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Gene expression analyses of hepatocellular adenoma and hepatocellular carcinoma from the marine flatfish *Limanda limanda*

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ABSTRACT: At selected sites around the UK, the offshore sentinel flatfish species dab *Limanda limanda* are found to contain elevated levels of macroscopic liver tumors. Previous proteomic and metabolomic studies have demonstrated that differences exist between tumor and non-tumor tissues; however, these differing features were not identified, and little is known about the changes at the gene expression level, or whether prognostic markers are present and can be identified. A flounder *Platichthys flesus* custom cDNA microarray and RT-PCR were used to investigate hepatic mRNA expression in the histologically confirmed tumors, hepatocellular adenoma (HA) and hepatocellular carcinoma (HC) from dab, and in adjacent normal tissue from the same fish. Differences in gene expression were observed between tumor and normal tissues, and between tumor types. A class-prediction approach using 50 transcripts revealed sufficient group-specific expression profiles to allow segregation of samples dependent on their tumor type or the sex of the host. Vitellogenins were found to display the greatest induction (up to 500-fold induction) in some HC tumors from female fish and in both HA and HC tumors from males. To the best of our knowledge, this is the first report of the association of vitellogenin expression with tumors of wild fish.

KEY WORDS: Cancer · Dab · Genomics · *Limanda limanda* · Liver · Tumor · Vitellogenins · Marine environment · Monitoring

INTRODUCTION

Fish have a long history of use in environmental carcinogenesis research and have served as valuable alternatives to conventional rodent models and to samples collected from human tumors. They have well described tumor pathologies, are sensitive to many classes of carcinogens, are responsive to many tumor promoters and inhibitors, and develop neoplasms that are histologically similar to human cancers (Bailey et al. 1996, Stern & Zon 2003). In the marine sentinel species dab *Limanda limanda*, liver lesions recorded have been histopathologically assigned to 5 main categories: non-specific inflammatory responses, non-neoplastic toxicopathic lesions, foci of cellular alteration, benign tumors, and malignant tumors (Feist et al. 2004). Additional sub-typing results in the classification of approximately 30 categories of liver lesions under the Biological Effects Quality Assurance in Monitoring (BEQUALM) program (Feist et al. 2004). The detection of liver lesions has been incorporated into several national and international monitoring programs (Meyers et al. 1990, Vethaak & Wester 1996, Lang 2002, Lyons et al. 2004, Stentiford et al. 2009, Vethaak et al. 2009) and serves as a reliable and sensitive indicator of the health status of fish populations and, as such, the health of the environment (Thain et al. 2008). Furthermore, the use of dab as a model

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species may lead to a better understanding of the environmental factors involved in the formation of cancer, particularly since patterns of occurrence in wild fish are consistent between distinct sites and years (Stentiford et al. 2009). Using the BEQUALM classification scheme for liver histopathology, specific sample groups can be identified (e.g. 'no liver pathology observed', 'pre-neoplastic lesions', 'benign tumor', 'malignant tumor') and used to phenotypically 'anchor' samples for further bioinformatic approaches (Stentiford et al. 2005).

Little research has been conducted to investigate gene expression profiles in naturally occurring tumors of wild fish. The expression changes that are likely to occur in and between tumor sub-types may well provide the foundation for molecular classification of tumors, and provide prognostic markers that can be used as indicators of pollution-induced damage. In addition, utilization of tumor tissues from wild animals will provide additional useful information for comparison with laboratory-induced tumors, since this will likely reflect the multi-factorial nature of cancer formation in natural populations (du Corbier et al. 2005, Stentiford et al. 2005, Ward et al. 2006). DNA microarrays have been applied in many areas of biological research, including human cancer (Rhodes et al. 2004), and are increasingly being used in studies of gene expression in fish (Douglas 2006), primarily as a tool for understanding development processes and basic physiology. Recently, a custom cDNA microarray for the European flounder *Platichthys flesus* was used to detect toxic stress responses in fish from polluted estuaries (Williams et al. 2003), oxidative stress and other responses in fish exposed to cadmium (Sheader et al. 2006, Williams et al. 2006), and gene expression in response to 17-β estradiol (Williams et al. 2007). These studies demonstrate the applicability of microarray technologies and real-time RT-PCR for investigating and understanding the transcriptional responses of environmentally important sentinel fish species.

Recent pilot studies from our group, which combined the use of histopathology, proteomics and metabalomics, identified specific protein and metabolite profiles in liver tumors from dab (Stentiford et al. 2005). Subsequent studies by Ward et al. (2006) reported that proteomic analysis of dab plasma samples could also indicate the presence or absence of liver tumors and the geographic origin of the host. We report here a proof-of-concept study in which we have investigated the transcriptional profiles of liver tumors from dab, and whether transcriptional differences exist between 2 different tumor types. Histopathologically confirmed specimens were selected to create sample groups of hepatocellular adenoma (HA) and hepatocellular carcinoma (HC) and sex of fish (male or female). Information relating to the geographical origin of the samples, sex, tumor type and secondary lesions/pathologies was also recorded. Transcriptional responses were measured using a custom cDNA microarray and RT-PCR primers developed for European flounder (Williams et al. 2003). Data are discussed in relation to transcript expression profiles and the combined use of histopathological and molecular techniques in environmental carcinogenesis research.

MATERIALS AND METHODS

Sample collection. Dab were captured at Clean Seas Environmental Monitoring Programme (CSEMP) sites in the UK during June and July of 2007 using a standard Granton trawl of approximately 30 min duration. Sampling is undertaken at this time to miss the main spawning season for dab, which around the British Isles falls between April and May (Rijnsdorp et al. 1992, Fox et al. 1997). Therefore, while the fish used in this study are all sexually mature, they have been sampled outside their breeding season. Upon landing, we immediately placed the fish in flow-through holding tanks containing aerated seawater. The fish were then measured, sexed (Table 1) and examined for indicators of external diseases according to standard procedures (Bucke et al. 1996, Anonymous 2004). Fish were then sacrificed by a blow to the head followed by immediate severing of the spinal cord. In selected fish where sizeable visible liver nodules were present, the liver was removed from the fish and a small section (approx. 2 to 3 mm^3) of the tumor and adjacent non-tumor tissue were excised and immediately frozen in liquid nitrogen, and later stored at −80°C. In addition, a section was made through the same liver so as to obtain both tumor and non-tumor tissues according to the methods of Feist et al. (2004). The section was fixed in 10% neutral buffered formalin (NBF) for 24 h, and then transferred to 70% industrial methylated spirit (IMS) for subsequent histological confirmation of the lesion type.

Histopathology. Fixed liver samples were processed to wax in a vacuum infiltration processor using standard procedures (Feist et al. 2004, Stentiford et al. 2009). Sections were cut at 3 to 5 µm on a rotary microtome and the resulting sections mounted onto glass slides before staining with hematoxylin and eosin (H&E) according to standard procedures (Feist et al. 2004). Histological confirmation of lesion types was carried out using guidelines set out in the BEQUALM program (Feist et al. 2004). Lesions relevant to the current study include the benign neoplasm HA and the malignant neoplasm HC.

