPGT, α-2HS-glycoprotein, dihydropyrimidine dehydrogenase, Cu/Zn SOD, aldehyde dehydrogenase and paraoxonase). Four transcripts (Elongation factor 1 (EF1), EF2, Int-6 and complement component C3) were found to be significantly less abundant in the Tyne male fish. Selected genes were assayed by real-time PCR, then normalised to α-tubulin. These assays confirmed the significance of the array results for CYP1A, UDPGT and EF1, but not for Cu/Zn SOD. This study provides a link between traditional single-gene biomarker studies and the emerging field of eco-toxicogenomics, demonstrating the utility of microarray studies on environmentally sampled, non-model organisms.

Keywords: Microarray; cDNA array; Flounder; Real-time PCR; CYP1A; Toxicogenomics

1. Introduction

The technique of DNA microarraying has been applied to many areas of biological research. In the field of toxicology, advances in transcript analysis have led to the recognition that altered gene expression is potentially an early, rapid and

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sensitive means of stress response detection. Toxicogenomics combines information and material from genomics and bioinformatics in order to identify and characterise mechanisms of toxicity from known or suspected toxicants. This has included high-throughput (in vitro) toxicity testing (Gore et al., 2000), medical diagnostics (DeRisi et al., 1996) and environmental toxicology using model organisms (Bartosiewicz et al., 2001). In fish, there is a lack of extensive sequence information for species other than fugu (*Takifugu rubipres*) or zebrafish (*Brachydanio rerio*). A number of strategies have been used to overcome this problem. Zebrafish clone sets have been used to study the statistical variation of differential expression (Herwig et al., 2001) and to elucidate developmental processes (Dickmeis et al., 2001; Ton et al., 2002). A cross-species approach has been taken to characterise the effects of zinc exposure, where a fugu array was used to identify differentially regulated genes from rainbow trout (*Oncorhyncus mykiss*; Hogstrand et al., 2002). To construct specific arrays for other species, clones from fish cDNA libraries have been used. Responses of the channel catfish (*Ictalurus punctatus*) to cold acclimation were studied via this route (Ju et al., 2002) and the variation within and between natural populations of *Fundulus* was investigated using a microarray constructed by a similar method (Oleksiak et al., 2002). Differential display (DD-RTPCR) has provided differentially regulated clones to elucidate the effects of estrogen on minnows (*Cyprinodon variegatus*) by arraying (Denslow et al., 2001; Larkin et al., 2002a). Suppressive subtractive hybridisation (SSH) with goby (*Gillichthys mirabilis*) tissue has similarly provided clones for microarraying to profile hypoxia-related gene expression (Gracey et al., 2001). Clones produced using a variety of these techniques were used to analyse gene expression in largemouth bass (*Micropterus salmoides*) exposed to estrogenic compounds (Larkin et al., 2002b). Our approach was similar in that we combined clones from specific, selected, stress-related genes, random clones and some genes identified by SSH as differentially expressed in flounder from polluted sites.

In the field of marine environmental monitoring, molecular biomarkers (including gene and protein expression changes and enzyme induction) have been shown to aid the recognition of pollutant exposure and impact (Malins and Ostrander, 1994). The use of a range of biomarkers is preferable to minimise misinterpretation of results (Sole et al., 1996). Our aim was to integrate recognised biomarkers of exposure and effect with the DNA microarray-based study of pollutant-induced gene expression changes in an environmentally relevant fish species, European flounder. This could extend the biomarker approach to create, potentially, a powerful technique for environmental research and monitoring. *Platichthys flesus* is a teleost flatfish common in estuarine environments throughout Western Europe, many of which are significantly impacted by toxicants including xenoestrogens, heavy metals, polycyclic aromatic hydrocarbons (PAHs) and dioxins, usually present as complex mixtures closely associated with the sediment. This species feeds mostly on invertebrates from the estuarine sediment, making it a good candidate for pollution monitoring. A number of researchers have used flounder in biomarker studies (Goksoyr et al., 1996; Sandvik et al., 1997; Besselink et al., 1998; Bogovski et al., 1998; Lyons et al., 1999). *P. flesus* and a closely related species, the dab (*Limanda limanda*), are key species in the National Marine Monitoring Programme (NMMP), run by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), which samples fish from 70 sites in UK waters.

2. Materials and methods

P. flesus adult liver samples, from the polluted Tyne estuary (1,431,583 m^3 effluent per day) and the reference Alde estuary (145 m^3 effluent per day) (Lyons et al., 1999) were the kind gift of Dr. B.P. Lyons (CEFAS). Liver cDNA Smart-RACE (Clontech) libraries were synthesised from these samples, as was genomic DNA. *P. flesus* lambda ovary cDNA and genomic libraries had previously been constructed in this laboratory (Williams et al., 2000).

Candidate genes were selected by reference to previous literature on biomarkers in fish species and components of mammalian 'toxicology arrays'. The Bioinformatics Project, an MRC-funded facility within the School of Biosciences, provided access to DNA sequence analysis software (GCG, Madison, WI). Vertebrate homologues of the candidate genes were aligned and conserved regions identified. Degenerate PCR primer pairs encompassing, typically, 400 bp were designed using Primer3 software (Rozen and Skaletsky, 2000). These primer sequences are available via the Web site http://www.genipol.stir.ac.uk. Primers of 18–22 bases were synthesised by Alta Biosciences, MWG-Biotech, or Bio-Rad.

DNA fragments were amplified with *Taq* polymerase (Bioline) using conventional (Techne) or 96-well temperature gradient (Eppendorf) PCR machines, with *P. flesus* liver cDNA, ovary cDNA, or genomic DNA as a target. After optimisation, PCR products were excised from agarose gels, purified, then cloned into pBluescript II SK+ (Stratagene) or pCR2.1 (Invitrogen). Some initial DNA fragments from SSH study were also cloned. Briefly, liver Smart-RACE cDNA from 10 adult female flounders from the Tyne and 10 from the Alde estuaries was pooled and subtracted libraries in pCR2.1 were produced by PCR-select subtraction method (Clontech). Details of the procedure are given in Sheader et al. (2003). PCR product sequencing was carried out by Birmingham Biosciences Genomics Laboratory with an ABI 3700 automated sequencer. Sequences obtained were compared with DNA and protein databases using BLASTN and BLASTX analysis software, respectively (Altschul et al., 1990). Novel sequences were submitted to the European Bioinformatics Institute database (EMBL-EBI).

The required clones were re-amplified using vector primers (M13 Rev and M13–20), purified by Multiscreen 96-well PCR purification plates (Millipore) and loaded into a 384-well plate (Genetix) in 50% dimethylsulfoxide (DMSO), 0.3 × SSC (Ausubel, 1995) at ~100 ng/μl in preparation for arraying. An MGII arraying robot (Biorobotics) with split pins was used to array samples (six spots per sample per slide) onto GAPS-II coated glass slides (Corning), which were then baked for 2 h at 80 °C.

