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Differential gene expression in anthracene-exposed mummichogs (*Fundulus heteroclitus*)

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Abstract

The polycyclic aromatic hydrocarbon (PAH) anthracene is present in many estuarine systems at concentrations believed to cause sublethal adverse effects, although its exact mode of toxicity remains unclear. Knowledge of the induction or suppression of specific genes as a result of exposure may be useful in explaining these effects. We have generated a fingerprint of anthracene exposure using the mummichog (*Fundulus heteroclitus*), a non-migratory estuarine fish species. The fish were exposed in 7-day static renewal tests to environmentally relevant concentrations of 0, 27, 50, and 80 μg/l of anthracene. Total RNA was extracted from the livers and differential display reverse transcription polymerase chain reaction (DD RT-PCR) was used to recover 26 differentially expressed cDNA fragments. These cDNAs were isolated, sequenced, and compared to sequences of known genes in order to identify possible physiological consequences of exposure to anthracene. We then constructed macroarrays using these fragments and probed them with RNA from both anthracene-exposed fish and fish from a known PAH-impacted site. Three genes appear to be good indicators of exposure to anthracene in the range of concentrations tested, which included CYP2N2 and two expressed sequence tags (ESTs) termed 15C1 and 18C2. The expression of nine genes was altered in fish collected from a site with multiple PAHs. Band 15C1 and CYP2N2 again showed statistically significant upregulation in the field-caught fish, while a trypsin precursor and fatty acid-binding protein (FABP) all showed similar trends in induction as the laboratory-exposed fish. Further insight into the mechanism of toxicity of contaminants will be gained by the ability to identify and use differentially expressed genes as markers of exposure and effects.

Keywords: Mummichogs; Anthracene; *Fundulus heteroclitus*; Polycyclic aromatic hydrocarbon; Arrays; Gene expression

1. Introduction

Anthracene is a low molecular weight polycyclic aromatic hydrocarbon (PAH) that is a product of the incomplete combustion of wood and fossil fuel, is present in charbroiled foods, and is used in dye-making and paint production (Faust, 1997). It is present in marine and aquatic ecosystems primarily as a result of run-off from former wood-treating operations and coal gasification plants, and as a result of other types of petrochemical pollution (Mueller et al., 1989; Brenner et al., 2002). Although anthracene is on the US Environmental Protection Agency's (US EPA) list of Priority Pollutants (EPA, 1995), its

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sublethal effects are not particularly well characterized (Faust, 1997).

For example, exposure of 1 mg/ml anthracene to Chinese hamster ovary cells produced no differences in cellular viability and no differences in DNA synthesis when compared to untreated cells (Garrett and Lewtas, 1983). In addition, anthracene does not appear to be carcinogenic in laboratory rodents (IARC, 1983) and is currently not classifiable as a human carcinogen (EPA, 1998). However, anthracene may disrupt signal transduction, as T-lymphocytes incubated with 10 μM anthracene induced intracellular calcium mobilization within 3 min (Krieger et al., 1994). Most of the information in aquatic organisms concerns its effects on reproduction. For example, adult fathead minnows exposed to 12 μg/l anthracene for 9 weeks had a reduction in the number of eggs laid, in the percentage of hatched eggs, and in fry survival (Hall and Oris, 1991). Exposure of medaka (*Oryzias latipes*) to 20 μg/l anthracene for 8 weeks halved mating success (Cheek et al., 1999), while exposure of *Daphnia magna* to 8.2 μg/l anthracene for 21 days significantly reduced the number of neonates produced (Holst and Giesy, 1989).

We were interested in investigating the mechanisms of action of anthracene in adult organisms to determine differences that might indicate the effects of long-term exposure. To this end, we used mummichogs (*Fundulus heteroclitus*), an estuarine fish species with a small home range (Lotrich, 1975). It has contact with contaminated sediment over the full course of its lifetime, making it an excellent model for chronic exposure. The fish are plentiful in estuaries along the entire eastern seaboard and easily reared in the laboratory, making them an often-used study organism (Abraham, 1985).