Expression analysis by cDNA microarray. The genomic tool for bio-monitoring of pollutant coastal

Table 1. *Limanda limanda.* Sample information and lesion categories. HC: hepatocellular carcinoma; HA: hepatocellular adenoma; bFCA: basophilic focus of cellular alteration; eFCA: eoisonophilic focus of cellular alteration; vFCA: vacuolated focus of cellular alteration; CN: coagulative necrosis; MMA: melanomacrophage aggregates; LMI: lymphocytic/monocytic infiltration; Re: regeneration; Li: lipoidosis; Fi: fibrosis; Pl: Phospholipidosis; FI: fibrilar inclusions; HNP: Hepatocellular and nuclear pleomorphism; Cefas: Centre for Environment, Fisheries and Aquaculture Science; Y: yes; N: no

Fish ID	Cefas reference	Sample site	Sex	Fish size (cm)	Principal lesion	Associated secondary lesions/pathology	Microarray analysis	RT-PCR analysis
1	RA07046-60	West Dogger	F	21	HA	CN, MMA, LMI	Y	Y
2	RA07046-64	West Dogger	F	26	HA	Pl, FI, MMA, Re	Y	Y
3	RA07046-65	West Dogger	F	25	HA	CN, MMA, LMI	Y	Y
4	RA07045-1200	North Dogger	F	23	HC	eFCA, MMA, LMI, Re	N	Y
5	RA07046-36	Off Humber	F	22	HC	vFCA, MMA, LMI	Y	Y
6	RA07046-62	West Dogger	F	27	HC	HA, bFCA, CN, Li, MMA	Y	Y
7	RA07045-470	SE Isle of Man	M	20	HA	CN, MMA	Y	Y
8	RA07046-38	Off Humber	M	22	HA	MMA	Y	Y
9	RA07046-66	Amble	M	20	HA	MMA	Y	Y
10	RA07045-111	Red Wharf Bay	M	20	HC	HNP	Y	Y
11	RA07046-52	Central Dogger	M	23	HC	vFCA, eFCA, bFCA, MMA, Re	Y	Y
12	RA07046-59	West Dogger	M	20	HC	MMA, LMI	Y	Y

impact (GENIPOL) cDNA microarray for European flounder was employed to determine mRNA expression differences between tumor and non-tumor liver samples from dab, and has been described previously (Williams et al. 2003, 2006, 2007, Diab et al. 2007). We have used this array to investigate cross-species hybridization efficiency in different flatfish species (plaice *Pleuronectes platessa* and sole *Solea solea*) (Cohen et al. 2007) and experimentally determined 79% intra-family hybridization efficiency (percentage of non-differentially, or up-regulated, transcripts). In addition, we have successfully employed the flounder array for transcriptomics in Senegalese sole *S. senegalensis*, a species that is considerably more evolutionarily distant from European flounder than the dab (Osuna-Jiménez et al. 2009).

RNA was extracted from the frozen tumor and non-tumor samples using an RNeasy Mini Kit (Qiagen) and quantified using a NanoDrop-1000 (Thermo Fisher Scientific). In selected samples, RNA quality was assessed using an Experion Bioanalyzer (Bio-Rad). Microarray experiments were carried out as previously (Williams et al. 2003, 2006, 2007, Diab et al. 2008). Briefly, 20 µg total RNA was treated to remove DNA contamination using a DNAfree kit (Ambion) and reverse transcribed using Oligo(dT) 12-18 primers (Alta Bioscience). cDNA (500 ng) was labeled with Cy5-dCTP or Cy3-dCTP (Amersham). Cy5-labeled dab tumor cDNA (60 pmol incorporated) was mixed with Cy3-labeled dab non-tumor cDNA (60 pmol incorporated). Each of the 12 hybridizations consisted of tumor cDNA and non-tumor cDNA from 1 individual fish. These were hybridized, washed and scanned (Axon 4000B; Molecular Devices). Data were captured using Genepix software (Molecular Devices).

The Genespring v7.2 software package (Agilent) was used to analyze microarray data. Clones corresponding to the same gene were considered as replicate spots. After Lowess normalization, low-trust spots were discarded. Lists of apparently differentially expressed genes were generated by finding genes that differed by more than 1.5-fold in expression in samples grouped by sex and tissue-type parameters. Statistically significant differences were determined using a parametric Welch *t*-test. The p-value cut-off was 0.05, and a Benjamini and Hochberg (Benjamini & Hochberg 1995) multiple testing correction was used, for a false discovery rate (FDR) cut-off of 0.05. For class-prediction, the K-nearest neighbour approach was employed with gene selection by Golub's method (Golub et al. 1999) incorporated within the Genespring software package. The output of this was 50 genes the expression of which was most closely associated with membership of the designated sample groups. Principal component analysis and clustering were carried out within Genespring; clustering employed a Spearman algorithm. Heat maps were generated using Genespring. Gene expression values (tumor/'normal') were compared between samples grouped by other parameters (sampling site, sex, length and secondary pathologies shown in Table 1) by *t*-test with a Benjamini and Hochberg multiple testing correction for FDR < 0.05.

Expression analysis by real-time RT-PCR. Selected transcripts (e.g. vitellogenins) were assayed by quantitative real-time RT-PCR to confirm the significance of the array results, and also to investigate other genes suggested to have biological significance in tumor formation or progression for which sequence information or primers were available (e.g. thioredoxin [THX] and glutathione-S-transferase [GST]). Total RNA was

again extracted from frozen liver tumor and non-tumor samples (see Table 1) using an RNeasy Mini Kit, as described above. cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen). A 1:10 dilution of the synthesized cDNAs was used in all subsequent real time PCR reactions. Primers previously designed for several genes from the closely related flatfish, the European flounder (Williams et al. 2003, Sheader et al. 2006) were used to measure vitellogenin-B (VTG), THX, GST, and 2 commonly used normalization genes, 18S rRNA (18S) and α-tubulin (ATUB). Samples were assayed in duplicate on a StepOne Real-Time thermocycler (Applied Biosystems). Each 20 µl PCR reaction contained the following: 10 µl Power SYBR-Green 2× PCR Master Mix (Qiagen), 400 nM of each primer, and 2 µl of a 1:10 dilution of cDNA. Thermocycling conditions consisted of an initial denaturation temperature of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 52°C for 20 s, and 72°C for 20 s. Following amplification, a dissociation profile (95°C for 15 s, 52°C for 1 min, +0.3°C min^{-1} increasing to 95°C for 15 s) was added to generate a melt-curve thermal profile to examine the amplification products. Reaction conditions and reagent concentrations were the same for the 5 primer pairs used. Threshold cycle (C_t) values obtained for 18S and ATUB transcripts from HA, HC and matching normal tissues, were compared to identify a suitable internal control for quantification. Relative quantification (Livak & Schmittgen 2001) was employed to analyze the RT-PCR data. Means (±SD) were calculated for each duplicate sample for both tumor and normal hepatic tissues, and then pooled to obtain relative expression data for each tumor type.

To further confirm the amplification of the correct target, each RT-PCR product from 2 select dab VTG/GST/THX and ATUB amplifications were purified with the Wizard SV gel and PCR Clean-Up System (Promega). Amplicons were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and products electrophoresed on an ABI PRISM 3100-Avant genetic analyser (Applied Biosystems) following standard procedures. Consensus sequences were constructed from the 2 sequencing reactions using Vector NTI Advance v10.3.0 software (Invitrogen) and the resulting sequences compared to the European flounder sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990).