Five adult male and five adult female feral *P. flesus* fish were caught from each of the Tyne and Alde estuaries during autumn 2000, their livers were immediately removed and snap frozen in liquid nitrogen and none exhibited any gross pathology. Tyne sediment has been found to contain a range of organic and inorganic contaminants, for example, values relative to sediment dry weights were: PAHs: up to 43 mg/kg (Lyons et al., 1999), Cd: up to 7 mg/kg, Hg: up to 2 mg/kg and Cu: up to 180 mg/kg (Matthiessen et al., 1998) with similar heavy metal concentrations being found in suspended particulate samples (Laslett, 1993).

Messenger RNA was prepared from *P. flesus* liver samples using Poly-Attract (Promega). The quality of mRNA was assessed using an RNA 6000 nano-assay with the Bioanalyser 2100 (Agilent). We then synthesised cDNA using SuperscriptII reverse transcriptase (Invitrogen) with random primers (Alta Bioscience). cDNA was labelled with Cy3-dCTP or Cy5-dCTP (AP Biotech) using the Bioprime random priming kit (Invitrogen) with non-biotinylated dNTPs. Labelled cDNA was purified with QIA-prep spin columns (Qiagen); the amount of dye incorporated into each cDNA sample was determined by spectrophotometry at 550 nm (Cy3) and 650 nm (Cy5). Thirty to fifty picomoles of each dye (labelled cDNA) was mixed for each hybridisation and concentrated to 10 μl in a YM30 spin filter (Amicon). Array slides were prehybridised, then hybridised overnight with a mix of control and test probes under a plastic coverslip (Sigma hybrislip) in formamide buffer at 42 °C (Corning DMSO protocol). All male samples were individually hybridised against an Alde male control sample and all female samples were individually hybridised against an Alde female control sample. Slides were washed in 0.1 × SSC at room temperature, then dipped briefly into water before drying by centrifugation. Arrays were scanned at 532 and 635 nm using a confocal scanner (Axon) at photomultiplier tube voltage of 600 V. The images were analysed using GenePix software (Axon) and

data were subsequently input to Genespring software (Silicon Genetics) for further analysis.

The signal intensity for each gene was divided by its control channel value in each sample. When the control channel value was below 50, the data point was considered unusable. Intensity-dependent normalisation was also applied, where the ratio was reduced to the residual of the Lowess fit of the intensity versus ratio curve. The 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 200. Only genes marked as present were used.

Data derived from the five fish of the same sex from each sampling site were considered as biological replicates, giving four data sets, Tyne and Alde male and Tyne and Alde female. Each of these data sets consisted of the results of separate array experiments for each of the five individual fish. Tyne and Alde data sets from each sex were compared using Genespring software. Statistically significant differences were determined by a parametric Welch *t*-test using global error model variances derived by combining measurement variation and inter-sample variation for array data from the five replicates (Silicon Genetics). The *P*-value cut-off was 0.05. A multiple testing correction (Benjamini and Hochberg, 1995) was performed, which forecast that approximately 5% of the identified genes would be expected to pass the test by chance.

To determine whether α-tubulin expression was similar in Tyne and Alde samples, total RNA was extracted from male liver samples using the SV Total RNA kit (Promega). Five micrograms of each RNA sample was used for Northern blotting, probed with a labelled α-tubulin PCR product (clone TUB-o4) using the North-2-South kit (Pierce). Densitometry was performed using ImageQuaNT software (Molecular Dynamics). Real-time quantitative PCR was performed on four male Alde and five male Tyne cDNA samples. For validation, specific primer pairs were designed for four genes identified as differentially regulated from the microarrays and for the control gene α-tubulin. These primers were: for CYP1A, CYP-F 5′-CTGGAGGAACACATCTGCAA-3′, CYP-R 5′-CTCATCACTGAGGGTCACCA-3′; for α-tubulin, ATUB-F 5′-CACAGCCTCACTTCGTTTTG-3′, ATUB-R 5′-AGATGACAGGGGCATAGGTG-3′, for UDPGT, UDPGT-F 5′-ATGACCTTCCCGCAGAGAGT-3′ and UDPGT-R 5′-GATGAGCCAGAGAGCCCC-3′; for Cu/Zn SOD, CZSOD-F 5′-TGGAGACAACACAAACGGG-3′, CZSOD-R 5′-CATTGAGGGTGAGCATCTTG-3′, for elongation factor 1 (EF1), EF1-F 5′-TGTCCCATCTGCTAAGGCTG-3′ and EF1-R 5′-CTTGAGGCGTTCTGTCTCCT-3′. Standards were amplified for each gene, quantified by spectrophotometry at A_{260} and serially diluted. Samples and standards were assayed in triplicate. Assays were performed with SYBR-Green PCR mix (Bio-Rad) on an iCycler Real-Time thermocycler (Bio-Rad). Data were calculated from the standard curve and then divided by the mean reading for α-tubulin for each sample. The *t*-test was performed to compare the Alde and Tyne groups for each gene.

3. Results

71 primer pairs and 16 additional primers for semi-nested PCR were designed for different target genes. Primers included those previously used in our laboratory to amplify flounder Ha-ras, Ki-ras, p53 and β-actin (Lee and Chipman, 1998; Lee et al., 2000; Franklin et al., 2000). Due to the necessary use of degenerate primers, as anticipated, many non-specific PCR products were amplified and some primer pairs produced no product. Different PCR products were cloned and sequenced, 89 of 111 showed similarity to sequences available in the databases (Table 1). In addition, 22 clones produced by SSH between Tyne and Alde *P. flesus* Smart-RACE cDNA (Sheader et al., 2003) were sequenced and analysed. In total, 128 novel DNA sequences for *P. flesus* were submitted to EMBL-EBI.