To determine the sublethal effects of anthracene exposure, we examined changes in gene expression using differential display reverse transcription polymerase chain reaction (DD RT-PCR) (Liang and Pardee, 1992), which has been used successfully in isolating cDNAs from mummichogs (Bain, 2002) and other aquatic organisms (Morgan et al., 2001; Denslow et al., 2001; Lee and Goetz, 1998). In DD RT-PCR, the sequences of interest do not need to be known a priori; literally, hundreds of genes can be screened simultaneously. Since changes in gene expression are known to precede the manifestation of morphological alterations, one can use differential gene expression for early detection of exposure to a contaminant (Steiner and Anderson, 2000).

Mummichogs were exposed in the laboratory to several concentrations of anthracene and 26 differentially expressed fragments were discovered. An array was constructed to confirm induction or repression of gene transcription in both the laboratory and fish caught from a PAH-impacted site. Several genes of known function were found to be differentially expressed in both the laboratory and field-caught fish. Thus, the arrays can potentially be used to assess exposure and effects in contaminated sites.

2. Materials and methods

2.1. Collection and laboratory exposure of mummichogs

Adult male mummichogs (>5 cm) were collected near Georgetown, South Carolina at the North Inlet-Winyah Bay National Estuarine Research Reserve (referred to as the Georgetown site) using baited minnow traps. After transport to Clemson University's aquatic facility, they were maintained in 22 °C water with a salinity of 18 ppt (CoraLife Synthetic Sea Salt, Carson, CA), on a light:dark cycle of 14:10 h, and were fed TetraMin flake fish food (Blacksburg, VA). The fish were allowed to acclimate for 7 days and a water change was done every 48 h.

After the acclimation period, forty fish were randomly assigned to one of four treatment groups, each containing 10 fish. Individual fish were placed in a 3.75 l glass jar, and exposed to 0 (ethanol vehicle only), 27, 50, or 80 μg/l anthracene in a 7-day static renewal test. A complete water change was performed every 48 h. The mummichogs were euthanized in MS-222 (1 g/l) after the 7-day exposure. The livers were removed, placed in tubes containing Tri-Reagent (Sigma Chemical Co., St. Louis, MO), and total RNA was extracted using Tri-Reagent. The samples were stored at −80 °C.

2.2. HPLC analysis of anthracene concentrations

Water samples (1 l) were taken from each tank after 48 h of exposure, during the first water change. The

anthracene was extracted using methylene chloride, solvent-exchanged into acetonitrile, and then analyzed by HPLC (Hodgeson et al., 1990) using a Bondpak C_{18} reverse-phase column (3.9 mm × 150 mm). The mobile phase was 100% acetonitrile and anthracene was detected using a Waters scanning fluorescence detector with an excitation wavelength of 251 nm and an emission wavelength of 405 nm. One sample from each treatment group was spiked with tritiated fluoranthene before extraction to determine the extraction efficiency. The fluoranthene signature was detected at 450 nm. The results are presented as the average concentration of anthracene (μg/l) recovered from the water for each treatment group. Significant differences were determined by ANOVA followed by Fisher's PLSD ($P < 0.05$).

2.3. Differential display

To identify novel gene expression as a result of anthracene exposure, differential display was performed using the RNAimage Kit (GenHunter Corp., Nashville, TN) as modified from Liang and Pardee (1992). Differential display was chosen for these studies because it requires a very low amount of starting material, compared to other techniques that analyze differential gene expression, such as subtractive hybridization. Two control samples were compared to two samples each from the low, medium, and high treatment groups. Reverse transcription was performed using 0.2 μg total RNA, one of three 0.2 μM oligo-dT primers (H-T_{11}A: 5′-AAGCTTTTTTTTTTA-3′, H-T_{11}C: 5′-AAGCTTTTTTTTTTC-3′, H-T_{11}G: 5′-AAGCTTTTTTTTTTG-3′), 20 μM dNTPs, and 100 U MMLV-RT. Tubes were incubated at 37 °C for 1 h. Each reverse transcription was followed by a PCR reaction using 20 ng of the reverse transcribed product, 0.2 μM of the original oligo-dT primer, 0.2 μM of an arbitrary primer (H-AP1, H-AP2, H-AP3, H-AP9, and H-AP10 from the GenHunter RNAimage kit), 2 μM dNTPs, 2 μCi [^{33}P]dATP (3000 Ci/mmol, New England Nuclear, Boston, MA), and 1 U Taq polymerase. The PCR reactions were performed for 40 cycles at 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 60 s. The RNAspectra Kit (GenHunter), which uses rhodamine red-labeled oligo-dT primers instead of radiolabeled dATP, was used for fluorescently tagged differential display. This method has been reported to increase the specificity of the differential display reactions. In these reactions, six control samples were compared to four each of the treatment groups.