RESULTS

Liver histopathology

Grossly visible liver nodules (principal lesion) were observed as masses of ≥2 mm in diameter on the upper and lower surface of the dissected liver. Liver lesions in resected dab samples were characterized as either HA or HC according to the diagnostic criteria set out in the BEQUALM program (Feist et al. 2004). HA were characterized by their distinctive border and compression of surrounding parenchyma, enlargement and increased number of blood vessels, thickened hepatocellular trabecular cords and a lack of nuclear and cellular atypia compared to the surrounding parenchyma (Fig. 1A). HC were characterized by a generally less distinctive border with the surround-

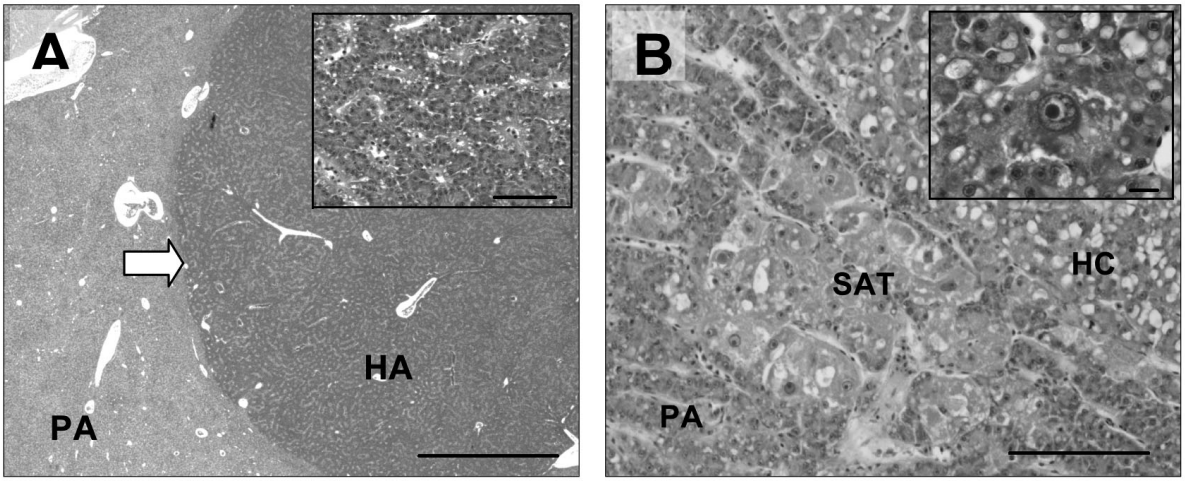

Fig. 1. *Limanda limanda.* Histopathology of liver neoplasia in dab. Discrimination of hepatocellular adenoma (HA) from hepatocellular carcinoma (HC). (A) HA with distinctive border (arrow) and compression of surrounding parenchyma (PA). Scale bar = 1 mm. Inset: architectural alteration caused by enlargement and increased number of blood vessels and thickened hepatocellular trabecular cords. Scale bar = 100 µm. (B) HC with satellite lesion at periphery (SAT) and a less distinctive border with surrounding PA. Scale bar = 100 µm. Inset: Atypical hepatocyte with hypertrophic nucleus within HC. Scale bar = 10 µm

ing parenchyma, atypical hepatocytes with hypertrophic nuclei, and the presence of multinucleate hepatocytes (Fig. 1B). In all cases, other non-specific pathologies were recorded in the surrounding non-tumor tissues (see Table 1).

Microarray analysis of mRNA expression

Data from the microarray experiments were grouped by sex and lesion type (HA or HC). Mean expression of each group of samples is shown in Tables 2 & 3 for those

Table 2. *Limanda limanda.* Transcripts significantly (false discovery rate, FDR < 0.05) induced at least 1.5-fold (fold changes in gene expression between tumor and non-tumor) in one or more groups of tumor samples. This employed the K-nearest neighbors approach with gene selection by Golub's method. GenBank: example flounder GenBank accession number; Common name: putative identity of gene. Those samples with FDR < 0.05 are shown in ***bold italics***; those with single *t*-test p-values < 0.05 are shown in **bold**. F-HA: female hepatocellular adenoma; F-HC: female hepatocellular carcinoma; M-HA: male hepatocellular adenoma; M-HC: male hepatocellular carcinoma

GenBank	Common name	F-HA	F-HC	M-HA	M-HC
DV570392	60S ribosomal protein L10	***1.62***	**2.20**	1.64	**1.57**
DV567581	3β beta-hydroxysteroid dehydrogenase type VII	**1.53**	***2.05***	1.65	**1.51**
DV565518	40S ribosomal protein S12	***1.32***	***2.19***	1.57	***1.42***
DV565793	60S acidic ribosomal protein P0 (L10E)	**1.23**	***1.88***	1.63	***1.44***
AJ605265	60S ribosomal protein L36a	***1.31***	**1.88**	1.53	***1.39***
DV566503	60S ribosomal protein L18	**1.32**	***1.88***	1.51	**1.34**
AJ605263	60S ribosomal protein L3	1.38	1.84	1.39	***1.42***
DV566031	40S ribosomal protein S20	**1.14**	**2.10**	1.52	***1.26***
DV568038	60S ribosomal protein L7a	**1.23**	1.86	1.58	***1.35***
DV570097	SSR alpha subunit	1.14	2.28	**1.24**	***1.35***
AJ605290	40S ribosomal protein S7	1.21	***1.93***	1.47	**1.39**
DV565339	40S ribosomal protein S11	**1.09**	***1.91***	1.61	***1.35***
DV565525	40S ribosomal protein S16	**1.25**	***1.89***	1.47	**1.34**
DV565753	40S ribosomal protein S25	***1.33***	***1.97***	1.42	**1.23**
DV568176	cDNA clone sim to selenoprotein P, plasma, 1a	1.22	***2.68***	0.92	1.11
AJ578009	60S Ribosomal protein L35	***1.25***	***1.79***	1.52	***1.34***
DV566652	40S ribosomal protein S19	**1.17**	**1.83**	1.57	***1.31***
DV565724	60S ribosomal protein L15	1.18	***1.80***	1.61	**1.27**
DV565343	60S ribosomal protein L29	**1.30**	***1.85***	1.47	**1.23**
AJ305219	40S ribosomal protein S8	1.15	***1.81***	1.54	***1.34***
DV568255	40S ribosomal protein S27a	**1.21**	***1.86***	1.51	***1.25***
DV565726	40S ribosomal protein S5	1.25	***1.71***	1.45	**1.42**
AJ310439	40S ribosomal protein S26	**1.28**	***1.88***	1.44	**1.22**
DV565683	60S ribosomal protein L11	***1.25***	***1.77***	1.48	***1.30***
DV565605	40S ribosomal protein S17	**1.26**	***1.87***	1.42	**1.21**
DV567339	MAP kinase-interacting serine/threonine kinase 2	1.04	***1.30***	1.67	1.73
DV565775	60S ribosomal pProtein L9	**1.15**	***1.89***	1.47	**1.22**
AJ300776	Eukaryotic translation elongation factor 2	1.11	***1.70***	1.50	**1.39**
DV565606	60S ribosomal protein L41	**1.16**	***1.71***	1.53	***1.30***
DV566097	Ubiquitin-like FUBI/ S30 ribosomal fusion protein	**1.20**	***1.84***	1.42	**1.21**
DV565723	40S ribosomal protein S28	***1.23***	**1.59**	1.43	1.37
AJ305223	60S ribosomal protein L27a (L22)	1.09	***1.80***	1.42	***1.25***
DV565446	40S ribosomal protein S18	**1.18**	***1.66***	1.39	1.32
DV565839	60S ribosomal protein L17	**1.18**	***1.68***	1.44	**1.23**
DV566429	40S ribosomal protein S21	1.15	***1.86***	1.32	1.21
DV565305	60S ribosomal protein L13A	**1.24**	***1.69***	1.28	**1.29**
DV565555	60S ribosomal protein L28	1.08	***1.72***	1.47	**1.24**
AJ606078	60S ribosomal protein L10a	1.12	***1.72***	1.39	**1.25**
DV565296	60S ribosomal protein L22	1.10	***1.82***	1.32	1.23
DV565427	60S ribosomal protein L37a	**1.30**	***1.63***	1.27	***1.26***
DV565885	60S acidic ribosomal protein P1	***1.26***	1.74	1.33	**1.13**
DV565344	40S ribosomal protein S27; metallopanstimulin 1	**1.10**	***1.67***	1.35	***1.26***
DV566231	40S ribosomal protein S23	***1.25***	**1.62**	1.31	**1.17**
DV565359	60S ribosomal protein L34	0.99	1.55	1.49	***1.30***
DV565926	16S mitochondrial ribosomal RNA	**1.58**	***1.59***	**1.32**	0.76
DV565533	40S ribosomal protein S14	1.14	***1.58***	1.19	***1.21***
DV565731	40S ribosomal protein S27	0.99	***1.52***	1.34	1.16
DV565372	Protein transport protein SEC61 gamma subunit	1.05	***1.59***	1.05	1.05