We arrayed 160 samples six times each per slide for a total of 960 spots; these included the DNA species detailed above, extra duplicates and negative controls of plasmid DNAs (pUC18, pBluescript, pGEM, pRL,) and luciferase. We also included a 400-bp exon fragment of our *P. flesus*

Table 1
PCR products cloned and sequenced from *P. flesus* DNA

Clone	Primers	Anneal	Size	Accession	Most similar to	Accession	Species	%ID	*E*-value
ACT-g2	BACT-3′, BACT-EX3	50C	1158	[AF135499]	β-Actin	AF135499	*P. flesus*	100	
AHR-o2	AHR-F2, AHR-R2	55C	221	[AF034412]	Ahr2	AF034412	*P. flesus*	100	
ALAS-l6	ALAS-F, ALAS-R	50C	612	AJ302076	5-Aminolevulinic acid synthase	L35915	*Opsanus tau*	90	1E−102
ALAS-o26	ALAS-F, ALAS-R	50C	140	AJ291834	NADP-menadione oxidoreductase	AF128816	*O. latipes*	72	8E−46
ALD-o1	ALD-F, ALD-R	45C	381	AJ298325	Aldehyde dehydrogenase 1	X05409	*Homo sapiens*	85	3E−60
BAX-o4	BAX-F, BAX-R	59C	173	AJ409362	Zinc finger 234	X78927	*H. sapiens*	54	7E−01
CAT-o7	CAT-F, CAT-R	45C	105	AJ298326	60S Ribosomal protein L7	L16558	*H. sapiens*	76	6E−09
CAT-l1	CAT-F, CAT-R	55C	244	AJ409363					
CAT-o21	CAT-F, CAT-R	45C	631	AJ508544	Nuclear movement protein PNUDC	AF259800	*Pleurodeles waltl*	*86*	*1E−24*
CB5-l1	CB5-F, CB5-F	45C	307	AJ409365	ets variant 6	AF340230	*T. rubipres*	100	2E+00
CB5-l21	CB5-F, CB5-R	45C	296	AJ508535	Dihydropyrimidine dehydrogenase	U20938	*H. sapiens*	76	1E−37
CB5-l4	CB5-F, CB5-R	45C	255	AJ298328	Transketolase	AF270484	*Xenopus laevis*	73	1E−27
CLU-o1	CLU-F, CLU-R	50C	550	AJ300775	Peptide release factor	AF072934	*H. sapiens*	62	1E−61
CLU2-o1	CLU-F, CLU-R2	40C	625	AJ409366	*H. sapiens* predicted ORF	XM_114126	*H. sapiens*	60	3E−40
COL-o5	COL-F, COL-F	45C	409	AJ300776	Elongation factor 2	AF000576	*Rattus norvegicus*	84	9E−66
COX-l4	COX-F, COX-R	50C	353	AJ300777	Transferrin	AF219998	*Paralichthys olivaceus*	71	2E−32
CPR-l1	CPR-F, CPR-R	47C	395	AJ300778	Peroxin (Pex11)	AB018080	*H. sapiens*	60	8E−06
CY3-o5	CY3-F, CY3-R	55C	385	AJ310471	CYP3A1	M10161	*R. norvegicus*	67	3E−48
CY4-l4	CY4-F, CY4-R	54C	425	AJ409367	*Tetraodon* GSS CNS02464	AL180373	*Tetraodon nigroviridis*	*87*	*4E−06*
CY4-l5	CY4-F, CY4-R	54C	354	AJ409356					
CY4-l7	CY4-F, CY4-R	54C	287	AJ310424	E1beta-55 kDa-associated protein	AJ007509	*H. sapiens*	65	1E−08
CY4-l8	CY4-F, CY4-R	54C	334	AJ409357	Cysteine-rich protein	U63332	*H. sapiens*	86	7E−05
CY4-l9	CY4-F, CY4-R	54C	325	AJ409358	*Battrachocottus baicalensis* ORF2	U18939	*B. baicalensis*	47	4E−06
CYP-o049	Subclone		400	AJ132353	CYP1A	AJ132353	*P. flesus*	100	
D25-l1	DX25-F, DX25-R	55C	361	AJ310425	Membrane progesterone receptor	Y12711	*H. sapiens*	73	2E−46
EPX-o7	EPX-F, EPX-R	45C	578	AJ409359	RIKEN cDNA clone 0610025L17	AK002667	*Mus musculus*	62	8E−15
FEN-g4	FEN-F, FEN-R	50C	709	AJ310712	UDP-GalNAC	AJ133523	*M. musculus*	47	2E−38
FIB-l1	FIB-F, FIB-R	50C	351	AJ310418	Gamma-fibrinogen	J02894	*X. laevis*	79	3E−54
FMO-l4	FMO-F, FMO-F	50C	459	AJ310419	Glucose-6-phosphatase	AF005946	*Ptyochromis xenagnathus*	80	1E−68
FMO-l5	FMO-F, FMO-F	50C	609	AJ409360	*Caenorhabditis elegans* hypothetical C24B9.6	AF068709	*C. elegans*	39	1E−01
FMO2-l21	FMO-F, FMO-R2	40C	270	AJ508536					
FMO2-ll0	FMO-F, FMO-R2	40C	638	AJ310420	ITI H chain/SHAP	AB050592	*Oryctolagus cuniculus*	64	2E−77
FOS-o3	FOS-F, FOS-R	50C	265	AJ310422	RNA pol III subunit RPC II	AF051316	*H. sapiens*	82	9E−39
FOS-l1	FOS-R, FOS-R	41C	147	AJ310435	Adducin-related protein	AF166167	*C. elegans*	66	4E−10
H27-l2	H27-F, H27-R	45C	388	AJ310423	Apolipoprotein AI	AF013120	*Sparus aurata*	53	7E−25
FYN-l4	FYN-F, FYN-R	56C	374	AJ310436	c-fyn	X54971	*Xiphophorus helleri*	90	1E−63
G6D-l2	G6D-F, G6D-R	50C	395	AJ310437	TIF3/P42	AF108214	*M. musculus*	89	2E−56
G6D-l4	G6D-F, G6D-R	50C	613	AJ310438	Glucose-6-PO4 dehydrogenase	X83611	*T. rubipres*	94	1E−113
G6D-l24	G6D-F, G6D-R	50C	196	AJ508537	Nucleic acid binding protein	AF086712	*T. rubipres*	50	8E−05

Table 1 (*Continued*)