The PCR products were electrophoresed on a 6% urea-polyacrylamide gel. For the radiolabeled cDNA, the gels were transferred to chromatography paper, dried, and placed on autoradiography film. For fluorescently labeled samples, image analysis of the gel was done using the Bio-Rad Molecular Imager FX (Hercules, CA). We visually inspected the bands to determine whether there was differential expression. Differentially expressed bands were eluted and the cDNA was re-amplified by PCR. The PCR products were cloned into the pCR 2.1 Vector (Invitrogen, La Jolla, CA), inserted into competent *E. coli* cells, and sequenced. BLAST searches were then performed to search for homologies to known genes (Altschul et al., 1997).

2.4. Creation of the cDNA macroarrays

A series of macroarrays was created using a total of 48 different fragments. Of these 48, 26 fragments were discovered by differential display, including insulin-like growth factor I (IGF-I), CYP2N2, trypsin precursor, a chaperonin containing T-complex, and fatty acid-binding protein (FABP). Also incorporated on the macroarray were seven fragments that were upregulated in response to pyrene and three genes that were upregulated in response to trivalent chromium. In addition, there were eleven genes on the array that are generalized stress response genes and one housekeeping gene. The cDNA of the genes included on the array, and the primers used to make them, were CYP1A1 (#AF026800, Morrison et al., 1998) (forward 5′-cctcttcggagctggtttcg-3′, reverse 5′-cgtttcgtgcgataacctcac-3′), CYP3A30 (#AF105068, Celander and Stegeman, 1997) (forward 5′-ccttttgtgacgaacatcaag-3′, reverse 5′-catggattgagagaggatctc-3′), CYP2P1 (#AF117341, Oleksiak et al., 2003) (forward 5′-gtgacaagcagttccagac-3′, reverse 5′-ctcggtgatgacgggtcaagg-3′), CYP2P3 (#AF117343, Oleksiak et al., 2003) (forward 5′-gctacgaatacaatgacaaac-3′, reverse 5′-caagaagcttctgtagtatc-3′), MDR (#AF099732) (forward 5′-ggagaaggctgggaagattgc-3′, reverse 5′-gtcctcgaagctcacgttgc-3′), Na/K-ATPase (#AY057072, Semple et al., 2002) (forward 5′-gtrgaagacattgctgchcg-3′, reverse 5′-cacaccrtcaccwgtyacagc-

3′), CFTR (#AF000271, Singer et al., 1998) (forward 5′-cttcggagtggtgccgcag-3′, reverse 5′-caggacctgcagtg-ttatgg-3′), AhR1 (#AF024591, Karchner et al., 1999) (forward 5′-cgagccaaacacttcttctctgtc-3′, reverse 5′-gctgtcaggttccatctcttgc-3′), AhR2 (#U29679, Karchner et al., 1999) (forward 5′-ggtcaagagctacttta-aagc-3′, reverse 5′-cacagccctgagagaattctg-3′), and ARNT (#AF079311, Powell et al., 1999) (forward 5′-caaagcctggcccccgcag-3′, reverse 5′-gacccttcagtttc-accacctgt-3′). The housekeeping gene was LDH (#L23786, Bernardi et al., 1993) (forward 5′-gcatggc-gtgtgccgtcagc-3′, reverse 5′-gctcaggttcttcacgatgc-3′). As with the differential display products, these cDNAs were cloned into the pCR2.1 vector (Invitrogen), inserted into competent *E. coli* cells, and their sequences verified. The cloned cDNA fragments were amplified by PCR using M13 and T7 primers. The PCR products were denatured with 0.5N NaOH/0.5 mM EDTA at 95 °C for 5 min, and neutralized with 0.6 M sodium acetate. The denatured PCR products were then spotted onto a nylon membrane using the Bio-Dot Microfiltration Apparatus (Bio-Rad), and UV-crosslinked to it.