Table 3. *Limanda limanda*. Transcripts significantly (FDR < 0.05) repressed at least 1.5-fold (fold changes in gene expression between tumor and non-tumor) in one or more groups of tumor samples. GenBank: example flounder GenBank accession number; Common name: putative identity of gene. Those samples with FDR < 0.05 are shown in ***bold italics***; those with single *t*-test p-values < 0.05 are shown in **bold**. See Table 2 for abbreviations

GenBank	Common name	F-HA	F-HC	M-HA	M-HC
DV565619	Apolipoprotein A-IV	***0.65***	1.61	0.99	0.96
DV568183	Cytochrome b5	1.11	***0.62***	1.13	1.15
DV566197	Alanine-glyoxylate aminotransferase	1.06	***0.60***	1.39	0.81
DV567002	Similar to onzin	1.00	***0.63***	**1.25**	0.89
DV565297	C-type lectin 2-1	**1.19**	***0.66***	0.89	1.01
DV569985	Actin-related protein 2/3 complex	***0.66***	**1.04**	1.07	0.95
EC378548	Glyceraldehyde 3-phosphate dehydrogenase	0.89	***0.57***	0.84	1.13
DV567117	Ceruloplasmin precursor (fFerroxidase)	0.97	***0.60***	0.94	0.81
DV566228	Beta-hexosaminidase alpha chain precursor	0.94	***0.46***	1.03	0.89
DV565380	High choriolytic enzyme 1 precursor	***0.76***	1.00	0.51	1.03
AJ578044	Cytochrome c oxidase polypeptide II	0.96	0.50	0.99	***0.84***
DV566124	Cytochrome c oxidase polypeptide III	0.97	0.60	0.93	***0.77***
DV567956	Selenide water dikinase 2	1.01	***0.64***	0.79	***0.83***
DV565511	Putative ISG12 protein	0.91	0.72	0.98	***0.63***
AJ580013	Cathepsin L	***0.49***	0.86	0.90	0.95
DV567061	Prothrombin precursor	1.05	***0.66***	0.72	0.76
DV565348	Hepcidin-like precursor	0.92	***0.73***	0.61	0.91
DV565688	Hepcidin precursor	0.91	***0.69***	0.65	0.91
DV565968	Chymotrypsinogen 2	***0.74***	1.14	0.48	0.69
AJ508542	MHC II invariant chain	1.22	***0.40***	0.73	0.69
DV570186	UDP-glucuronosyltransferase 2A1	***0.66***	1.05	0.68	0.59
AJ310423	Apolipoprotein AI	***0.80***	***0.51***	0.79	0.83
AJ310418	Fibrinogen gamma	0.80	***0.57***	0.68	0.87
AJ508737	Fibrinogen alpha	0.84	***0.61***	0.60	0.83
DV565399	Kininogen 1	**0.82**	***0.55***	0.65	0.82
AJ605266	Alpha-1-antitrypsin	0.73	***0.50***	0.76	0.83
AJ605271	Cytochrome P450 monooxygenase CYP2K6	0.87	0.56	***0.64***	0.73
DV565529	Cytochrome c oxidase subunit I	0.88	0.36	**0.85**	***0.68***
DV567824	NADH dehydrogenase subunit 4	0.84	0.56	**0.70**	***0.66***
DV565300	Carboxypeptidase A1	***0.58***	0.89	0.33	0.92
DV566497	Vacuolar-proton-APTase subunit M9.2	**0.62**	***0.66***	0.66	**0.75**
AJ132353	CYP1A Cytochrome P450 1A	0.74	***0.36***	0.83	0.72
AJ508743	Alpha-2-macroglobulin	***0.62***	**0.55**	0.65	0.79
DV567349	cDNA clone lithmor74 similar to fFibronectin 1b	**0.70**	***0.44***	0.72	**0.60**
DV565675	Liver-expressed antimicrobial peptide 2A	***0.65***	**0.61**	0.59	0.58
AJ543346	Bile salt-activated lipase (BAL)	0.96	**0.63**	**0.34**	***0.40***
DV567212	cDNA clone similar to microsomal GST-3	0.33	***0.31***	**0.59**	0.51

transcripts statistically significantly induced or repressed at least 1.5-fold in one or more comparisons between tumor and non-tumor tissues (see Figs. 2 & 3 for heat maps displaying variation within sample groups). Fish ID no. 4 (see Table 1) showed high hybridization to negative controls in the HC tumor sub-sample and was therefore eliminated from further analysis as it was considered to be contaminated. For female HA samples, 107 genes changed by 1.5-fold or more, and 35 were statistically significant at FDR < 0.05, for female HC 574 1.5-fold and 77 significant, male HA 199 1.5-fold and 7 significant, male HC 84 1.5-fold and 45 significant; these included unidentified expressed sequence tags (ESTs) not shown in Tables 2 & 3.

Of those transcripts analyzed, the expression of vitellogenins A and B showed the greatest induction in tumor compared to non-tumor tissue; however, the expression of these genes was highly variable between individuals (see Table 4, Fig. 6), and not statistically significant at FDR of 0.05. Choriogenin L and reticulon 1 transcripts, which we have previously found induced in response to estrogen treatment in flounder (Williams et al. 2007) were induced in female HC samples, though again not significantly due to high variability (Table 4). The group of transcripts that showed most statistically significant changes was those encoding several ribosomal proteins, which were generally, but not highly, induced.

Class prediction via principal component analysis allowed us to find a subset of genes the expression of which could be used to classify samples into their respective tumor 'type' (either HA or HC; see Fig. 4). The genes that were most predictive of group membership are shown in Table 5. Similarly, samples could