Clone	Primers	Anneal	Size	Accession	Most similar to	Accession	Species	%ID	*E*-value
G45-l1	G45-F, G45-R	50C	162	AJ310439	40S Ribosomal protein S26	X69654	*H. sapiens*	98	2E−23
G45-l3	G45-F, G45-R	50C	144	AJ310440	Sec-63-like protein	AF100141	*H. sapiens*	87	2E−18
G78-g1	G78-F, G78-R	50C	353	AJ305218	GRP-78 (BiP)	AJ002387	*M. musculus*	95	6E−37
G78-o2	G78-F, G78-R	48C	298	AJ409352					
G78-o23	G78-F, G78-R	48C	323	AJ508545	*O. latipes* EST OLc20.03f	AU240482	*O. latipes*	*94*	*1E−19*
G78-o5	G78-F, G78-R	50C	230	AJ305219	40S Ribosomal protein S8	AJ266243	*G. mirabilis*	94	8E−34
G94-o1	G94-F, G94-R	48C	324	AJ305220	Chromobox protein	AJ238107	*O. latipes*	90	9E−44
G153-o8	G153-F, G153-R	57C	259	AJ305221	30 kDa Splicing factor	AF083385	*H. sapiens*	64	3E−27
G153-o71	G153-F, G153-R	48C	300	AJ409353					
PARP-l1	PARP-F, PARP-R	50C	368	AJ310714					
GAP-l1	GAP-F, GAP-R	52C	613	AJ305222	GAPDH	AB029337	*P. olivaceus*	94	1E−109
GAP-o6	GAP-F, GAP-R	50C	448	AJ305223	60S Ribosomal protein L22	X64207	*X. laevis*	89	7E−34
GCL-o26	GCL-F, GCL-R	45C	375	AJ508538	*O. latipes* EST MF01SSA	BJ015474	*O. latipes*	*81*	*2E−26*
GCL-o15	GCL-F, GCL-R	45C	270	AJ409354	*P. americanus* EST	AW013109	*P. americanus*	*83*	*4E−40*
GCL-l1	GCL-F, GCL-R	50C	244	AJ292040	Complement component C3	AB0231653	*P. olivaceus*	84	8E−29
GCL-l2	GCL-F, GCL-R	47C	232	AJ310715					
GCL-l5	GCL-F, GCL-R	47C	246	AJ409355					
GLR2-o21	GLR-F, GLR-R2	59C	409	AJ508539					
GLR2-o22	GLR-F, GLR-R2	60C	180	AJ508540					
GPX-o1	GPX-F, GPX-R	45C	342	AJ292042	Glutathione peroxidase	AF281338	*O. mykiss*	86	3E−51
GRX-l3	GRX-F, GRX-R	45C	98	AJ310511					
GRX-l4	GRX-F, GRX-R	45C	75	AJ310512					
GRX-l6	GRX-F, GRX-R	45C	129	AJ292043	Glutaredoxin	P12864	*O. cuniculus*	47	1E−03
GST-o1	GST-F, GST-R	55C	381	AJ310428	Glutathione-S-transferase	X95200	*P. platessa*	97	1E−67
H27-l1	H27-F, H27-R	45C	320	AJ310717	NADH ubiquinone oxidoreductase	AF115416	*S. salar*	85	2E−41
H27-l3	H27-F, H27-R	45C	297	AJ306230	Chromosome condensation protein	AF111423	*X. laevis*	46	7E−05
H60-l2	H60-F, H60-R	50C	370	AJ310718					
H60-l4	H60-F, H60-R	50C	402	AJ306231	α-2HS-glycoprotein	AB038690	*Pan troglodytes*	47	5E−23
H60-o14	H60-F, H60-R	45C	382	AJ306232	Histone H3.3	M11667	*Gallus gallus*	98	2E−45
H70-g1	H70-F, H70-R	50C	430	AJ306233	HSP-70	AB010871	*P. olivaceus*	99	1E−75
H90-o1	H90-F, H90-R	55C	361	AJ306234	HSP-90-beta	AF135117	*S. salar*	88	5E−33
HEM-o2	HEM-R, HEM-R	45C	343	AJ306236	T-complex protein 1	AB027708	*Carassius auratus*	92	4E−55
HEM-l2	HEM-F, HEM-R	50C	312	AJ306235	GP36B	X76392	*Canis familiaris*	89	7E−46
HEM2-l23	HEM-F2, HEM-F2	55C	485	AJ508541	Transglutaminase	S79761	*Pagrus major*	80	4E−04
HEM2-l6	HEM-F2, HEM-F2	55C	201	AJ306237	Microtubule aggregate protein	AF085251	*Perca flavescens*	55	1E−14
HRAS-g1	HR-9, HR-11	55C	206	[X90910]	Ha-ras	X90910	*P. flesus*	100	
HRAS-g3	HR-9, HR-11	55C	167	AJ310513					
JUN-l1	JUN-F, JUN-R	45C	312	AJ306239	Complement component C8 beta	AB020962	*P. olivaceus*	90	4E−43
KRAS-g1	KR6, KR7	60C	206	[Y17187]	Ki-ras	Y17187	*P. flesus*	100	
MSD-l5	MSD-R, MSD-R	50C	374	AJ291832	ADP/ATP Carrier Protein	Q09073	*R. norvegicus*	87	4E−42

Table 1 (*Continued*)

Clone	Primers	Anneal	Size	Accession	Most similar to	Accession	Species	%ID	*E*-value
MTT-o41	MTT-F, MTT-R	55C	123	AJ291833	Metallothionein	X56743	*P. platessa*	97	7E−23
MYC-o1	MYC-F, MYC-R	50C	409	AJ310514	*P. americanus* EST	AW013487	*P. americanus*	*95*	*1E−123*
NMO-o1	NMO-F, NMO-R	50C	343	AJ291834	NADP-menadione oxidoreductase	AF128816	*O. latipes*	72	8E−46
OGG-o2	OGG-F, OGG-R	45C	478	AJ310719	Zona pellucida protein C	AF128809	*O. latipes*	74	4E−49
OGG-o6	OGG-R, OGG-R	45C	445	AJ310720					
OGG-l1	OGG-F, OGG-R	45C	459	AJ291835	NMP 200	AJ131186	*H. sapiens*	86	3E−76
ORN-l2	ORN-F, ORN-R	40C	350	AJ306293	ODC	AF290981	*B. rerio*	87	2E−57
P53-g1	P53-1, P53-3	50C	294	[Y08919]	p53	Y08919	*P. flesus*	100	
P53-g3	P53-1, P53-3	50C	201	AJ306294	Cytochrome B (mitochondrial)	AF090775	*Scardinius acarnanicus*	77	1E−21
PARA-o2	PARA-F, PARA-R	50C	489	AJ292086	Paraoxonase 2	L47573	*G. gallus*	56	8E−51
PARP-o4	PARP-F, PARP-R	48C	487	AJ292087	ARD-1 *N*-acetyltransferase	X77588	*H. sapiens*	75	4E−44
PGP-g1	PGP-F, PGP-R	52C	138	AJ292082	P-glycoprotein A	X72067	*P. americanus*	100	8E−19
POL-o1	POL-F, POL-R	41C	257	AJ292083	DNA polymerase beta	D29013	*H. sapiens*	75	4E−35
PPR-o8	PPR-F, PPR-R	50C	474	AJ310515	E3-ubiquitin ligase (SMURF1)	AF169310	*X. laevis*	97	3E−20
PRX-o4	PRX-F, PRX-R	55C	337	AJ292084	Peroxiredoxin	AB010959	*C. carpio*	84	6E−50
RHO-o1	RHO-F, RHO-R	50C	382	AJ292085	Rho	AF098514	*G. gallus*	99	3E−69
SOD-o1	SOD-F, SOD-R	50C	320	AJ291980	Cu/Zn SOD	Y12236	*B. rerio*	79	3E−46
SRC-o2	SRC-F, SRC-R	45C	495	AJ310516					
SRC-l3	SRC-F, SRC-R	45C	363	AJ291981	c-src	AF052430	*X. laevis*	89	1E−59
TGF-o6	TGF-F, TGF-F	50C	313	AJ291982	40S Ribosomal protein S15a	AF220553	*P. olivaceus*	98	4E−39
TGF-l1	TGF-F, TGF-R	45C	430	AJ291984	Translation elongation factor 1-d	X66837	*X. laevis*	66	4E−46
TGF-o1	TGF-F, TGF-R	50C	237	AJ291983	Acetyl-CoA acetyltransferase	D00512	*R. norvegicus*	85	8E−32
TGF-o2	TGF-F, TGF-R	50C	180	AJ310517					
TGF-o3	TGF-F, TGF-R	50C	141	AJ310518					
TRX-o1	TRX-F, TRX-R	50C	137	AJ310519					
TUB-o4	TUB-F, TUB-R	55C	247	AJ291985	α-Tubulin	K00557	*H. sapiens*	100	5E−42
UGT-l3	UGT-F, UGT-R	50C	432	AJ291986	UDPGT	X74116	*P. platessa*	97	6E−79
UGT-o1	UGT-F, UGT-F	50C	403	AJ310721					
VIM-o1	VIM-F, VIM-F	50C	432	AJ310722	ATIC/PURH	S64492	*G. gallus*	69	8E−39
VIT-g15	VIT-R, VIT-R	48C	173	AJ309702					
VIT-o2	VIT-F, VIT-R	50C	119	AJ309703					
VTG-1	cDNA clone		1480	[AJ416327]	Vitellogenin	AJ416327	*P. flesus*	100	
HEM2-l21	HEM-F2, HEM-R	55C	563	AJ508542	*P. olivaceus* EST M12	AU090264	*P. olivaceus*	*85*	*2E−33*
HEM2-l24	HEM-F2, HEM-R	55C	92	AJ508543	Ferritin H3	AJ238012	*O. latipes*	94	2E−03
SHA1A3	SSH clone		281	AJ508725	Elastase 1 precursor	AB029755	*P. olivaceus*	*88*	*6E−52*
SHA1C8	SSH clone		381	AJ508726	NADH dehydrogenase subunit 1	AP002951	*P. bicoloratus*	96	1E−48
SHA1C9	SSH clone		476	AJ508727	Egr-1	AY029282	*Clarias gariepinus*	*91*	*2E−53*
SHA1E8	SSH clone		629	AJ508728	Int-6	AF162775	*X. laevis*	*81*	*3E−53*
SHA1G2	SSH clone		263	AJ508729					
SHA1H1	SSH clone		473	AJ508730	c-fos	U40757	*Fugu rubipres*	*93*	*1E−23*
SHA2F7	SSH clone		344	AJ508731	Antifreeze glycoprotein precursor	M55000	*Notothenia coriceps*	*91*	*7E−03*