2.5. Verification of differential gene expression using the cdna macroarrays

Total RNA (20 μg) from six 50 μg/l anthracene-exposed fish and from six control fish was reverse transcribed with 50 μCi [^{33}P]dATP, 75 μM dNTPs, 0.2 μM oligo-dT primer, and 800U MMLV-RT for use as probes against the macroarrays. The membranes were pre-hybridized at 42 °C for 4 h in hybridization buffer (5× SSPE, 5× Denhardt's solution, 2.5% SDS, and 50% formamide) containing 100 μg/ml of denatured salmon sperm (GenHunter Corporation, 2001). The cDNA probes were denatured, added to the hybridization tubes along with additional denatured salmon sperm, and incubated overnight. The arrays were rinsed twice at room temperature in 1× SSC/0.1% SDS for 15 min, and once at 60 °C in 0.25× SSC/0.1% SDS for 15 min. The macroarrays were wrapped in plastic and detected using the Bio-Rad Molecular Imager FX. The resulting dots were then analyzed by densitometry corrected by normalization to an array-wide average. Statistical differences were determined using a Student's *t*-test.

2.6. Assessing utility of the arrays using field-caught fish

Adult male mummichogs were collected from Diesel Creek, a PAH contaminated site in Charleston, SC. Sampling conducted by Sanger et al. (1999) has shown an average anthracene concentration in the creek sediments of 445 ng/g dry weight, along with high concentrations of other PAHs including pyrene, fluoranthene, phenanthrene, and chrysene, with total summed PAHs averaging 8131 ng/g. The fish were euthanized on site, their livers were removed, placed in Tri-Reagent, and then snap-frozen in liquid nitrogen. The same procedure was used to collect additional fish from the Georgetown site. Total RNA was extracted and six RNA samples from each group of fish were used as probes against the macroarrays as described above.

3. Results

3.1. Anthracene concentrations in water samples

Mummichogs were exposed to 0, 27, 50, and 80 μg/l anthracene for 7 days. Water samples taken from the exposure tanks after 48 h showed concentrations significantly lower than the nominal concentrations (Table 1). The average anthracene concentration recovered ranged from 10.2 to 15.9%. The anthracene recovered in the control tanks was due to an artifact, as 0.1 μg/l is at the detection limits of anthracene using this procedure. The recovery of the surrogate spike averaged about 50%, indicating that the recoveries using the methylene chloride were low. Presumably some

Table 1
Anthracene concentrations in the exposure water

Nominal concentration (μg/l)	Measured concentration (μg/l)
0	0.17 ± 0.12 a
27	4.28 ± 2.06 b
50	7.01 ± 3.43 c
80	8.15 ± 4.92 c

Water samples were collected after 48 h of exposure and analyzed by HPLC. The values are the average ± standard deviation of 10 samples.: the letters (a–c) denote that the concentrations were statistically different from one another using ANOVA followed by Fisher PLSD ($P > 0.05$).

of the anthracene was absorbed by the fish, although much of the anthracene may have remained in an emulsion between the layers of water and methylene chloride, or it might have absorbed to the food, feces, or to the container wall. However, the results show that all concentrations, except for the 50 and 80 μg/l groups, were statistically different from one another.

3.2. Identification of differentially expressed genes

To identify genes that would respond to anthracene exposure, we analyzed fish from the laboratory exposures by DD RT-PCR. A total of 26 cDNA bands were identified from DD RT-PCR gels. Only cDNA bands that appeared in the majority of the fish from a treatment group were considered to be differentially expressed. These fragments were isolated, inserted into plasmids, and sequenced. Eight of these fragments showed homology to genes of known function, including CYP2N2, hyaluronic acid-binding protein, trypsin precursor, chaperonin containing T-complex, FABP, and IGF-1 (Table 2). The remaining eighteen sequences have no known homology.