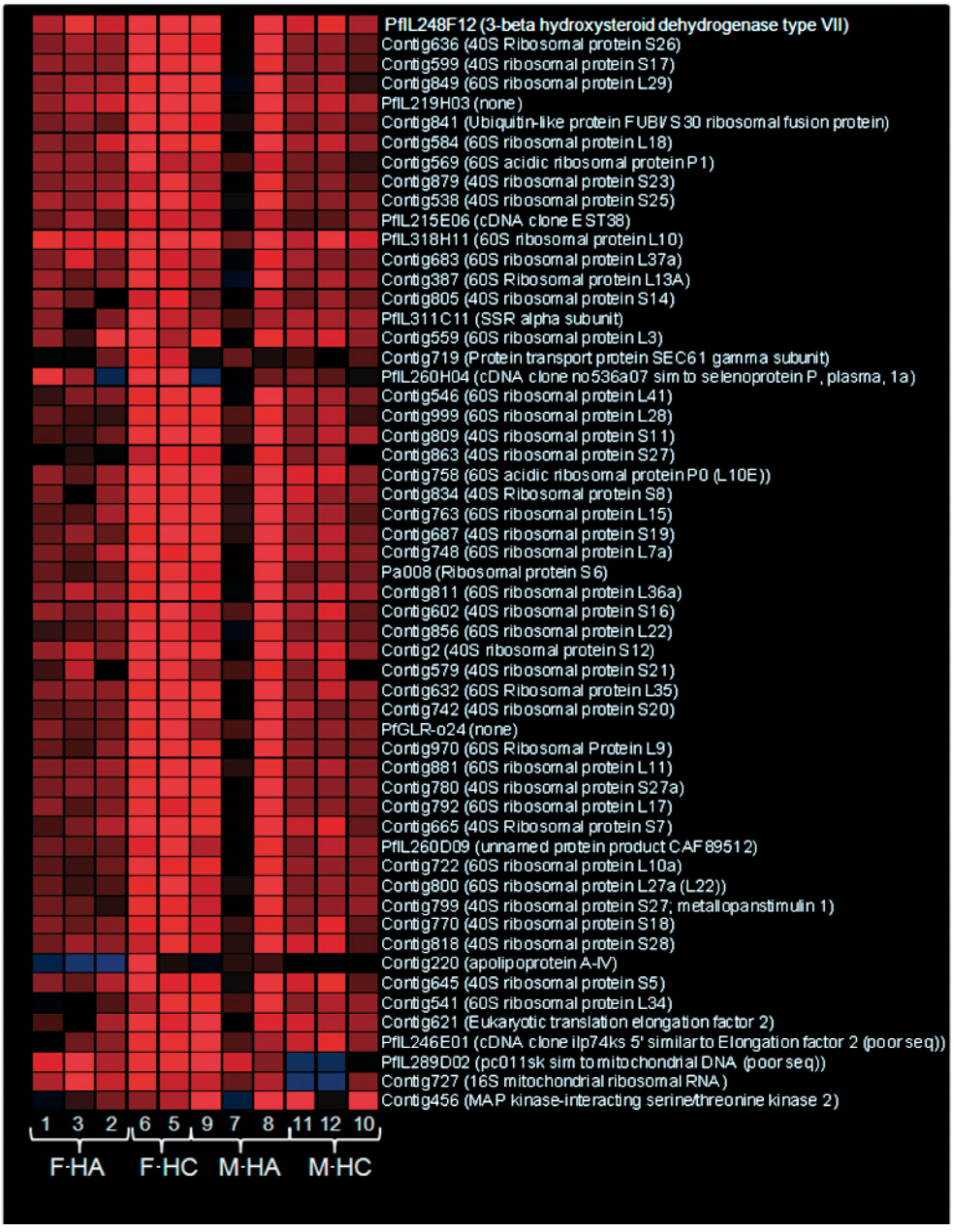

Fig. 2. *Limanda limanda*. Pattern of transcript expressions from Table 2 (induced at least 1.5-fold; false discovery rate, FDR < 0.05). Red coloration indicates a higher expression in tumor than normal tissue; blue indicates a lower expression in tumour than normal. Intensity of color illustrates fold change with maximum color intensity at 2-fold change. Unchanging genes are shown as black. 1–12: Fish ID nos., see Table 1; F-HA: female hepatocellular adenoma; F-HC: female hepatocellular carcinoma; M-HA: male hepatocellular adenoma; M-HC: male hepatocellular carcinoma

also be grouped by sex of the host or by both sex and tumor type (data not shown).

Six transcripts, including 3 unidentified ESTs, were found to be significantly influenced (fold changes in gene expression between tumor and non-tumor; FDR < 0.05) by the presence or absence of select secondary pathologies present in the liver tissues surrounding the principal lesion (Table 6). Fish sex, size and sample location had no significant influence on transcript expression.

RT-PCR analysis of mRNA expression

Melt curve and agarose gel analysis of dab RT-PCR products indicated that, as expected, only a single reaction product was produced using the European

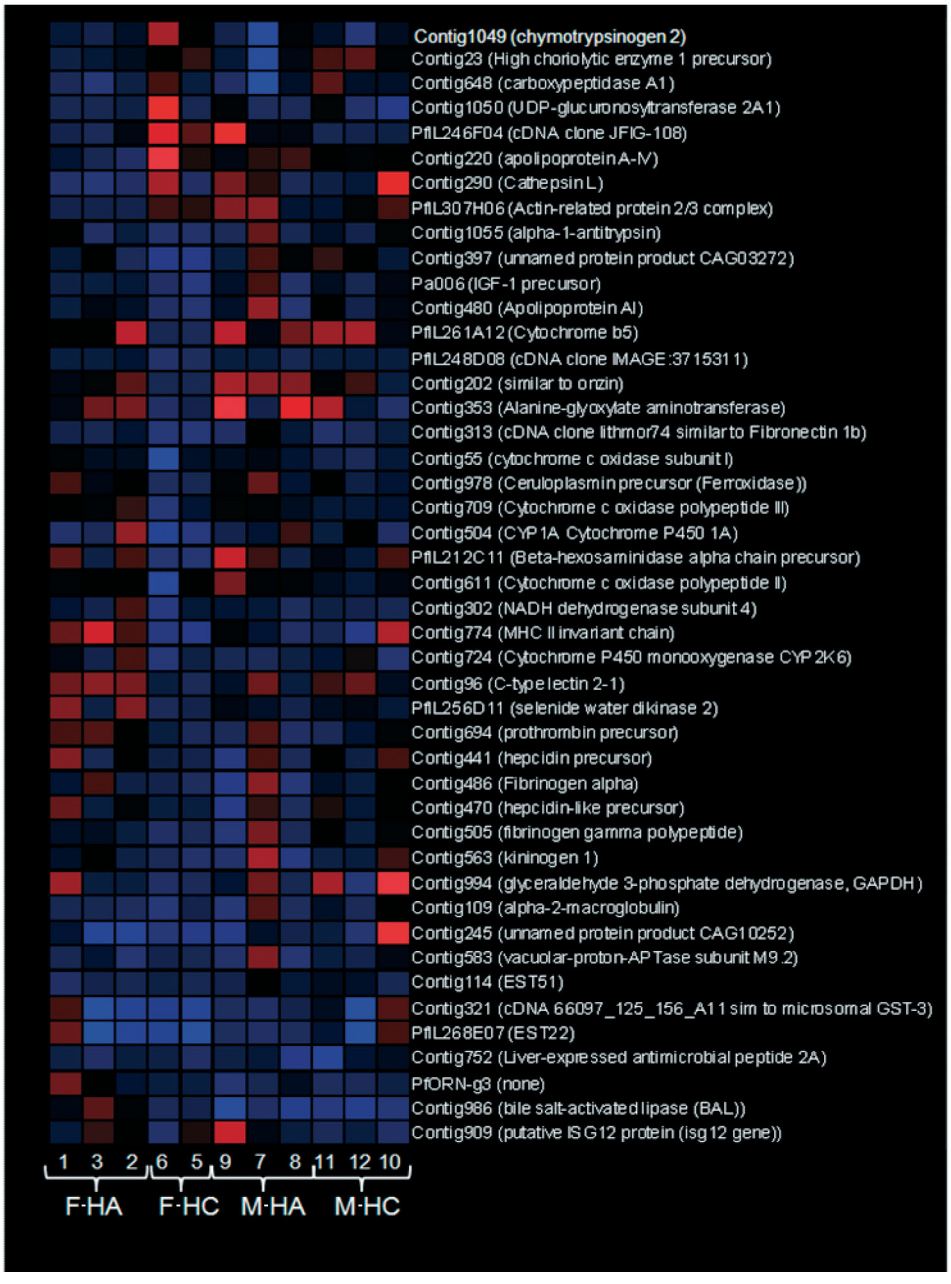

Fig. 3. *Limanda limanda.* Pattern of transcript expressions from Table 3 (repressed at least 1.5-fold; false discovery rate, FDR < 0.05). See Fig. 2 for explanations and abbreviations

flounder primers for VTG, THX, GST, and ATUB. Sequencing and BLAST analysis of select dab RT-PCR products confirmed that the correct target was amplified (VTG: 98% similar to *P. flesus*; THX: 94% similar to *P. flesus*; GST: 84% similar to *P. flesus*; ATUB: 89% similar to *P. flesus*). Comparison of C_t values from ATUB and 18S amplifications for HA, HC, and matching normal samples showed that ATUB transcripts were markedly up-regulated in both HA and HC tumor samples, compared to matching normal tissues (Fig. 5). Expression values were therefore normalized using a single reference gene (18S mRNA), a method well accepted in systems lacking a panel of well characterized multiple reference genes (Bustin et al. 2005). RT-PCR results for VTG-B confirmed the microarray findings. There was agreement in the array and RT-PCR data, in terms of increased or decreased VTG expression between sample groups (Fig. 6), and in some samples considerable induction of VTG (up to 262-fold induction when compared to surrounding