Table 1 (*Continued*)

Clone	Primers	Anneal	Size	Accession	Most similar to	Accession	Species	%ID	*E*-value
SHA2G1	SSH clone		206	AJ508732	Type 1 keratin	Y14289	*O. mykiss*	61	2E−04
SHA2G4	SSH clone		193	AJ508733	Complement component C3	AB021653	*P. olivaceus*	*88*	*5E−08*
SHA2G7	SSH clone		171	AJ508734	*T. nigroviridis* genomic 037O01	AL250342	*T. nigroviridis*	*100*	*5E−15*
SHA3A6	SSH clone		562	AJ508735	Chitin binding protein b04	AB051629	*Bos taurus*	52	3E−06
SHA3E3	SSH clone		236	AJ508736	Trypsinogen 1 precursor	AF012462	*P. americanus*	*94*	*1E−37*
SHA3G3	SSH clone		208	AJ508737	*P. flesus* EST 19	BE638429	*P. flesus*	*100*	*8E−26*
SHT1F12	SSH clone		118	AJ508738	*P. olivaceus* EST LC11(8)	C23339	*P. olivaceus*	*92*	*1E−20*
SHT1H6	SSH clone		409	AJ508739	*F. rubipres* scaffold 421	01000421	*F. rubipres*	*90*	*5E−71*
SHT2B6	SSH clone		132	AJ508740	*T. rubipres* MBF298945.y1.gz	119288133	*T. rubipres*	*92*	*7E−17*
SHT2B11	SSH clone		370	AJ508741	*O. latipes* EST MF01SSB027G23	BJ539353	*O. latipes*	*100*	*5E−16*
SHT2E4	SSH clone		236	AJ508742	*S. salar* EST SS1-0327	BG935991	*S. salar*	*93*	*4E−31*
SHT3H2	SSH clone		534	AJ508743	α-2-Macroglobulin-2	AB026129	*Cyprinus carpio*	*88*	*8E−07*
SHT4A9	SSH clone		353	AJ508744	Cystinosin	Y15924	*H. sapiens*	39	1E−18
SHT4B5	SSH clone		352	AJ508745	Fatty-acid-binding protein	AY034789	*Fundulus heteroclitus*	*97*	*1E−07*
SHT4B12	SSH clone		295	AJ508746	*T. rubipres* MBF753429.y1.gz	113141553	*T. rubipres*	*91*	*4E−10*

Accession numbers in brackets denote *P. flesus* sequences submitted by others; primers show the names of primers used to amplify these clones; SSH clones were derived from subtractive hybridisation. In clone names, 'o' clones were derived from ovary cDNA, 'l' clones from liver cDNA and 'g' clones from genomic DNA. 'Size' denotes lengths in base pairs of the products excluding primer-derived sequences. %ID and *E*-value refer to conceptual amino acid sequences unless in italics which refer to nucleotide sequence. CYP-o049 was a subclone of our previous clone, VTG-1 was kindly donated by Prof. J.A. Craft.

cytochrome P450 1A (CYP1A) genomic clone (Williams et al., 2000) and a 1480-bp fragment of *P. flesus* vitellogenin cDNA, kindly provided by Prof. J.A. Craft.

The arraying process required optimisation with respect to labelling method, target concentration and spotting buffer composition. Comparisons were made between Smart-RACE liver cDNA populations produced from 10 Tyne-caught fish and from 10 Alde-caught fish. Data were compared from different arrays; the mean coefficient of variation using the same samples but different labelling reactions and hybridisations was 11% with a standard deviation of 4% (data not shown). Selected mRNA samples were analysed, there was no evidence of degradation, with average transcript sizes being greater than 2 kb in all cases.