Table 2
Summary of expressed bands in *Fundulus* after anthracene exposure

Band name	GenBank number	Primer	Expression	Putative identity	GenBank number, species, and *E*-value of identity
2G1	CF542183	H-AP1	+	IGF-I	AF061278 from *Paralichthys olivaceus*: *E*-value $= 1 \times 10^{-22}$
3G1	CF542259	H-AP1	+	CYP2N2	AF090435 from *F. heteroclitus*: *E*-value $= 6 \times 10^{-7}$
4A2		H-AP1	+	CYP2N2	AF090435 from *F. heteroclitus*: *E*-value $= 6 \times 10^{-7}$
5A2-1	BM056132	H-AP1	−	EST	
5A2-2	CF542180	H-AP1	−	EST	
6A2	BM056134	H-AP1	−	EST	
8C1	BM056135	H-AP1	+	EST	
8C2	CF542181	H-AP1	+	Hyaluranic acid-binding protein/trypsin precursor	XP217361 from *Rattus norvegicus*: *E*-value $= 9 \times 10^{-27}$: S49489 from *Paranothothenia magellanica*: *E*-value $= 4 \times 10^{-19}$
9C2	BM056133	H-AP1	−	EST	
10C1	BM056136	H-AP1	−	EST	
11C2-2	CF542182	H-AP1	−	EST	
14C1	BM056137	H-AP2	+	Chaperonin containing T-complex	AAH44997 from *Homo sapiens*: *E*-value $= 2 \times 10^{-17}$
15C1	BI993600	H-AP2	+	EST	
18C1	CF542181	H-AP2	+	Hyaluranic acid-binding protein/trypsin precursor	XP217361 from *R. norvegicus*: *E*-value $= 9 \times 10^{-27}$: S49489 from *P magellanica*: *E*-value $= 4 \times 10^{-19}$
18C2	BM056138	H-AP2	+	EST	
20C1	BI993600	H-AP2	+	EST	
22G1	Bi993602	H-AP3	+	EST	
24G1	CF542184	H-AP3	+	EST	
25A1	AY034789	H-AP3	−	FABP	AY034789 from *F. heteroclitus*: *E*-value $= 1 \times 10^{-167}$
26G1	CF542185	H-AP9	−	EST	
28G1	CF542186	H-AP9	+	EST	
30G1	CF542187	H-AP9	+	EST	
32G1	CF542188	H-AP9	+	EST	
42C2	CF542189	H-AP9	−	EST	
44G1	CF542190	H-AP10	+	EST	
48G1	FG542260	H-AP10	+	EST	

Bands were generated by differential display, cloned into the pCR2.1 vector, and sequenced. Blast searches were performed to ascertain sequence identity. In the expression column, (+) indicate upregulation while (−) indicate downregulation.

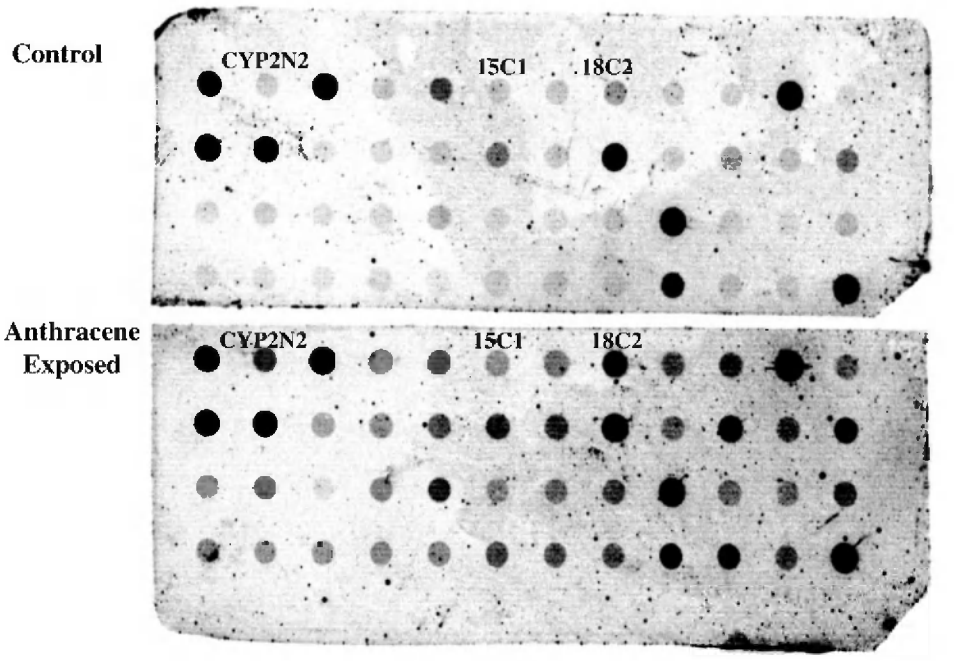

Fig. 1. Duplicate macroarrays probed with cDNA from a control fish (top) and an anthracene-treated fish (bottom). These blots are representative of all six arrays. Several spots that displayed differences between the control fish and the treated fish are labeled according to Table 2.