Table 4. *Limanda limanda.* Transcripts induced or repressed 5-fold or more (fold changes in gene expression between tumor and non-tumor) in one or more groups of tumor samples. GenBank: example flounder GenBank accession number; Common name: putative identity of gene. Those samples with single *t*-test p-values < 0.05 are shown in **bold**. See Table 2 for abbreviations

GenBank	Common name	F-HA	F-HC	M-HA	M-HC
DV567998	Vitellogenin A	3.43	343.32	8.73	9.57
DV567625	Vitellogenin A	3.11	208.33	6.99	7.39
AJ416327	Vitellogenin B	**5.19**	187.91	7.15	3.77
DV565941	Choriogenin L	1.30	10.32	0.96	0.88
DV567933	Reticulon 1	1.06	6.96	0.86	1.27
DV568108	C-type lectin 1	1.13	0.18	1.03	0.68
DV568963	C-type lectin 1	1.11	0.16	0.95	0.63
DV565329	Glutathione S-transferase GST-A	0.79	0.14	1.00	**0.64**
X95199	3′ untranslated region of GST-A	0.64	0.19	0.99	0.66

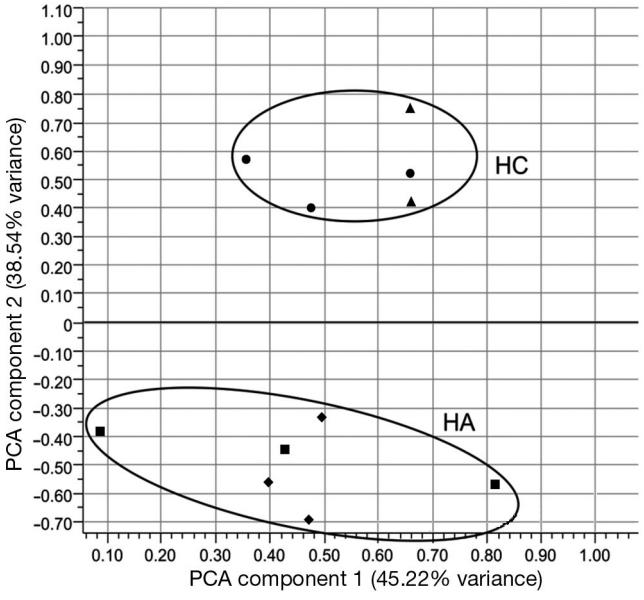

Fig. 4. *Limanda limanda.* Principal components analysis of the expression of 50 mRNA transcripts best able to predict the tumor type of the hepatocellular carcinoma (HC; n = 5) and dab hepatocellular adenoma (HA; n = 6) samples (see Fig. 1 for abbreviations). ■: M-HA; ◆: F-HA: ●: M-HC; ▲: F-HC

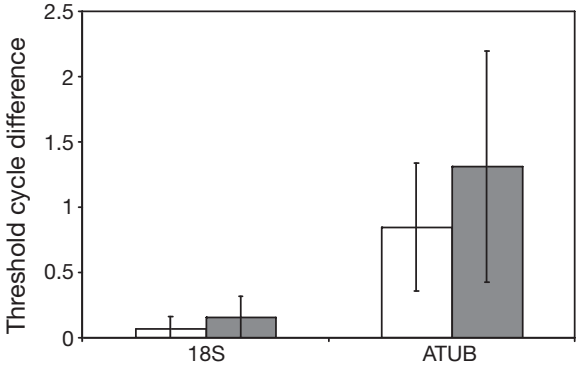

Fig. 5. *Limanda limanda.* Comparison of threshold cycle (C_t) differences observed between matched normal and tumor samples HA (open columns) and HC (filled columns) for 2 commonly used control genes (18S rRNA and α-tubulin, ATUB). The graph shows the mean C_t (±SD) for each lesion type (n = 6)

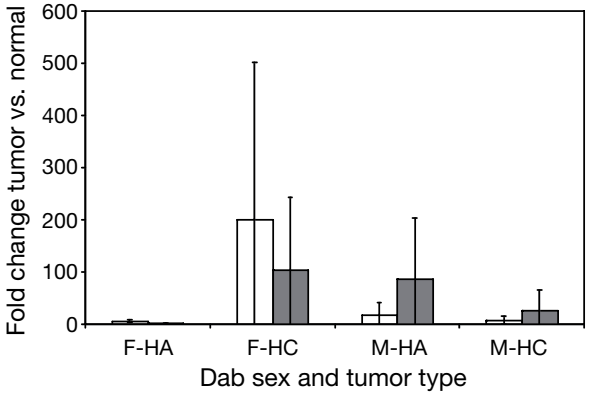

Fig. 6. *Limanda limanda.* Vitellogenin-B (VTG-B) mRNA expression analysis in female (F) and male (M) dab HA and HC by microarray (open columns) and RT-PCR (filled columns). See Fig. 1 for abbreviations. n = 3 per group (±SD), apart from microarray analysis of F-HC where n = 2

normal hepatic tissue). However, as observed with the array data, there was substantial variation in VTG expression between samples from the same group (sex of host and tumor type). There was a considerable increase in mean VTG expression in 2 out of 3 female HC samples (262- and 50.3-fold increase, tumor vs. normal) and in 2 of the male HA (219.6- and 42.1-fold increase, tumor vs. normal) and HC (71.8- and 6.7-fold increase, tumor vs. normal) samples. Female HA samples showed little induction of VTG (2.6, 2.3 and 2.4). No significant differences were observed between tumor types in either female or male samples for GST or THX (Fig. 7).

Table 5. *Limanda limanda.* Transcripts whose expression (fold changes in gene expression between tumor and non-tumor) best predicts class membership when grouped by tumor type, with expression values in each group of samples. GenBank: example flounder GenBank accession number; Common name: putative identity of gene. NA: not available; none: clones with no similarity (at BLAST *E*-value < 1×10^{-6}) to known sequences. See Table 2 for abbreviations