Experimental comparisons were carried out using an Alde sample as the control in two-colour hybridisations. Each test sample was derived from separate individuals and was individually hybridised against the same Alde control sample. Subsequently, data derived from the Alde hybridisations were compared with data derived from the Tyne hybridisations. Male (Fig. 1) and female samples were compared separately. Differentially

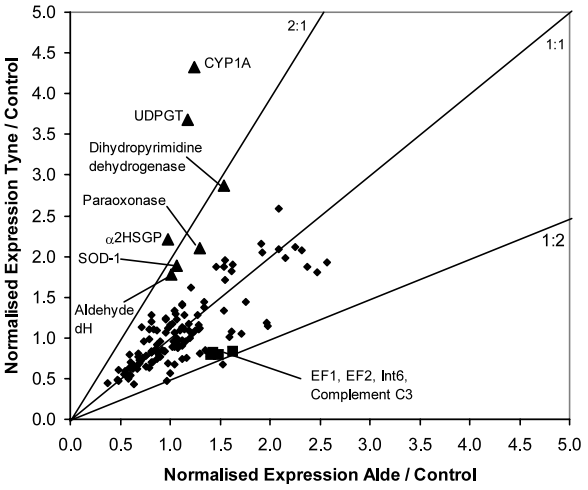

Fig. 1. Comparison of gene expression in Tyne and Alde male flounder liver by cDNA microarray. 2:1 denotes twofold expression in Tyne over Alde, 1:2 denotes twofold expression in Alde over Tyne. The labelled genes are those which showed a significant difference ($P < 0.05$) between the five Tyne liver samples and the five Alde liver samples.

expressed genes are shown (Table 2), where probability values were significant ($P < 0.05$). Although a number of genes appeared differentially expressed in the female comparisons, none of these were statistically significant, as there was a large variation in gene expression between female individuals. In comparisons using male fish, however, 11 genes showed significantly altered expression between flounder from the Tyne and Alde estuaries. Those more highly expressed in Tyne fish were CYP1A, UDP-glucuronosyltransferase (UDPGT), α-2HS-glycoprotein, dihydropyrimidine dehydrogenase, Cu/Zn superoxide dismutase (SOD), aldehyde dehydrogenase and paraoxonase. Of these, CYP1A, UDPGT and α-2HS-glycoprotein were greater than twofold more highly expressed in Tyne rather than Alde fish. Four genes showed significantly greater expression in Alde rather than Tyne fish. These were translation EF1, translation EF2, translation initiation factor 3 subunit 6 (Int-6) and Complement component C3. None of these showed greater than twofold differential expression.

The Benjamini and Hochberg multiple testing correction forecast that differences close to *P*-value of 0.05 could have been obtained by chance. We therefore selected two highly significant genes (CYP1A, $P = 9.86\text{E}-04$; UDPGT, $P = 6.74\text{E}-04$), one of lesser significance (EF1, $P = 0.002$) and one of significance close to the *P*-value of 0.05 (Cu/Zn SOD, $P = 0.038$). These were analysed by real-time quantitative PCR. As the mean ratio of Tyne/Alde α-tubulin expression was 1.002 as determined by densitometric analysis of Northern blots, the results for each sample were normalised to α-tubulin expression, then subjected to *t*-test analysis (Fig. 2). CYP1A and UDPGT were still identified as significantly more abundant and EF1 significantly less abundant in Tyne samples. Cu/Zn SOD, though higher in the Tyne samples, was not identified as statistically significant ($P = 0.46$; Table 2).

4. Discussion

Some of the degenerate primers for specific gene targets non-specifically amplified a range of dif-

Table 2
Genes differentially expressed between Tyne and Alde flounder, as determined by cDNA microarray (array) and real-time PCR analysis (PCR)

Clone	Accession	Putative identity	Array expression	Array *P*-value	PCR expression	PCR *P*-value
Upregulated > twofold in Tyne males, or significantly different from Alde males						
CYP-o049	AJ132353	CYP1A	3.5	9.86E−04	2.5	7.34E−04
UGT-l3	AJ291986	UDPGT	3.1	6.74E−04	3.1	8.84E−04
H60-l4	AJ306231	α-2HS-glycoprotein	2.3	6.74E−04	–	–
CB5-l21	AJ508535	Dihydropyrimidine dehydrogenase	1.9	0.002	–	–
SOD-o1	AJ291980	Cu/Zn SOD	1.8	0.038	1.4	ns
ALD-o1	AJ298325	Aldehyde dehydrogenase	1.8	0.007	–	–
PARA-o2	AJ292086	Paraoxonase	1.6	0.002	–	–
Downregulated > twofold in Tyne males, or significantly different from Alde males						
HEM2-l24	AJ508543	Ferritin H/M chain	2.3	ns	–	–
ORN-l2	AJ306293	ODC	2.0	ns	–	–
TGF-l1	AJ291984	EF1-delta	1.9	0.002	3.9	3.12E−05
COL-o5	AJ300776	EF2	1.8	0.030	–	–
SHA2G4	AJ508733	Complement C3	1.7	0.038	–	–
SHA1E8	AJ508728	Int-6	1.7	0.044	–	–
Upregulated > twofold in Tyne females						
UGT-l3	AJ291986	UDPGT	4.3	ns	–	–
COX-l4	AJ300777	Transferrin	4.0	ns	–	–
VTG-1	AJ416327	Vitellogenin	2.8	ns	–	–
H60-l4	AJ306231	α-2HS-glycoprotein	2.7	ns	–	–
PARA-o2	AJ292086	Paraoxonase	2.1	ns	–	–
SOD-o1	AJ291980	Cu/Zn SOD	2.0	ns	–	–
G78-g1	AJ305218	GRP-78	2.0	ns	–	–

ferent products. These were exploited to provide a greater range of targets on the array, allowing normalisation of each array to one. It is anticipated that in future, with the additional gene sequences now becoming available for fugu and zebrafish, and from various fish EST projects, the design of more specific primers for selected genes would be facilitated. Isolating gene fragments by this method is, however, a time-consuming process and, while necessary to link this study to previous biomarker investigations, our approach was supplemented by use of some initial clones produced by SSH (see Sheader et al., 2003).

For optimisation of the arraying process, we used Tyne and Alde Smart-RACE cDNA, made from pooled mRNA of 10 female fish per site. While the optimised conditions allowed a mean array-to-array coefficient of variation of 11 ± 4% standard deviation, the differential expressions derived from these experiments did not allow analysis of inter-individual variation, an essential factor to consider for environmental samples. We therefore chose to hybridise five individuals from each site against a control sample and then compare the data by group. Males and females were considered separately. Inter-individual variation was sufficiently high in females such that no statistically significant differential expression could be detected. This may be related to the observation that vitellogenin levels differed radically between the female fish, suggesting that individuals were at different stages of their reproductive cycles. Female flounder have also been found to be less responsive, in terms of biotransformation enzyme activity, to xenobiotic insult than males. Increased levels of 17-β-estradiol have been shown to downregulate benzo-(a)-pyrene (B(a)P)-induced activity of enzymes such as CYP1A (Winzer et al., 2002b). It has been recognised that the use of adult female fish in biomonitoring studies is more problematic than the use of male fish (Goksoyr et al., 1996), and our