3.3. Control versus treated and Georgetown versus Diesel Creek cDNA macroarrays

In order to verify that the gene products recovered from the gels were differentially expressed, and determine whether similar patterns of expression would be found in fish collected from a PAH-impacted site, we constructed cDNA macroarrays. Twelve identical macroarrays of 48 fragments, containing both our isolated fragments as well as other cDNAs previously shown to be responsive to toxicant stress, were probed with cDNA from either laboratory-exposed fish or fish collected from the two field sites.

Two representative macroarrays comparing RNA from control fish versus RNA from 50 μg/l anthracene-exposed fish are shown in Fig. 1, while arrays comparing Georgetown versus Diesel Creek fish are shown in Fig. 2. In total, three fragments showed statistically significant differences in expression in anthracene-exposed fish when compared to the control fish (Table 3). No other bands showed statistically significant differential expression. Of the three differentially expressed fragments in the laboratory exposures, both CYP2N2 (band 3G1) and 15C1, an EST, were also significantly induced in the Diesel Creek fish. Two different cDNA fragments of CYP2N2 representing different sections of the RNA were spotted on the array as fragments 3G1 and 4A2. Although band 3G1 was not significantly induced in Diesel Creek fish, 4A2 was statistically different. Presumably these differences are simply due to high variability in the 3G1 samples, and CYP2N2 is truly induced in both anthracene-exposed and Diesel Creek fish. Alternatively, the two fragments could represent splice variants. Although this has not been shown in the CYP2N family, there are splice variants in CYP27 (Chen et al., 1998) and CYP2D (Huang et al., 1997). The third differentially expressed fragment in anthracene fish, 18C2, an EST, showed almost two times the expression of the control fish but showed no differential expression in the Diesel Creek fish.

In the macroarrays comparing the Diesel Creek fish gene expression to the Georgetown fish expression (Fig. 2), nine bands were significantly

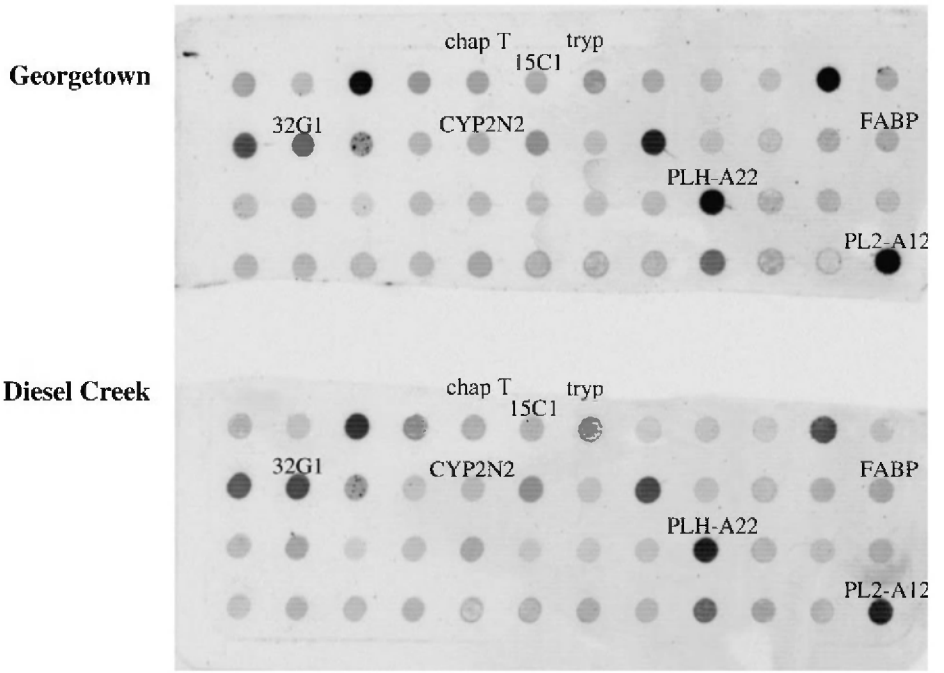

Fig. 2. Duplicate macroarrays probed with cDNA from a Georgetown fish (top) and a Diesel Creek fish (bottom). cDNA spots that showed differential expression are labeled as in Table 2 with the following abbreviations: tryp, trypsin precursor; chap T, chaperonin containing T-complex; FABP, fatty acid-binding protein; PL2-A12, hepatocyte growth factor activator.