GenBank	Common name	F-HA	F-HC	M-HA	M-HC
DV569643	Translocon-associated protein, delta subunit precursor	0.94	1.47	0.88	1.11
AY156727	GST alpha	0.97	0.63	1.00	0.78
DV565765	Apolipoprotein D precursor	0.79	1.41	1.02	1.24
DV565318	Trypsinogen 2 precursor	0.43	0.71	0.28	0.90
AJ306239	Complement component C8 beta chain precursor	0.93	1.18	0.69	1.26
DV566243	Isochorismatase domain containing 2	1.08	0.72	0.89	0.81
DV565628	cDNA clone CNB74-B11	0.97	0.71	0.94	0.81
DV568443	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase precursor	1.10	1.26	0.97	1.09
DV565380	High choriolytic enzyme 1 precursor	0.76	1.00	0.51	1.03
DV566614	NHP2 non-histone chromosome protein 2-like 1	1.16	0.87	1.43	1.07
DV567812	RNA 3′-terminal phosphate cyclase-like protein	0.91	1.00	0.67	1.03
DV565825	X-box binding protein 1B	0.75	3.35	0.98	1.35
DV569697	40S ribosomal protein S15A	0.96	1.69	1.17	1.44
DV570302	cDNA clone CGX46-A10	0.78	1.28	0.92	1.06
DV565349	Glutathione peroxidase 1	1.08	0.92	1.51	0.78
DV565962	cDNA clone CR726539	1.30	0.93	1.17	0.85
DV568963	C-type lectin 1	1.11	0.16	0.95	0.63
DV565529	Cytochrome c oxidase subunit I	0.88	0.36	0.85	0.68
DV565300	Carboxypeptidase A1	0.58	0.89	0.33	0.92
DV566124	Cytochrome c oxidase polypeptide III	0.97	0.60	0.93	0.77
DV565583	High choriolytic enzyme 1 precursor	0.96	1.12	0.54	1.05
DV568965	Trypsinogen 2 precursor	0.53	0.84	0.28	0.99
DV565421	Trypsin	0.66	0.89	0.40	0.91
NA	Complement component C4B	0.79	0.95	0.66	1.07
DV565298	Reticulon-1	0.82	3.30	0.77	1.19
DV565467	Bromodomain-containing 3	1.01	0.92	1.03	0.88
DV565448	Mitochondrial ATP synthase c-subunit (P3)	1.25	0.88	1.12	1.00
DV565632	FAD-synthetase (Pp591)	1.03	1.18	1.05	1.16
EC377685	STEAP family member 4	0.89	0.94	0.74	0.98
DV565984	Interleukin-8 variant	1.07	0.74	0.93	0.89
DV566096	None	1.24	1.06	1.13	1.12
DV566112	cDNA clone JFConA425F	0.99	1.13	0.92	1.02
DV566160	Survivin 1	1.15	0.91	1.13	0.94
DV566675	None	0.67	0.97	0.65	0.99
DV566685	Tryptophan 2,3-dioxygenase	1.24	1.57	0.94	1.75
DV567152	Hypothetical protein zgc:77713	1.07	1.50	0.83	1.02
DV567855	Hypothetical protein Q6PBK5	1.07	1.18	1.02	1.19
EC378563	Intraflagellar transport protein 20	0.98	1.23	0.91	1.10
DV568108	C-type lectin 1	1.13	0.18	1.03	0.68
DV568416	Mitochondrial citrate synthase precursor (CS)	1.54	0.74	1.05	0.96
EC378950	Very large inducible GTPase 1	0.92	1.26	0.99	1.11
DV568905	None	1.15	0.73	1.03	0.85
DV569328	Rag C (Ras-related GTP binding C)	1.04	0.88	0.95	0.90
DV569295	AT rich interactive domain 1A	0.91	0.87	0.95	0.85
EC379169	Cut-like 1	0.97	1.22	1.03	1.20
DV569485	Leukotriene B4 12-hydroxydehydrogenase	1.13	1.26	1.05	1.26
EC379219	RNA binding motif protein 25	1.09	0.89	0.96	0.86
DV569644	Aldose reductase-related protein 2	0.86	0.70	0.85	0.70
DV570097	SSR alpha subunit	1.14	2.28	1.24	1.35
DV570352	Hypothetical protein zgc:73259	1.24	0.87	1.31	1.12

DISCUSSION

In this proof-of-concept study, the microarray and RT-PCR data were characterized by high biological variability between samples within the same group (host sex and tumor type), which is not unexpected, as no 2 environmentally induced tumors are expected to have exactly the same etiology, especially as the fish used for the trial were of different lengths and captured from different locations around the UK coastline. Nevertheless, the analyses did indicate that gene expression differences exist between HA and HC from

Table 6. *Limanda limanda.* Gene expression differences (alterations at FDR < 0.05 in fold changes between tumor and non-tumor samples) associated with (–) absence or (+) presence of certain secondary pathologies in the liver tissue surrounding the principal lesion (HA or HC). GenBank: example flounder GenBank accession number; Common name: putative identity of gene; T: tumor; N: normal; none: clones with no similarity (at BLAST *E*-value < 1×10^{-6}) to known sequences. See Tables 1 & 2 for abbreviations

Secondary lesion/ pathology	GenBank	Common name	Mean expression T/N without pathology (–)	Mean expression T/N with pathology (+)
CN	DV570132	None	0.893	1.304
vFCA	EC378836	Small nuclear RNA activating complex, polypeptide 3	1.093	1.303
	EC378277	Cross-immune reaction antigen PCIA1	0.972	1.232
	DV567954	RNA polymerase I polypeptide D	1.048	1.627
	EC378552	Unnamed protein product CAG00637	1.028	1.275
bFCA	DV567398	cDNA clone CLJ77-B05	1.077	0.793
	DV568855	None	1.003	0.636

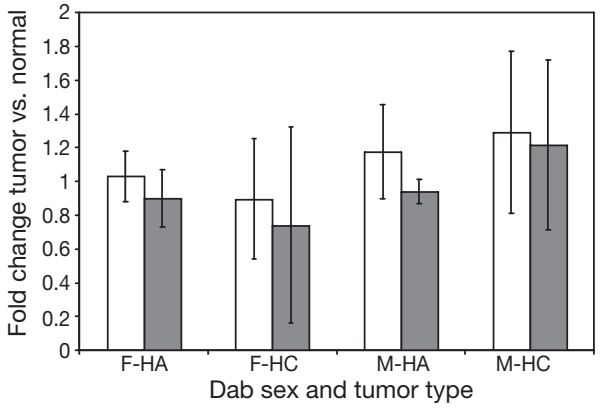

Fig. 7. *Limanda limanda.* Glutathione-S-transferase (GST; open columns) and thioredoxin (THX; filled columns) mRNA expression analysis in female (F) and male (M) dab HA and HC by RT-PCR. See Fig. 1 for abbreviations. n = 3 per group (±SD)

both female and male dab, and, conversely, that the tumors were correctly classified according to histopathological diagnostic criteria. As such, the data may begin to unearth clues as to the environmental pressures driving flatfish tumor progression. Understanding such drivers is essential when using fish disease as a prognostic marker to environmental health (Thain et al. 2008, Stentiford et al. 2009). In addition, the present study highlighted candidate mRNA transcripts for further study (see Table 5), such as VTG, choriogenin L, and reticulon 1, whose association with tumors of wild fish has, to the best of our knowledge, not been previously documented.

The class prediction approach using the cDNA array results indicated that within this particular data set there were sufficient group-specific transcript expression profiles to allow segregation of samples dependent on their tumor type (see Table 5). The differences between benign (HA) and malignant (HC) liver tumor expression profiles are likely due to differences in their growth and invasive properties. Recent studies assessing retinoblastoma allele status in tumors (HC and HA) and normal liver tissues of dab sampled from similar geographical locations as the present study have also indicated that it is possible to use genetic information to predict sample phenotype (Rotchell et al. 2009). Due to the relatively low replicate numbers (n = 5, 6) and high inter-individual variability, the specific groups of 'predictive genes' will require further testing with an increased number of additional samples to fully validate these transcriptional signatures. Future studies may also include an analysis of whether the numbers of predictive genes can be reduced, or whether early non-neoplastic lesions such as foci of cellular alteration, which are considered early stage indicators of tumor formation in flatfish (Kohler et al. 1992), display similar transcriptional profiles to those of HA or HC. Nonetheless, our study implies that mRNA expression profiles are of utility in determining and delineating between hepatic tumors in dab, and potentially other cancers of fish.

Six transcripts were found to be significantly influenced by the presence or absence of select secondary pathologies present in the liver tissues surrounding the principal lesion (see Tables 1 & 6). It would appear that in the presence of either coagulative necrosis or a vacuolated focus of cellular alteration 5 transcripts are induced, while the presence of a basophilic focus of cellular alteration results in a repression of 2 transcripts. However, the 'non-tumor' materials were sampled from the tissues surrounding the principal lesion and may have contained secondary lesions, thus influencing the results. In addition, other factors, pathologies and parasites not present in the original tissues section, but present in the remaining liver tissues, may also have influenced gene expression prior to sample resection.