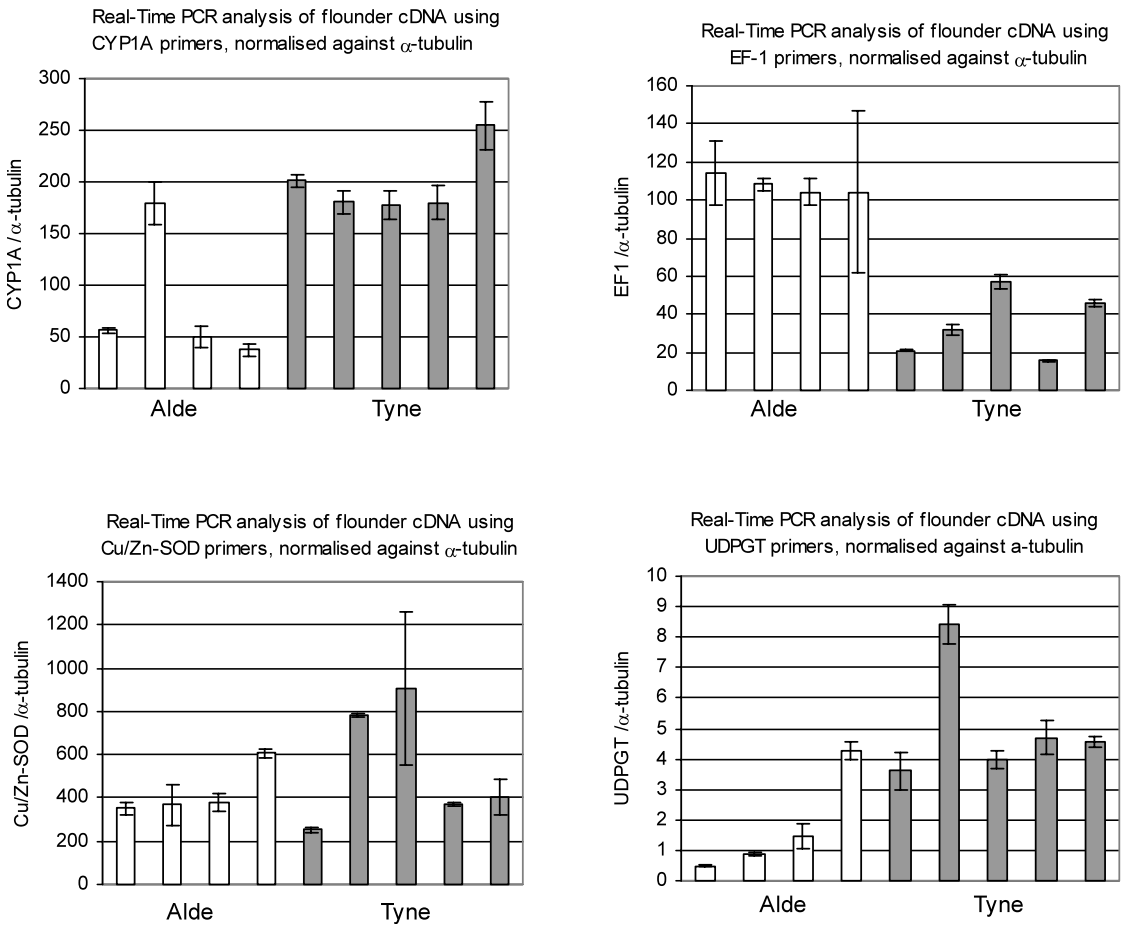

Fig. 2. Real-time PCR analysis of male flounder liver samples using four primer pairs, all normalised to α-tubulin. Clear boxes denote individual Alde samples, shaded boxes denote individual Tyne samples. Standard errors of the mean of three replicates for each sample are shown. Results are summarised in Table 2.

results concur with this finding. Of our SSH clones, Int-6 and complement component C3 showed less expression in the Tyne than the Alde fish both by array analysis and by subtractive hybridisation; the lack of reproducible response from other SSH-derived clones may be due to their isolation from female fish. We are currently using immature flounder in laboratory-based exposures.

Comparison of the results derived from microarraying and the more accurate real-time PCR showed a qualitative agreement in that the differentially expressed genes in male fish were correctly assigned as up- or downregulated. Real-time PCR also allowed us to test the Cu/Zn SOD array result of borderline significance and assign it as not statistically significant.

Of the differentially expressed genes detected in male fish, several are known to respond to organic pollutants. Cytochrome P4501A (CYP1A) is a mono-oxygenase that catalyses the phase I metabolism of a wide range of planar aromatic compounds. Its gene has been cloned and sequenced from European flounder (Williams et al., 2000) and a range of other fish species (Leaver et al., 1993; Berndtson and Chen, 1994; Roy et al., 1995; Morrison et al., 1995, 1998; Stien et al., 1998). In particular, CYP1A enzyme activity, protein quantification and mRNA quantification

have been extensively used as biomarkers in wild fish (Hahn and Stegeman, 1994; Craft et al., 2001), in which induction has been noted at polluted sites, although the extent is species-dependent (Wirgin et al., 1996; Anulacion et al., 1998). Laboratory studies have shown induction by PAHs and polychlorinated biphenyls, regulated via the Ah receptor, and the flounder CYP1A promoter has been shown to confer inducibility by 3-methylcholanthrene in a reporter gene assay in cultured cells (Williams et al., 2000). Our finding of CYP1A mRNA induction in Tyne fish by the microarray and confirmation by real-time PCR gives us confidence that classical biomarker responses are being detected by these methods. We have also shown that flounder UDPGT is elevated in male fish from the polluted site, though the possibility exists that this reflects the induction of a number of closely related UDPGT transcripts. UDPGT was also upregulated in female Tyne fish, though this was not statistically significant. Our flounder clone is most similar to UGT1B1 from plaice, which showed greatest similarity to mammalian UDPGT family 1 (George et al., 1998). The plaice enzyme is inducible by PAH compounds; however, there may be six or more UDPGT isoforms in plaice and they display considerable polymorphism (George and Leaver, 2002). The aldehyde dehydrogenases are another multigene family (Hsu et al., 1994). Our clone is most similar to human class 1 aldehyde dehydrogenase. The human class 3 gene is inducible by dioxins via the Ah receptor (Safe, 1995). In the dab (*L. limanda*), increased aldehyde dehydrogenase activity has been used as a marker for preneoplastic hepatocyte foci (Winzer and Kohler, 1998). In European flounder, aldehyde dehydrogenase enzyme activity has been used as a biomarker and was found to be inducible by B(a)P, a PAH (Winzer et al., 2002a), though this induction was less marked in females than males. This sex difference was reflected in our array data.

Paraoxonase (PON) is an esterase catalysing the hydrolysis of organophosphates and aromatic carboxylic acids, with an involvement in protection of low-density lipoprotein from oxidative stress (Primo-Parmo et al., 1996). Our transcript was most closely related to PON2 gene and was expressed at higher levels in the polluted Tyne than the Alde in both male and female fish, though only the male result was statistically significant. In immunohistochemical investigations of rat tissue, PON protein was found to be inducible by 3-MC treatment, though the isoform induced was uncertain (Rodrigo et al., 2001).