different (Table 3). No other bands showed statistically significant differential expression. A trypsin precursor/hyaluronic acid-binding protein (bands 8C2 and 18C1), 15C1, and CYP2N2 all showed similar trends in induction as the laboratory-exposed fish, while a hepatocyte growth factor activator (band PL2-A12) was suppressed in both Diesel Creek and anthracene-exposed fish. Three additional fragments,

Table 3
Fold differences in expression for selected fragments on the macroarrays

Fragment name	Identity	Fold difference, treated vs. control	Fold difference, Diesel Creek vs. Georgetown
3G1	CYP2N2	1.89 ± 0.73*	1.39 ± 0.63
8C2	Hyaluranic acid-binding protein/trypsin precursor	2.20 ± 1.91	2.22 ± 1.05*
14C1	Chaperonin containing T-complex	1.05 ± 0.40	1.34 ± 0.21**
15C1	EST	1.42 ± 0.34*	1.81 ± 0.41**
18C1	Hyaluranic acid-binding protein/trypsin precursor	2.32 ± 1.89	2.04 ± 0.79*
18C2	EST	1.85 ± 0.78*	−1.08 ± 0.43
32G1	EST	−1.13 ± 0.47	1.64 ± 0.41*
4A2	CYP2N2	1.48 ± 0.56	1.35 ± 0.17**
25A1	FABP	1.33 ± 1.60	1.27 ± 0.24*
PL2-A12	Hepatocyte growth factor activator	−1.18 ± 0.32	−1.50 ± 0.20*
PLH-A22	EST	1.13 ± 0.44	−1.40 ± 0.19**

The intensity of each dot on the macroarray was quantitated by phosphorimaging and corrected using an array-wide normalization. Values are the average fold difference ± standard deviation of the treated fish expression versus the control fish expression, or the Diesel Creek fish expression versus the Georgetown fish expression. Negative values indicate down-regulation of a particular fragment. Statistical differences were determined by Student's t-test with $n = 6$ for each group.

* $P < 0.05$.

** $P < 0.01$.

a chaperonin containing T-complex (band 14C1), band 32G1, and band PLH-A22, were differentially expressed in the Diesel Creek fish but their expression was not altered by anthracene exposure. Thus, we found three genes that appear to be good indicators of anthracene exposure and nine genes that are altered in response to complex PAH mixtures.

4. Discussion

Our goals were to discover genes whose transcription was altered by the environmental contaminant anthracene and to develop cDNA arrays that could be used to assess impacts at PAH-impacted sites. We developed a cDNA macroarray containing 48 fragments, 26 of which were thought to be differentially expressed due to anthracene. Of these 48 fragments, nine showed statistically significant differential expression in the field-caught fish, while the laboratory fish showed statistically significant levels of expression for three fragments (Table 3).

There are several explanations for why other anthracene-responsive fragments found by differential display did not show differential expression using the macroarrays. The fragments were probably false positives, a phenomenon known to occur with DD RT-PCR (Debouck, 1995). Indeed several groups have reported false positive rates in excess of 50% (Aiello et al., 1994; Sun et al., 1994). Investigators have suggested that this may be due to co-migration of cDNA bands in the differential display gel (Miele et al., 1998), or to genetic differences in the populations being studied (Pienta and Schwab, 2000). Alternatively, because the fragments and probes all come from feral fish there can be a wide range of expression for a particular gene. It is possible that there were significant increases or decreases in transcription in individual fish after exposure to anthracene but that these changes were masked by the intra-group variability. Significant differences in mummichog gene expression have been described in microarray studies by Oleksiak et al. (2002), in which the animals were unexposed to an toxicant and were all housed in the laboratory under identical conditions. Statistically different gene expression was observed in 161 of the 907 genes that they examined within the same population at a P-value of 0.01, with the expression change typically being between 1.5 and 2-fold, or greater. Thus, it is not surprising that differences in gene expression from exposed fish might have been masked by variability within the population. In addition, because we were using macroarrays, rather than microarrays, the size of the spot is much larger than normal. Thus, when the densitometry is performed, it probably underestimates the magnitude of the differences between the spots.