The array data indicated that there was high statistical confidence in induction (though to a modest extent)

of several ribosomal proteins in tissues of both tumor types. This is not unexpected, since induction of the protein synthetic apparatus would be required in proliferating cells within tumors. Nonetheless, we were able to show that normalization of RT-PCR data to 18S mRNA rather than ATUB (the only 2 reference genes used in prior flatfish RT-PCR studies) was appropriate, and gave comparable data to the array results, albeit with some variation (discussed below). Other orthologous genes significantly changing in expression and known to be involved in carcinogenesis included cathepsin L, 3β-hydroxysteroid dehydrogenase type VII, and a cDNA clone similar to selenoprotein P. Cathepsin L is a protease that is believed to be secreted by cancerous cells to facilitate tumor invasion and metastasis by degrading the components of the extracellular matrix. It has been documented to be up-regulated in a range of human cancers (Chauhan et al. 1991) and in HC in rainbow trout (Tilton et al. 2005). In humans, the closely related 17β-hydroxysteroid dehydrogenase is responsible for sex steroid metabolism (conversion of active estradiol to the less potent estrone) in normal and tumor tissues. It has been found to be up-regulated in breast tumors (Oduwole et al. 2004) and, conversely, has also been found to be reduced in colonic tumors (English et al. 1999). The apparent increase in expression of 3β-hydroxysteroid dehydrogenase in HC from female fish may indicate estrogen exposure and subsequent estrogen inactivation, and may be an important mechanism in the pathogenesis of HC. Selenoprotein P is known to bind selenium and protect against oxidative stress, and has previously been found to be down-regulated in murine and human tumors (Calvo et al. 2002); however, in our studies a transcript similar to this was moderately up-regulated in HC from female fish and may have a different function in hepatic tumors in dab.

VTG is a large serum glyco-lipo-protein and serves as the major precursor to the egg yolk proteins of oviparous vertebrates. It is synthesized and secreted by the liver, and is normally undetectable in the plasma of immature females and male fish. Classically, VTG has been measured as a biomarker for estrogen exposure (Christiansen et al. 1998, Denslow et al. 2001, Larkin et al. 2003), and elevated levels have recently been documented in flounder populations from some estuaries (Kleinkauf et al. 2004) and in dab from offshore sites (Scott et al. 2007), indicating that these fish may well suffer from endocrine disruption. Further support for this hypothesis comes from Stentiford & Feist (2005), who reported intersex (ovotestis) in dab from the North Sea, and suggest that these fish may be exposed to endocrine-disrupting chemicals. In addition, elevated levels of VTG have also been recorded in the serum from brown bullhead *Ameiurus nebulosus* with liver tumors (HA and HC) collected from a contaminated industrial site (Heppell et al. 1995). Interestingly, Tilton et al. (2005) observed that VTG transcription was not elevated in aflatoxin B_1-induced liver HC from rainbow trout. However, in a subsequent study investigating the effects of 17β-estradiol upon gene expression in aflatoxin B_1-induced liver HCs, they recorded substantial up-regulation of VTG transcripts (Tilton et al. 2007). In addition, several other genes that have previously been found in response to estrogen treatment of flounder (Williams et al. 2007) were also up-regulated in female dab with HC. These included choriogenin L (a precursor protein of the egg envelope) and reticulons (a group of integral membrane proteins that are implicated in cellular processes including apoptosis and axonal regeneration). In combination with VTG induction (above), these transcriptional responses indicate that hepatocarcinogenesis of wild dab is influenced through estrogenic mechanisms, as has been suggested for hepatocarcinogenesis of flounder inhabiting Dutch and German waters (Vethaak & Jol 1996, Köhler 2004, Vethaak et al. 2009). Whether anthropogenic (exogenous) endocrine-disrupting sources can contribute to the high prevalence of liver tumors observed in European flatfish warrants further study, particularly since the highest tumor prevalence in dab observed at offshore sites around the UK (e.g. Dogger Bank) coincides with those sites where intersex and elevated VTG expression have previously been reported (Stentiford & Feist 2005, Scott et al. 2007, Stentiford et al. 2009).

Differences observed between the array and RT-PCR data (such as the VTG results) are most likely explained by the simple fact that RT-PCR is more sensitive than the microarray, especially with regard to quantification of low-level transcripts (such as VTG in male fish). Other contributing factors may include our use of separate RNA preparations (from the same tumor and normal liver samples) for the array and RT-PCR analysis. The possibility therefore exists that RNA extracted from different subpopulations of the same tumor sample had different expression profiles. Several studies investigating human melanoma and breast cancer cell populations have reported heterogeneity of genetic alterations and gene expression in different regions from the same tumors (Wild et al. 2000, Goidin et al. 2001, Zhu et al. 2003), and it is reasonable to assume that different portions of hepatic tumors contain dissimilar expression profiles, such as at the periphery of an invasive tumor versus the centre of the same lesion. Alternatively, as 2 different methods were used in the reverse transcription step (the array used Oligo(dT), whereas the QuantiTect Reverse Transcription Kit used a mixture of Oligo(dT) and random primers), this may have influenced the

efficiency of priming, leading to an imbalance of certain transcripts.

The differences observed in VTG expression between samples from the same tumor groups are probably due to the use of fish sampled from different locations. Although sample sizes were small, it is worth noting that the HA from female dab sampled from the same geographical location (West Dogger; see Table 1) had the smallest within-group expression and variation in VTG induction, when compared to the other sample groups (F-HC, M-HA, M-HC) from dab captured from different locations. This may suggest that geographical location of capture (and therefore potential exposure to carcinogens and/or promoters, or differences in the genetics of the host) may be important factors for tumor initiation and progression, and the resulting transcriptional profiles obtained in this study, and warrants further study. This hypothesis is supported by the recent demonstration that not only are disease profiles in dab distinctive between geographic locations, but also that these patterns are maintained over time, suggesting some biological basis to the pattern (Stentiford et al. 2009). Alternatively, exposure to potential carcinogens and/or promoters may occur while juvenile dab inhabit coastal waters until they reach 2+ yr when they may move offshore to join adult populations (Henderson 1998). Recently developed microsatellite markers for dab will undoubtedly add to our understanding of the underlying genetic differences between geographically isolated populations (Tysklind et al. 2009).

VTG transcripts detected in female European flounder have previously been shown to vary widely between individuals (Williams et al. 2003). Ward et al. (2006) also reported considerable variation in plasma proteomic features in dab with HC when compared to dab with HA tumors. In addition, these authors noted significant differences in the plasma proteome in dab from different geographical locations (the North and Irish Seas). Likewise, Stentiford et al. (2005) reported that unsupervised analysis of proteomic and metabolomic profiles from dab liver tumors and normal tissues indicated that fish-to-fish variability exceeded the differences between non-tumor and tumor samples from the same fish. Using supervised analysis (using partial least-squares regression) the authors were able to separate both the proteomic and metabolomic non-tumor and tumor datasets; however, even in this large dataset, fish tumors showing atypical proteomic and metabolomic profiles were still identified. This finding is in agreement with the present study, in which certain tumor samples showed substantially different expression profiles compared to other tumors (see fish ID no. 7, Figs. 2 & 3). All of these features strongly support the need for co-collection of important life history data when attempting to decipher cause-and-effect relationships in onset of disease in wild aquatic animal populations (Hines et al. 2007, Bignell et al. 2008, Stentiford et al. 2009).

Other possible explanations for the within-group variation include use of tumor samples that may have been resected from different parts of nodules expressing different transcriptional profiles (as discussed above). Laser Micro-Dissection (LMD) has the potential to address these issues and to allow researchers to harvest pure populations of cells for genetic analysis. Originally developed by Emmert-Buck et al. (1996) for the analysis of tumor cells, the technique is now used extensively in the many fields of cancer biology (Maitra et al. 2001, Stoehr et al. 2003, Player et al. 2004) and has recently been applied to the investigation of parasitic disease in aquatic species (Small et al. 2008). Future studies in our laboratory are being directed at using LMD coupled with downstream molecular techniques to analyze gene expression in specific tumors and pre-neoplastic lesions such as foci of cellular alteration (FCA) in liver sections exhibiting no gross lesion pathologies. We predict that such an approach will lead to a greater understanding of the etiology of liver tumors in marine sentinel flatfish, and that these improvements will be required to justify the continued usage of marine flatfish as sentinels for marine pollution monitoring.

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