Several genes not directly related to xenobiotic metabolism were also found to be differentially regulated in male flounder. Dihydropyrimidine dehydrogenase is the rate-limiting enzyme of uracil and thymidine catabolism. It is of interest in cancer therapy where it degrades the chemotheraputic drug 5-fluorouracil. The relevance of its induction in polluted fish is uncertain, as although dihydropyrimidine dehydrogenase mRNA levels are found to increase with tumour progression in human colorectal cancer (Shirota et al., 2002); they also vary in a circadian fashion in healthy controls (Raida et al., 2002).

Three genes related to translation were found to be significantly downregulated in male Tyne flounder as compared with Alde fish. These were EF1-delta (translation elongation factor 1-delta), EF2 (translation elongation factor 2) and translation initiation factor 3 subunit 6 (Int-6). The downregulation of these transcripts in pollutant stressed fish may be related to the nutritional status of the fish. Alternatively, cellular stresses inhibit protein synthesis (Patel et al., 2002), leading to the formation of stress granules, consisting of untranslated mRNAs (Dunand-Sauthier et al., 2002). This may represent a mechanism whereby the majority of transcripts requiring cap-dependent translation are 'delayed' whereas transcripts undergoing cap-independent translation are processed preferentially (Holcik et al., 2000). Cap-independent translation is facilitated by IRES elements found in stress-linked proteins such as chaperones. Reduced expression of protein translational genes has been found in skeletal muscle of the goby under hypoxic stress, but this effect was not found in the liver (Gracey et al., 2001). EF1-delta and Int-6 have also been identified as oncogenes (Joseph et al., 2002; Crane et al., 2000).

Two components of the acute phase response were differentially expressed in male fish. Complement component C3 is a hepatocyte-derived serum

acute phase protein, known to be induced in teleosts in response to bacterial infection (Bayne et al., 2001). The activity of the complement pathway is especially high in fish (Ellis, 2001). In this study, less transcript was detected in polluted fish, which may indicate either a higher rate of infection in fish from the reference Alde site, or a suppression of the acute phase response in the Tyne fish. There is some evidence that stresses such as the heat shock response can downregulate the expression of acute phase genes (Moon et al., 1999). α-2HS-glycoprotein, a fetuin, is another major serum protein produced in the liver. Although it has also been implicated as a key protein in a variety of biological processes, in humans and rat it is downregulated during the acute phase response, mediated by interleukin-1β (Banine et al., 2000). The promoter of mouse fetuin is known to possess several metal-response elements (MREs), binding sites for metal-responsive transcription factor 1 (MTF-1), raising the possibility of its induction by heavy metal exposure (Lichtlen et al., 2001). It has not been extensively studied in fish, and differs in regulation even amongst the mammals. Its increased expression in Tyne as compared with Alde fish is of a high significance for males, it appears to have increased in Tyne females as well, but not to the level of statistical significance. Whether this is due to a suppression of the acute phase response, heavy metal exposure, or another mechanism, remains to be determined.

Cu/Zn SOD is an antioxidant enzyme, catalysing the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Its induction has been used as a biomarker of oxidative stress in fish (Bainy et al., 1996); however, its induction is modest or variable between fish species dosed with PAHs (Lemaire et al., 1996). Our Cu/Zn SOD transcript appeared elevated in both male and female fish from the polluted site, though statistically this was not significant for the female fish and was shown not to be significant for the male fish when analysed by real-time PCR.

Other transcripts upregulated in Tyne female fish, but not statistically significant, were transferrin, vitellogenin and glucose regulated protein 78 (GRP-78). Transferrin expression may be controlled at the transcriptional level and by RNA stability. This can be affected by a number of factors including oxidative stress (Gehring et al., 1999), estrogen (Denslow et al., 2001) and the acute phase response to infection (Biro et al., 1998). Vitellogenin is the classical biomarker for estrogen or xenoestrogen exposure (Christiansen et al., 1998; Denslow et al., 2001). The levels of vitellogenin transcript detected in female fish varied very widely between individuals both at the control and polluted sites. In male Tyne flounder, the expression of vitellogenin was detectable, but at too low a level to allow confidence in the data. GRP-78 is an endoplasmic reticulum chaperone related to heat shock protein 70 (HSP-70), known to be stress inducible (Halleck et al., 1997; Liu et al., 1997) and found to be induced in sponges (*Suberites domuncula*) exposed to cadmium (Schroder et al., 1999). Two transcripts expressed in lower amounts in Tyne male fish than Alde male fish, but not significantly, were ferritin and ornithine decarboxylase (ODC). Elevated ODC activity has been used as a marker of liver neoplasia in winter flounder (*Pleuronectes americanus*; Koza et al., 1993). However, brown trout (*Salmo trutta*) sampled from a site contaminated with heavy metals showed lower hepatic ODC than those from an uncontaminated site (Norris et al., 2000). Our result for ODC was not significant, which may be related to the high variation in ODC activity found between individual fish, such as medaka (*Oryzias latipes*; Calabrese et al., 1993). Our fish also exhibited no gross liver pathologies. Ferritin protein synthesis is part of the acute phase response, and oxidative stress has been found to downregulate ferritin synthesis (Gehring et al., 1999).

Before studying the effects of individual toxicants, we aimed to test the array using feral fish in order to assess the potential of this technique for environmental monitoring. The fish sampled from the polluted site have been chronically exposed to a complex mixture of toxicants, potentially developing tolerance to certain stresses, a very different situation from that of an acute laboratory exposure to a single compound. In addition, the gene expression of environmentally sampled fish may be affected by many different factors including tem-

perature, diet, salinity, reproductive status, capture stress and genetic polymorphism (Hylland et al., 1998; Rotchell et al., 2001; Belfiore and Anderson, 2001; Oleksiak et al., 2002). These complicating factors could potentially mask true biomarker responses.

The inductions of CYP1A, UDPGT, aldehyde dehydrogenase and perhaps paraoxonase suggest that flounder from the polluted Tyne are responding to PAH contamination. This corresponds well with previous studies (Lyons et al., 1999) that reported a higher incidence of bile metabolites, DNA adducts and strand breaks in Tyne flounder as compared with Alde flounder. Gene expression changes potentially related to other factors, such as metal, xenoestrogens and organophosphates, have been detected, though with less confidence. The downregulation of translation-related genes and differential regulation of acute phase genes may be indicative of a general stress response, though this is one of a number of possibilities. We are currently extending our flounder microarray with 10,000 clones derived from a normalised flounder liver cDNA library and characterising the responses of juvenile flounder to individual toxicants in laboratory exposures.

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