The patterns of induction or repression were the same in both the laboratory-exposed and field-caught animals. However, there were more fragments that were statistically differentially expressed between the Georgetown and Diesel Creek fish than between control and anthracene-exposed fish, even though many of fragments had been generated from the laboratory exposures. This could be a factor of the duration of the exposure as a gene may be more strongly expressed in the fish after a lifetime of exposure in the field, rather than to a 1-week exposure in the laboratory. The difference may also have been due to the type of exposure since we performed aqueous exposures while the mummichogs in Diesel Creek are exposed to anthracene both in the water and in the sediment. Finally, mummichogs in Diesel Creek are exposed to a complex mixture of PAHs rather than just one. Indeed, two of the down-regulated fragments on Diesel Creek fish arrays, PLH-A22 and hepatocyte growth factor activator (PL2-A12), were discovered in mummichogs exposed in the laboratory to pyrene. Neither one was significantly repressed in anthracene-exposed animals. Hence, these fragments appear to be able to discriminate between at least these two PAHs.

Bands 3G1 and 4A2, which are both portions of CYP2N2, were induced in both the anthracene fish and the Diesel Creek fish. CYP2N2 is an arachidonic acid epoxygenase and hydroxylase, and is potentially involved in xenobiotic metabolism (Oleksiak et al., 2000). PAHs such as benzo[a]pyrene have been shown to increase the P450-mediated arachidonic acid metabolism in the liver of scup (*Stenotomus chrysops*) (Schlezinger et al., 1998), although it is not known if this is due to an increase in CYP2N2 activity. It has also been demonstrated that exposure of mice to dimethylbenzanthracene (Nair et al., 2000) or exposing NIH3T3 cells to benzo[a]pyrene stimulates arachidonic acid metabolism (Ikegwuonu and Jefcoate, 1999).

Band 15C1 is an EST whose function is not known, but it was significantly upregulated in both the laboratory-exposed and the field-caught fish. The induction levels are similar in both groups and thus this sequence could tentatively be considered a biomarker of exposure to anthracene. Further investigation into whether this fragment is similarly upregulated in response to other PAHs or to other forms of stress would be necessary before it could be determined whether its expression is unique to anthracene exposure. Band 18C2 is another EST that was induced in the treated fish but its expression did not change in the field site fish. This fragment could perhaps represent a gene that is induced by exposure to anthracene alone but whose expression is suppressed upon exposure to multiple PAHs or other contaminants.

In Diesel Creek fish, a trypsin precursor was significantly induced, while the anthracene-exposed fish displayed the same trend, although their expression was not statistically significantly different. Trypsinogen, or trypsin precursor, is the inactive form of trypsin, a member of the serine protease family that is involved in generalized protein degradation. A number of trypsinogen genes are expressed not only in the pancreas, but also in the liver (Tanaka et al., 1986), lung (Imamura et al., 2003), and other organs where they are thought to play a role in apoptosis (Lumelsky and Schwartz, 1996). Potentially, trypsinogen was upregulated after exposure to anthracene and in Diesel Creek fish as a means to cope with damage caused by the toxicant.

Fatty acid-binding protein was also upregulated in the Diesel Creek fish, and showed a similar trend in the laboratory-exposed fish, although this was not statistically different. FABP is involved in the metabolism of intracellular lipids (Storch and Thumser, 2000) as well as in the binding and trafficking of arachidonic acid and its metabolites (Widstrom et al., 2001). This suggests that FABP could modulate the metabolism, activities, and targeting of these compounds in conjunction with CYP2N2. We have previously shown that pyrene can also modulate the expression of mummichog FABP (Bain, 2002).

In total, we found three genes that appear to be good indicators of anthracene exposure and nine genes that are altered in response to complex PAH mixtures. In general, we see an upregulation of genes that are involved in protein turnover and arachidonic acid metabolism. The ability to identify and use these genes as markers of exposure and effects should give us further insight into the mechanism of toxicity of contaminants.

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